1521-0103/355/3/506–515$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.115.227249
J Pharmacol Exp Ther 355:506–515, December 2015

The Journal of PHARMACOLOGY And Experimental Therapeutics

Integrated Pharmacokinetic/Pharmacodynamic Analysis for Determining the Minimal Anticipated Biological Effect Level of a Novel Anti-CD28 Receptor Antagonist BMS-931699

Zheng Yang, Haiqing Wang, Theodora W. Salcedo, Suzanne J. Suchard, Jenny H. Xie, Lumelle A. Schneeweis, Catherine A. Fleener, James D. Calore, Rong Shi, Sean X.Y. Zhang, A. David Rodrigues, Bruce D. Car, Punit H. Marathe, and Steven G. Nadler

Department of Metabolism and Pharmacokinetics, Pharmaceutical Candidate Optimization (Z.Y., H.W, A.D.R., B.D.C., and P.H.M.), Department of Exploratory Clinical and Translational Research (C.A.F., R.S., S.X.Y.Z.), Department of Protein Structures and Sciences (L.A.S.), Department of Immunology Discovery (S.J.S., J.H.X., and S.G.N.), Department of Bioanalytical Sciences (J.D.C.), Bristol-Myers Squibb Research and Development, Princeton, New Jersey; and Department of Drug Safety Evaluation (T.W.S.), Bristol-Myers Squibb Research and Development, New Brunswick, New Jersey

Received June 25, 2015; accepted October 6, 2015

ABSTRACT

BMS-931699 (lulizumab pegol), a domain antibody (dAb) conjugated with 40-kDa branched polyethylene glycol, is a human anti-CD28 receptor antagonist under development for the treatment of inflammatory and autoimmune diseases. In the present work, the minimal anticipated biologic effect level (MABEL) was determined for BMS-931699 by integrating all the available preclinical data. The relevance of the in vitro mixed lymphocyte reaction (MLR) assay to a whole blood CD28 receptor occupancy (RO) assessment, as well as the relationship between the CD28 RO and the inhibition of T-cell-dependent antibody response to keyhole limpet hemocyanin in vivo, was demonstrated through an integrated pharmacokinetic/pharmacodynamic analysis using anti-hCD28 dAb-001 (differing from BMS-931699 by two additional amino acids at the N-terminus) and a mouse surrogate. Based on this analysis, the EC_{10} value (0.32 nM) from the human MLR assay and the human plasma volume (0.04 l/kg) were employed to calculate the MABEL (0.01 mg) of BMS-931699 in humans, with a CD28 RO predicted to be ≤10%. The estimated MABEL dose was threefold higher than the value derived from the binding constant and twofold less than the MABEL converted from animal efficacy studies based on the body surface area. Furthermore, it was 2900-fold lower than the human equivalent dose derived from the no observed adverse effect level in monkeys (15 mg/kg/week for 5 doses, intravenous dosing) with a 10-fold safety factor applied. Therefore, the MABEL dose represented a sound approach to mitigate any potential risk in targeting CD28 and was successfully used as the first-in-human starting dose for BMS-931699.

Introduction

The CD28-B7 (CD80/CD86) costimulation pathway is crucial in regulating the T-cell activation and tolerance (Lenschow et al., 1996; Sharpe and Freeman, 2002). Inhibiting this pathway may represent a promising therapeutic intervention for autoimmune diseases and organ transplant rejection. Unlike the blockade of CD80 and CD86, inhibiting CD28 preserves the regulatory signal mediated by CTLA-4, hence avoiding the unwanted effects associated with the blockade of this part of immune regulation (Poirier et al., 2010). To this end, a novel Vκ light chain domain antibody (dAb), the smallest functional binding unit corresponding to the variable regions of either the heavy or the light chains of a human antibody, was developed to monovalently bind to and antagonize the human CD28 (hCD28) receptor. BMS-931699 (lulizumab pegol, molecular weight = 12 kDa) was formatted with 40-kDa branched polyethylene glycol to extend its half-life in vivo and was a potent inhibitor of hCD28-mediated T-cell proliferation, with an EC_{50} of 2.9 nM in a human dendritic cell-driven mixed lymphocyte reaction (MLR) assay (Suchard et al., 2013). In addition, anti-hCD28 dAb-001, a molecule that differs from BMS-931699 by two additional amino acids (ST) at the N terminus, was also studied in the preclinical setting. The additional amino acids in anti-hCD28 dAb-001 did not significantly affect the behavior of the dAb as tested across multiple biochemical and in vitro cellular assays (Suchard et al., 2013).

Targeting CD28, however, is not without a risk. This was evident in the TeGenero incident, where TGN1412, a CD28

The work was supported by Bristol-Myers Squibb Research and Development.
dx.doi.org/10.1124/jpet.115.227249.

ABBREVIATIONS: APCs, antigen-presenting cells; dAb, domain antibody; EMA, European Medicines Agency; FIH, first in human; HED, human equivalent dose; HRP, horseradish peroxidase; MLR, mixed lymphopcyte reaction; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; MABEL, minimal anticipated biological effect level; NOAEL, no observed adverse effect level; PBS, phosphate-buffered saline; PK/PD, pharmacokinetic/pharmacodynamics; QC, quality control; RO, receptor occupancy; TDAR, T-cell-dependent antibody response.

superagonist monoclonal antibody (mAb), caused a cytokine storm and severe side effects at a starting dose of 0.1 mg/kg in humans (Suntharalingam et al., 2006), a dose that was 500-fold lower than the no observed adverse effect level (NOAEL, 50 mg/kg) observed in monkeys or 160-fold lower than the human equivalent dose (HED, 16 mg/kg) after the body surface area conversion of the NOAEL in monkeys (Expert Group on Phase One Clinical Trials, 2006). The incident highlighted the importance of estimating a safe starting dose in first-in-human (FIH) clinical trials. To that end, the Expert Scientific Group on Phase I clinical trials, led by Sir Gordon Duff, recommended the minimal anticipated biologic effect level (MABEL) as the safe starting dose for some FIH studies (Expert Group on Phase One Clinical Trials, 2006), which was subsequently adopted as part of the guideline from the Committee for Medicinal Products for Human Use at the European Medicines Agency (EMA). Specifically, the EMA guideline recommends that, for high-risk biologics, the human starting dose calculation be based on the totality of the available preclinical data (in vitro and in vivo from animal species and in vitro from humans), rather than sole application of a traditional toxicology-based NOAEL approach (EMA, 2007). The considerations, approaches, and challenges associated with the MABEL determination have been discussed in details by Muller et al. (2009). Essentially, a sound understanding of target pharmacology, including in vitro-to-in vivo connectivity on target engagement and functional activities as well as species differences between humans and animals, is key to an appropriate MABEL determination. To that end, a pharmacokinetic/pharmacodynamic (PK/PD) modeling-based approach is advocated as a tool to facilitate the data and knowledge integration in the preclinical setting. The quantitative PK/PD information generated is not only useful for determining the MABEL as a safe starting dose in humans but also valuable for projecting a human efficacious dose in the clinic.

Because of the previous TeGenero incident, CD28 is considered to be a high-risk target. Although all the available preclinical data support that monovalent anti-hCD28 dAbs are T-cell costimulation inhibitors with no agonistic activity, a MABEL-based approach must be considered for a safe starting dose in FIH trials (Muller et al., 2009; Muller and Brennan, 2009), particularly in light of the fact that human subjects have not been exposed to the agent previously. In the present work, an integrated analysis using a PK/PD modeling-based approach was employed to evaluate all the available preclinical data, including BMS-931699, anti-hCD28 dAb-001, and a mouse surrogate (anti-mCD28 dAb-001), from which in vitro and in vivo assay connectivity was established to determine the MABEL. Different approaches of estimating MABEL doses were also explored and compared. The MABEL dose established was subsequently used as a safe starting dose for BMS-931699 in a FIH trial successfully (unpublished data).

Materials and Methods

Materials

BMS-931699, anti-mCD28 dAb-001, and anti-hCD28 dAb-001 were generated at Bristol-Myers Squibb (Princeton and Hopewell, NJ). Recombinant monomeric human or mouse CD28 antigen tagged to biotin was also produced at Bristol-Myers Squibb. Rabbit anti-dAb polyclonal antibody was custom made and purified at Covance Research Products (Denver, PA). Horseradish peroxidase-labeled donkey anti-rabbit polyclonal secondary antibody was obtained from Jackson Immunoresearch (West Grove, PA). Keyhole limpet hemocyanin (KLH) was purchased from Pierce (Rockford, IL). All other reagents were obtained from commercial sources.

In Vitro Studies

Determination of Binding Affinity. The binding affinity, measured as the dissociation constant (K_d) to hCD28 or mCD28 receptors, was determined using a surface plasmon resonance method (Biacore T100, GE Healthcare Bio-Sciences, Pittsburg, PA). The experiment was conducted at 25°C in a buffer (pH 7.4) containing 10 mM NaH_2PO_4, 130 mM NaCl, and 0.05% Tween 20. After preconditioning the streptavidin chip surface with a solution of 1 M NaCl and 50 mM NaOH, hCD28- or mCD28-biotinylated monomer was immobilized at a concentration of 1 or 2 μg/ml using 9- to 15-second contact time at a flow rate of 10–20 μl/min to give approximately 180–270 resonance units of the immobilized receptors. Samples of dAbs, in a buffer of 20 mM $NaPO_4$ and 150 mM NaCl (pH 7.2), were then injected for 3 minutes at a flow rate of 30 or 100 μl/min followed by 10–15 minutes of dissociation using the "high performance" sample injection type in the Biacore T100 Software Method Builder. The K_d was estimated by a global fitting of the data using the 1:1 Langmuir binding model available in the Biacore T100 evaluation software.

Evaluation of MLR EC_{50} in Various Species. For the human MLR assay, dendritic cells were generated from monocytes isolated by adherence to plastic and then cultured in wells with granulocyte-macrophage colony-stimulating factor and interleukin 4 for 6 or 7 days. Twenty-four hours before the assay setup, lipopolysaccharide (1 μg/ml) was added to induce maturation. Anti-hCD28 dAbs were then titrated in half-log concentrations to evaluate their inhibition potential of the dendritic cell-T cell interaction (1:10 ratio). The incubations were conducted at 37°C for 5 days. Proliferation was determined based on ^{3}H-thymidine incorporation, and EC_{50} values were generated from the inhibition curves of each treatment using XLfit (Microsoft, Redmond, WA).

For the cynomolgus monkey MLR assay, peripheral blood mononucleated cells were isolated from acid citrate dextrose-treated monkey blood using Ficoll diluted with 10% phosphate-buffered saline (PBS). The human cell line PM-LCL, an Epstein-Barr virus-transformed human B cell line, was used as antigen-presenting cells (APC). PM-LCLs, irradiated with 10,000 rads, were combined with cynomolgous monkey peripheral blood mononucleated cells at a ratio of 1:25 in the presence or absence of anti-hCD28 dAbs and incubated at 37°C for 5 days. Proliferation was measured by ^{3}H-thymidine incorporation and EC_{50} values were estimated using XLfit.

For the mouse MLR assay, single cell suspensions from lymph nodes of BALB/c mice and spleens from DBA/2 mice were loaded onto nylon wool columns and incubated at 37°C for 60 minutes. The T cells were eluted from the columns (Wako Chemicals, Richmond, VA) with warm media (RPMI 1640, 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 50 μM 2-mercaptoethanol) and APC eluted with ice-cold media. The BALB/c T cells and the DBA/2 APC were resuspended in the media at a ratio of 2:1 in the presence or absence of anti-mCD28 dAb-001 and incubated at 37°C for 4 days. Proliferation was measured by 3H-thymidine incorporation, and EC50 values were estimated using XLfit.

Quantification of CD28 Receptor Occupancy. The extent of CD28 receptor occupancy (RO) in whole blood was determined using either a direct RO assay or an indirect receptor competition assay. Assessment of CD28 RO in monkeys and humans was performed using the direct RO assay. For in vitro human or monkey blood, samples (100 μl) were spiked with various known concentrations of dAbs to generate a dose curve up to saturating levels, followed by incubation at 37°C for 1 hour. For in vivo monkey studies, samples were divided into two 100-μl aliquots; to one aliquot, a saturating dAb concentration was added, and both samples were incubated at 37°C for 1 hour. Blood samples were then washed three times with 2 ml of FACS buffer

(0.5% fetal bovine serum and 0.1% sodium azide in PBS), placed on ice, and incubated with 20 μg of mouse IgG for 5 minutes to block Fc-mediated nonspecific binding. A biotinylated rabbit anti- polyethylene glycol mAb (B-47, 5 μg/ml final concentration, Epitomics, Burlingame, CA) was added and incubated for 30 minutes on ice protected from light. Samples were washed once with 2 ml of FACS buffer, decanted, and immunostained with anti-CD3 PECy7 and anti-CD4 FITC (10 and 2.5 μl, respectively; BD Biosciences, San Jose, CA) along with phycoerythrin (PE)-conjugated streptavidin (1 μg/ml final concentration, Life Technologies, Carlsbad, CA). A background sample was generated by staining an untreated (in vitro) or baseline (in vivo) sample with all assay reagents in the absence of dAbs. Samples were further incubated for 30 minutes on ice protected from light. Blood samples were then washed once with 2 ml of FACS buffer, and 2.5 ml of FACS Lysing Solution (BD Biosciences) was added for 15 minutes to lyse red blood cells and fix samples. After centrifugation and decanting, samples were resuspended in 200 μl of FACS Lysing Solution for flow cytometry analysis (BD FACSCanto II, gating on CD3+CD4+ lymphocytes).

For the CD28 RO in mice, the indirect receptor competition assay was used. The experimental procedure was similar to what was described above, except that a PE-labeled mouse anti-CD28 mAb (L293, BD Biosciences), at a final concentration of 5 μg/ml, was used to compete with dAbs for binding to the CD28 receptors. A blank mouse blood sample was used to determine the maximum occupancy in the absence of dAbs. In addition, a background control sample was generated by using the unlabeled anti-mCD28 mAb at a saturating concentration.

All flow cytometry data were analyzed using FACSDiva software (BD Biosciences). The percentage of the CD28 RO for the direct RO assay was calculated as follows:

$$\%RO = \left(\frac{MFI_{sample} - MFI_{background}}{MFI_{saturated\ \ sample} - MFI_{background}}\right) x100$$

where MFI is the median fluorescence intensity, and saturated sample refers to the sample treated with a saturating concentration of dAbs. The percentage of the CD28 RO for the indirect receptor competition assay was calculated as follows:

$$\%RO = \left(1 - \frac{MFI_{sample} - MFI_{background}}{MFI_{saturated\ \ sample} - MFI_{background}}\right) x100$$

where saturated sample is in reference to the blank blood sample saturated with an excess amount of the PE-labeled anti-mCD28 mAb. For the EC_{50} calculation, %RO data versus dAb concentration data were fitted with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

In Vivo Studies. All studies were conducted according to the study protocols approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee.

Mouse. The pharmacokinetics and whole blood CD28 RO of anti-mCD28 dAb-001 were investigated in mice. Three groups of female BALB/c mice (N = 4 per dose group, 20–25 g, Charles River, Wilmington, MA) received intraperitoneal doses of anti-mCD28 dAb-001 at 0.08, 0.4, and 2 mg/kg, respectively. Serial blood samples (~0.1 ml) were obtained by retro-orbital bleeding at 2, 4, 24, 48, 72, 96, 168, 216, 264 hours post dose. A portion of blood samples was used for the determination of CD28 RO. The remaining portion was collected into K_2EDTA tubes, from which plasma samples were harvested by centrifugation at 4°C (1500–2000 g) and stored at −70°C until further analysis for drug concentrations.

To evaluate the KLH-induced T-cell-dependent antibody response (TDAR), five groups of female BALB/c mice (N = 8 per dose group, 20–25 g) received anti-mCD28 dAb-001 three times a week at intraperitoneal doses of 0.004, 0.02, 0.1, and 0.5 mg/kg, respectively, along with PBS as a vehicle control. Twenty four hours after the first dose of the dAb, mice were immunized with 250 μg KLH in PBS via intraperitoneal injections. Blood samples were collected, from which whole blood CD28 RO was determined at 24 and 72 hours post last dose. In addition, serum samples were harvested and analyzed for anti-KLH IgG on day 14 post the KLH challenge.

Monkey. Anti-hCD28 dAb-001 was administered to 3 groups of protein therapeutic-naive female cynomolgus monkeys (2.6–4.5 kg, Charles River, Houston, TX) at the doses of 0.05 (N = 2), 0.5 (N = 3), and 5 mg/kg (N = 2), respectively, by subcutaneous injections in the posterior thorax. Serial blood samples were collected into K_2EDTA tubes from the femoral vein at predose and 2, 4, 8, 24, 48, 96, 168, 240, 336, 408, 504, and 672 hours post dose. A portion of whole blood samples was collected for the CD28 RO analysis. The remaining portion were centrifuged at 4°C (1500–2000 g), and resultant plasma samples were stored at −70°C until further analysis for drug concentrations.

In addition, to determine the subcutaneous bioavailability, two protein therapeutic-naive female cynomolgus monkeys (3.1 and 3.2 kg) received anti-hCD28 dAb-001 intravenously at a dose of 0.05 and 0.5 mg/kg, respectively, via a bolus injection into the saphenous vein. Serial blood samples were collected into K_2EDTA tubes from the femoral vein at predose and 0.05, 0.5, 2, 4, 8, 24, 48, 96, 168, 240, 336, 408, 504, and 672 hours post dose. The plasma samples were then obtained by centrifugation at 4°C (1500–2000 g) and stored at –70°C until further analysis for drug concentrations.

The KLH-induced TDAR, in the presence of anti-hCD28 dAb-001, were further evaluated at 0.05, 0.5 and 5 mg/kg after subcutaneous administration to three groups of protein therapeutic-naive cynomolgus monkeys (N = 2 males and 2 females, 2.7–4.5 kg). All monkeys were immunized with KLH 24 hours and 6 weeks after the day 1 dosing of the dAb. Serial blood samples were collected into K_2EDTA tubes at predose and 2, 4, 8, 24, 48, 96, 168, 240, 336, 408, 504, and 672 hours post dose. Plasma samples were then harvested and stored at −70°C until further analysis for drug concentrations. In addition, whole blood samples were collected at various time points for the CD28 RO analysis.

Sample Analysis

Determination of dAb Concentrations. An enzyme-linked immunosorbent assay using a recombinant human or mouse monomeric CD28 receptor to capture dAb was developed to quantify dAb concentrations in plasma samples. Briefly, biotin-tagged CD28 receptors, at a final concentration of 1 μg/ml, were bound to streptavidin-coated 96-well plates and incubated at 22°C for 1–2 hour. Study samples, along with calibrators and quality controls (QCs), were diluted 1:10 (mouse) or 1:100 (monkey) in an assay buffer (1× PBS, 1% bovine serum albumin, and 0.05% Tween 20). Subsequent dilutions of study samples were also made using the assay buffer containing either 10% mouse or 1% monkey plasma. Study samples were then added onto the plates and incubated at 22°C for an additional 2 hours with calibrators and QCs included in each plate. Subsequently, the unbound materials were washed off from the plates using 1× PBS with 0.05% Tween 20. The bound materials were then detected using a rabbit anti-dAb polyclonal antibody (0.5 μg/ml) after incubation at 22°C for 1 hour. Afterward, the plates were washed and incubated with horseradish peroxidase (HRP)-labeled donkey anti-rabbit polyclonal secondary antibody for 1 hour. The plates were washed again followed by the addition of a HRP substrate (3,3′,5,5′-tetramethylbenzidine). The reaction was stopped by 1 M phosphoric acid, and the absorbance was measured at a wavelength of 450 nm using a Tecan plate reader (Morrisville, NC). The calibration curve was fitted using a four parametric logistic fit regression model weighted by 1/x. The lower limit of quantification for the mouse and monkey dAb assays, back-calculated to the concentration in 100% plasma samples, was 0.6 and 1.6 nM, respectively. The inter- and intra-assay precision was less than 20% coefficient of variation, with the

accuracy also within 20% of the nominal concentration. In addition, the QC samples were found stable, when stored at 22°C for over 2 months.

Quantification of In Vivo CD28 Receptor Occupancy. The same approach described for the in vitro CD28 RO determination was used for in vivo RO measurements.

Determination of KLH-Induced IgG Titers. Ninety-six-well plates were coated with KLH in PBS, blocked, and serial dilutions of test serum samples were added. Captured anti-KLH IgG titers were detected using HRP-conjugated antibody specific for mouse or monkey IgG (Southern Biotechnology Associates, Birmingham, AL) and 3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The IgG titers were quantitated in a SpectraMax Plus enzyme-linked immunosorbent assay plate reader (Molecular Devices, Sunnyvale, CA). For the mouse KLH study, serum from BALB/c mice collected on day 14 after immunization with KLH was pooled and used as a positive comparator, and the data are expressed as a ratio of the titer of the test serum to the titer of the pooled KLH immunized BALB/c mouse serum.

Data Analysis

Data are expressed as mean ± standard deviation (S.D.).

Pharmacokinetic/Pharmacodynamic Model. A two-compartment model coupled with first-order absorption was used to describe the PK data after subcutaneous or intraperitoneal administration of dAbs (Fig. 1), with the differential equations shown below:

$$\frac{dA_{IP\ or\ SC}}{dt} = -k_a \times A_{IP\ or\ SC} \quad (1)$$

$$V_c\frac{dC_P}{dt} = k_a \times A_{IP\ or\ SC} - (k_{el} + k_{pt}) \times V_c \times C_p + A_T \times \ k_{tp} \quad (2)$$

$$\frac{dA_T}{dt} = k_{pt} \times V_c \times C_p - k_{tp} \times A_T \quad (3)$$

where $A_{IP\ or\ SC}$ and A_T are the amount of the drug at the intraperitoneal or subcutaneous site and the peripheral compartments, respectively; C_p is drug concentration in the central compartment; V_c is the volume of distribution in the central compartment; k_a is the absorption rate constant; k_{pt} and k_{tp} are the transfer rate constants between the central and peripheral compartment; k_{el} is the first-order elimination rate constant. It is important to note that, for fitting the mouse intraperitoneal data, the V_c in eq. 2 becomes the $V_{c,apparent}$ (equal to the V_c divided by the bioavailability after intraperitoneal administration).

At time zero, the following conditions exist for the intraperitoneal or subcutaneous route:

$$A_{IP,\ t=0} = Dose_{IP} \cdot (\text{mouse})$$

$$A_{SC,\ t=0} = Dose_{SC} \times F_{SC} \cdot (\text{monkey})$$

$$A_{P,t=0} = 0$$

where $Dose_{IP}$ and $Dose_{SC}$ are the dose administered via the intraperitoneal and subcutaneous route, respectively; F_{sc} is the bioavailability after subcutaneous administration.

The CD28 RO data in whole blood were linked with plasma drug concentrations using an E_{max} model shown below:

$$RO = \frac{E_{max} * C_p}{EC_{50} + C_p} \quad (4)$$

where E_{max} is the maximum RO (fixed at 100%); EC_{50} is the plasma concentration corresponding to 50% of the E_{max}.

The PD response, measured as the suppression of KLH-induced IgG response in the presence of the anti-hCD28 dAb-001, was fitted using a signal transduction model (Fig. 1) that links the whole blood CD28 RO with the IgG response. In this model, it was assumed that KLH acted on APC to produce KLH-specific APC that subsequently formed an active complex with T-cells. The complex then created an effect signal (E) that transduced to the production of IgG (E_{IgG}). The concentrations of KLH-specific APC and the effect signal over time are defined as follows:

$$\frac{dS_{KLH}}{dt} = -k_{KLH} \times S_{KLH} \quad (5)$$

$$E = k_{effect} \times S_{KLH} \quad (6)$$

where S_{KLH} is the hypothetical concentration of KLH-specific APCs, with an elimination following first-order kinetics (k_{KLH}); k_{effect} is a proportionality rate constant that links the S_{KLH} and the effect signal. At time zero, the following conditions exist for S_{KLH}.

$$S_{KLH,\ t=0} = 30,000$$

In this case, 30,000 is the arbitrary concentration value assigned to KLH-specific APCs. In the presence of anti-hCD28 dAb-001, the effect signal was suppressed due to the blockade of the CD28 receptors (i.e., CD28 RO):

$$E' = k_{effect} \times S_{KLH} \times (1 - RO) \quad (7)$$

The effect signal (E or E'), as a forcing function, then cascaded through a series of five transit compartments to produce the anti-KLH IgG response, which are described using the following equations:

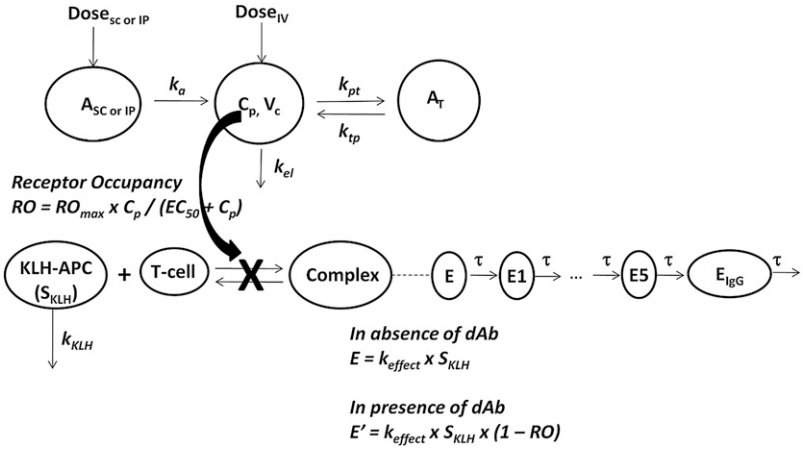

Fig. 1. Schematic representation of pharmacokinetic/pharmacodynamic model that links drug concentrations, receptor occupancy, and keyhole limpet hemocyanin-induced IgG response.

$$\frac{dE_1}{dt} = (E - E_1\ )/\tau \quad (8)$$

$$\frac{dE_2}{dt} = (E_1 - E_2\ )/\tau \quad (9)$$

$$\frac{dE_3}{dt} = (E_2 - E_3\ )/\tau \quad (10)$$

$$\frac{dE_4}{dt} = (E_3 - E_4\ )/\tau \quad (11)$$

$$\frac{dE_5}{dt} = (E_4 - E_5\ )/\tau \quad (12)$$

$$\frac{dE_{IgG}}{dt} = (E_5 - E_{IgG}\ )/\tau \quad (13)$$

where τ is the mean transit time between the transit compartments, E_1 to E_5 refer to the effect signals in various transit compartments. E_{IgG} is the anti-KLH IgG titer.

The PK and PD data from individual animals and various studies were pooled and simultaneously fitted using SAAM II (v1.2, Seattle, WA). Goodness of fit was assessed by the minimization of the objective function, Akaike and Schwarz-Bayesian information criteria, visual inspection of the fitting and residual plots, and the precision of the parameters estimated.

Results

Pharmacokinetic/Pharmacodynamic Modeling of Whole Blood CD28 Receptor Occupancy in Mice. Table 1 summarizes the PK/PD parameters estimated from the simultaneous fitting of the plasma drug concentration and RO data of anti-mCD28 dAb-001 after intraperitoneal administration of 0.08, 0.4, and 2 mg/kg to mice. The fitted versus observed profiles are shown in Fig. 2. The in vivo plasma EC_{50} corresponding to 50% of the CD28 RO in mouse whole blood was estimated to be 1.4 ± 0.088 nM.

TABLE 1
Pharmacokinetic and pharmacodynamic parameters of anti-mCD28 dAb-001 in mice or anti-hCD28 dAb-001 in monkeys

Parameter	Value Estimated (Mean ± S.D.)	
	Mouse	Monkey
PK		
k_a (hour^{-1})	0.28 ± 0.045	0.029 ± 0.0023
k_{el} (hour^{-1})	0.036 ± 0.0036	0.026 ± 0.0017
k_{pt} (hour^{-1})	0.0065 ± 0.0044	0.016 ± 0.0028
k_{tp} (hour^{-1})	0.040 ± 0.0075	0.022 ± 0.0026
V_c (l/kg)	0.077 ± 0.0088[a]	0.033 ± 0.0011
F (%)	–	100 ± 6.1 (SC)
RO		
E_{max} (%)	100 (fixed)	100 (fixed)
EC_{50} (nM)	1.4 ± 0.088	7.6 ± 0.60
PD (KLH-induced IgG)		
k_{KLH} (hour^{-1})	–	0.0033 ± 0.00033
k_{effect} (hour^{-1})	–	2.9 ± 0.39
τ (hour)	–	73 ± 3.7
Baseline IgG response	–	fixed at each dose level[b]

[a] $V_{c,apparent}$ for the mouse data.
[b] Based on the geometric mean of 4 animals at each dose group, the baseline IgG response was fixed at 42, 29, 33, and 121 for the vehicle group, 0.05, 0.5, and 5 mg/kg, respectively.

Pharmacokinetic/Pharmacodynamic Modeling of Whole Blood CD28 Receptor Occupancy and T-cell-Dependent Antibody Response in Monkeys Challenged with Keyhole Limpet Hemocyanin. Figure 3 shows the fitted versus observed drug concentration, RO, and TDAR data following SC administration of anti-hCD28 dAb-001 to cynomolgus monkeys, with the estimated monkey PK/PD parameters summarized in Table 1. The PK, RO, and TDAR data were well described using the PK/PD model developed. To mechanistically account for the delayed onset of the IgG production after the KLH challenge, the number of transit compartments ranging from 2 to 8 was evaluated during the model development, with five compartments yielding the lowest value in terms of the objective function, Akaike and Schwarz-Bayesian information criteria and selected as the final model. More importantly, the extent of the CD28 receptor blockade in whole blood was linked in a proportional manner to the TDAR inhibition using the signal transduction model (eq. 7). With the monkey TDAR data adequately described by the model, it demonstrated the relevance of the RO to the PD response, a sensitive measure of the CD28 receptor blockade in vivo. Consequently, the in vivo plasma EC_{50} for CD28 RO in monkeys was estimated to be 7.6 ± 0.6 nM.

Relevance of In Vitro Mixed Lymphopcyte Reaction Assay to Whole Blood CD28 Receptor Occupancy. Table 2 summarizes the comparisons of the in vitro binding affinity (K_d), in vitro EC_{50} from the MLR assay, and in vitro or in vivo EC_{50} for the CD28 RO generated for anti-mCD28 dAb-001, anti-hCD28 dAb-001, or BMS-931699 in mice, cynomolgus monkeys, or humans. In mice, the in vivo plasma EC_{50} (1.4 ± 0.1 nM) for the CD28 RO was in agreement with the in vitro MLR EC_{50} (1.4 ± 0.6 nM) and the in vitro K_d (1.2 nM, the average of two values) measured by Biacore. In monkeys, the in vivo EC_{50} (7.6 ± 0.6 nM) of anti-hCD28 dAb-001 for the CD28 RO was higher than the in vitro MLR EC_{50} (1.8 ± 1.9 nM), although they may not be meaningfully different given the relatively large variability associated with the MLR EC_{50}. Because cynomolgus monkeys and humans have the same extracellular domain of the CD28 receptor, it was reasonable to expect that the in vitro K_d (0.57 ± 0.43 nM) measured for humans was the same as that for monkeys. In this regard, both in vitro MLR EC_{50} and in vivo RO EC_{50} of anti-hCD28 dAb-001 in monkeys were higher than the in vitro binding affinity.

Similar to what was observed in mice and monkeys, the average human EC_{50} values (5.1 ± 2.3 and 4.4 ± 0.9 nM, respectively) of anti-hCD28 dAb-001 and BMS-931699 for the in vitro CD28 RO were comparable to the in vitro MLR EC_{50} (2.6 ± 1.1 and 2.9 ± 1.2 nM, respectively), but they were generally higher than the in vitro K_d values determined using Biacore (0.57 ± 0.43 and 0.41 ± 0.28 nM, respectively).

Translation of Whole Blood Receptor Occupancy to In Vivo Pharmacodynamic Response. The relationship between the whole blood CD28 RO and the in vivo pharmacodynamic response, measured as the KLH-induced IgG response (or TDAR), was examined in mice and cynomolgus monkeys (Table 3). In mice, after the repeat intraperitoneal dosing (3 times a week for 2 weeks) of anti-mCD28 dAb-001, a significant inhibition (78%) of the KLH-induced IgG response was observed at 0.02 mg/kg, where the average CD28 RO was 69% (99 and 39% at peak and trough, respectively). A complete inhibition was achieved at 0.1 mg/kg, when the average CD28

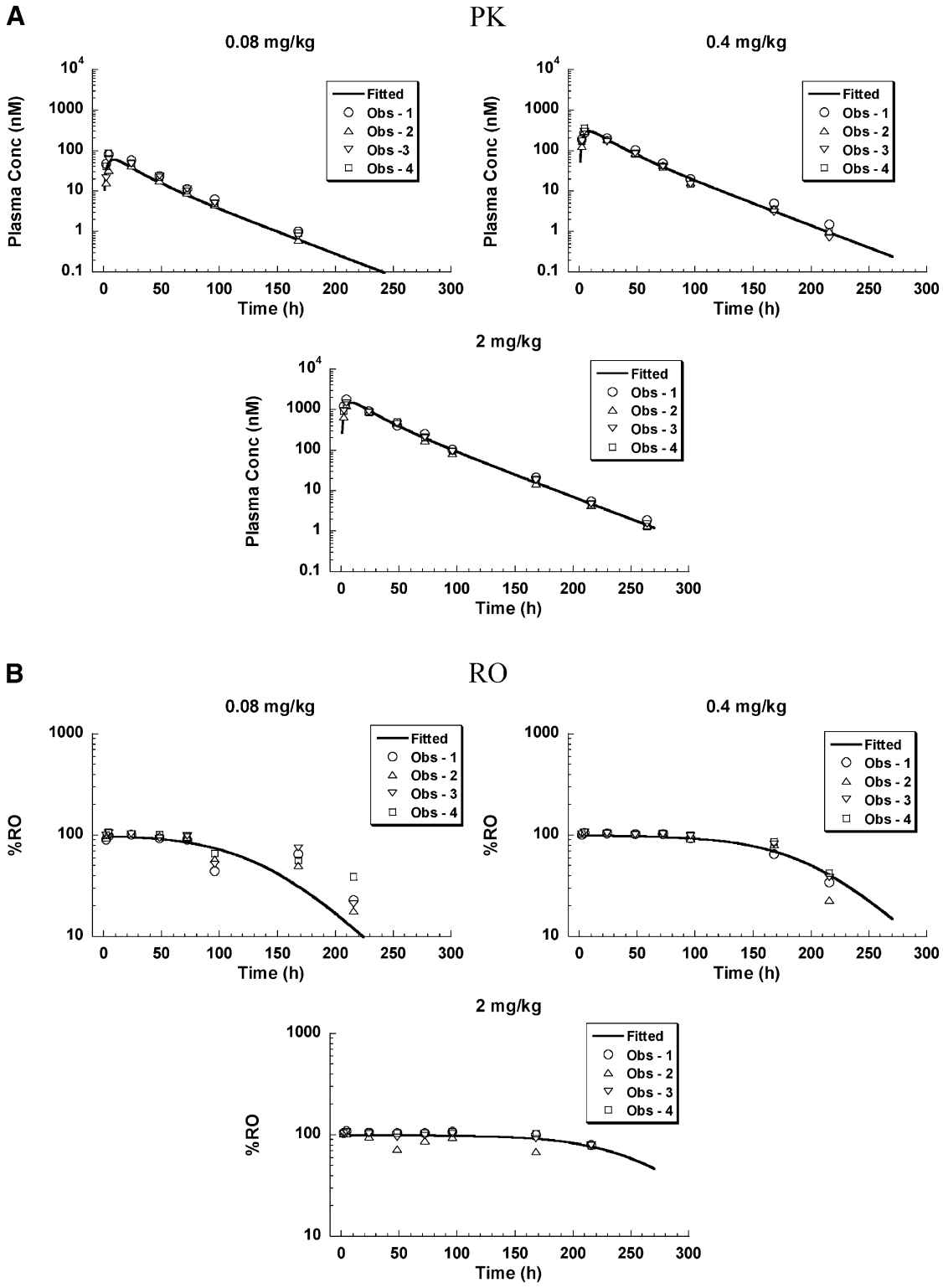

Fig. 2. Fitted versus observed time course data of drug concentrations and in vivo whole blood CD28 receptor occupancy after intraperitoneal administration of anti-mCD28 dAb-001 to mice.

RO was about 100% (102 and 97% at peak and trough, respectively). On the other hand, no inhibition was observed at 0.004 mg/kg when the average CD28 RO was 21% (41 and 0% at peak and trough, respectively). Similarly, after a single subcutaneous dose of anti-hCD28 dAb-001 to cynomolgus monkeys, the KLH-induced IgG response (estimated as the area under the average IgG response-time curve up to day 29) at 0.05, 0.5, and 5 mg/kg was inhibited by ~0, 79, and 97%, respectively. The corresponding average CD28 RO determined from the area under the CD28 RO-time curve over a

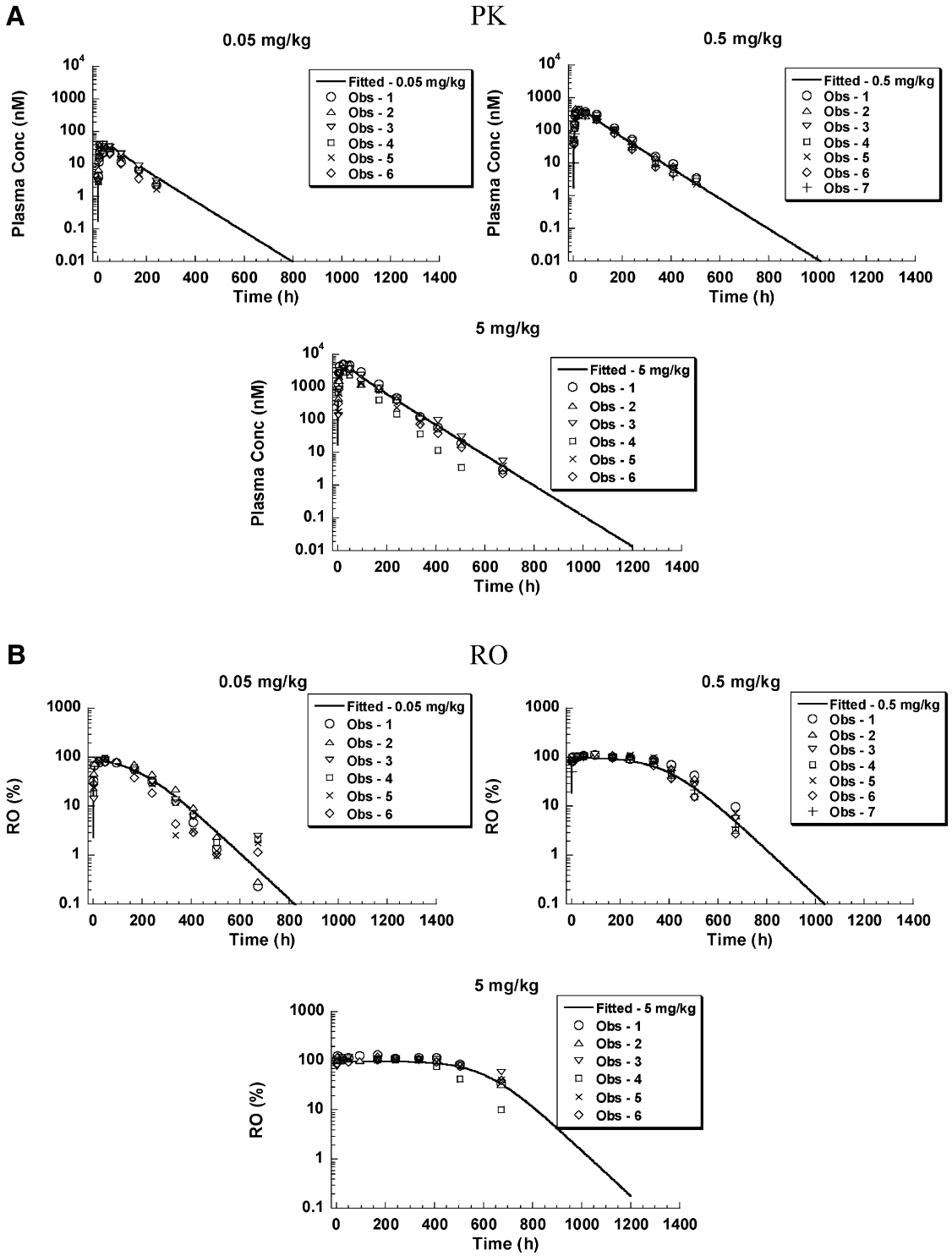

Fig. 3. Fitted versus observed time course data of drug concentrations, in vivo whole blood CD28 receptor occupancy, and keyhole limpet hemocyanin-induced IgG response after subcutaneous administration of anti-hCD28 dAb-001 to cynomolgus monkeys.

similar duration (up to day 28) was 26, 62, and 86%, respectively. Collectively, these data indicate that a CD28 RO of ≤30% is unlikely to produce a significant inhibition of the KLH-induced IgG response, a sensitive PD measure of the CD28 receptor blockade. Furthermore, using the PK/PD model described above, the effect of the CD28 RO in cynomolgus monkeys can be linked in a proportional manner to the inhibition of the KLH-induced IgG response, demonstrating a strong connection between the CD28 RO and the CD28-mediated PD response.

Calculation of MABEL. The aforementioned analysis of in vitro and in vivo preclinical data demonstrated alignment between the in vitro MLR and the CD28 RO as well as the relationship between the extent of the CD28 RO and the in

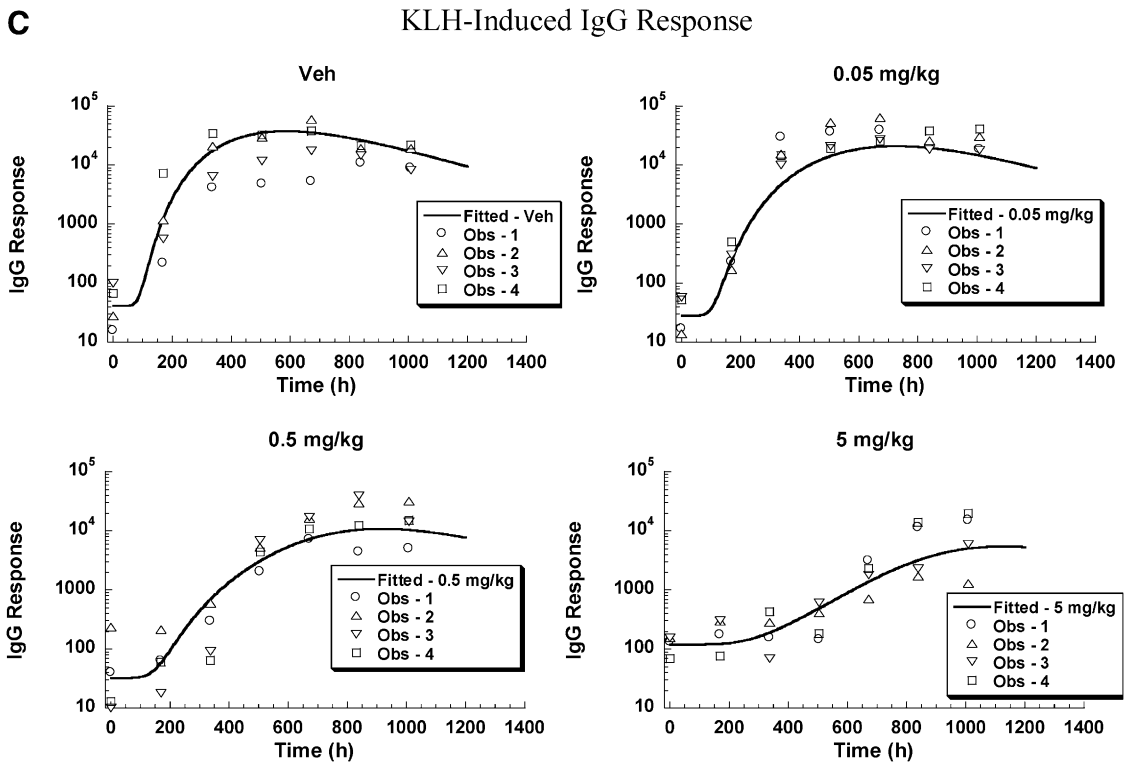

Fig. 3. *(continued)*

vivo pharmacodynamic response. A CD28 RO of ≤30% did not produce any significant inhibition of the KLH-induced IgG response in mice and cynomolgus monkeys. In addition, the CD28 superagonist TGN1412 did not cause a significant cytokine release in an in vitro setting until it reached a CD28 RO of approximately 20% (unpublished data). These data, taken together with the background information on the CD28 receptor as a high-risk target, led us to take a more conservative approach. As a result, an RO of ≤10% was chosen to define the MABEL dose for BMS-931699.

To estimate the CD28 RO of 10%, the EC_{10} value of BMS-931699 from the in vitro human MLR assay was used. The EC_{10} (0.32 nM) from the human MLR assay was then converted to a corresponding human dose using a plasma volume of 0.04 l/kg, which yielded a dose of approximately 0.16 μg/kg. For a 70-kg human subject, this corresponded to a dose of 11 μg (0.011 mg). Therefore, the MABEL dose for BMS-931699 was estimated to be approximately 10 μg (0.010 mg). At this dose, the RO was anticipated to be ≤10%, which would result in minimal or no meaningful biologic response.

Discussion

In the present work, a PK/PD modeling-based approach was employed to determine the in vivo EC_{50} for the whole blood CD28 RO in mice and monkeys as well as linking the CD28 RO to the KLH-induced IgG response in vivo. Through this integrated analysis of all the available in vitro and in vivo preclinical data, a MABEL dose was calculated to mitigate any potential risk associated with targeting CD28, despite the fact that BMS-931699 is an antagonist and monovalently binds to the target. The in vivo EC_{50} (7.6 ± 0.6 nM) of anti-hCD28 dAb-001 derived from the PK/PD modeling of the CD28 RO data in cynomolgus monkeys was comparable to its in vitro EC_{50} (5.1 ± 2.3 nM) in humans, demonstrating strong alignment between in vitro and in vivo despite the values generated in two different species. Furthermore, the human in vitro EC_{50} (4.4 ± 0.9 nM) of BMS-931699 was subsequently proven to be in excellent agreement with its in vivo EC_{50} (4.8 ± 0.8 nM) obtained from human Phase I clinical studies (unpublished data). Generally, it appears that there is a strong correlation in the CD28 RO between in vitro and in vivo.

TABLE 2
Summary of in vitro K_d, in vitro EC_{50} for inhibiting T-cell proliferation in mixed lymphopcyte reaction assay, and EC_{50} for whole blood CD28 RO

	Anti-mCD28 dAb-001	Anti-hCD28 dAb-001	BMS-931699
In Vitro K_d by Biacore (nM)	1.2[a] (mouse, n = 2)	—[b] (monkey)	0.57 ± 0.43 (human, n = 7)	0.41 ± 0.28 (human, n = 5)
In Vitro MLR EC_{50} (nM)	1.4 ± 0.6 (mouse, n = 15)	1.8 ± 1.9 (monkey, n = 16)	2.6 ± 1.1 (human, n = 70)	2.9 ± 1.2 (human, n = 10)
EC_{50} for CD28 RO (nM)	1.4 ± 0.1[c] (mouse, n = 12)	7.6 ± 0.6[c] (monkey, n = 19)	5.1 ± 2.3[d] (human, n = 3)	4.4 ± 0.9[d] (human, n = 10)

[a]Average of two K_d values (0.41 and 1.9 nM).
[b]The extracellular domain of human and monkey CD28 receptors is identical, thus the K_d value in monkeys is expected to be the same as that in humans.
[c]Estimated from PK/PD modeling of in vivo RO data.
[d]In vitro EC_{50}.

TABLE 3
Extent of CD28 RO versus inhibition of T-cell-dependent antibody response

	Mouse			Monkey	
Dose[a]	$RO_{average}$	TDAR Inhibition	Dose[b]	$RO_{average}$	TDAR Inhibition[c]
mg/kg	%	%	*mg/kg*	%	%
0.004	21	0	0.05	26	0
0.02	69	78	0.5	62	79
0.1	100	100	5	86	97

[a]Repeat intraperitoneal administration (3 times a week for 2 weeks).
[b]Single subcutaneous dose.
[c]Estimated as the area under the average IgG response-time curve up to day 29.

A number of methods, in addition to the one described in the *Results*, were also considered in the determination of the MABEL. In particular, the in vitro binding affinity, measured as the dissociation constant K_d from Biacore, could be used to calculate the RO in vivo (Muller and Brennan, 2009). Using the K_d value of 0.41 nM (Table 2) and the total CD28 expression of 0.65 nM (Expert Group on Phase One Clinical Trials, 2006), the plasma concentration that gave rise to 10% CD28 RO was calculated to be 0.1 nM. Assuming a human plasma volume of 0.04 l/kg, the MABEL dose calculated from the K_d was 3.1 μg (Table 4). This value was about threefold less than the MABEL estimated using the in vitro EC_{10} value from the MLR assay. This is not unexpected, given that there is no natural ligand present to compete with binding in the Biacore system, which could theoretically result in a larger in vivo RO EC_{50} than the in vitro K_d value. Also, the Biacore assessment is not a biologically functional measurement (e.g., CD28 receptors immobilized on a chip for binding affinity measurement). In contrast, the in vitro MLR assay may represent a reasonable approximation to the in vivo situation where cell-cell interactions are preserved and the inhibition of the T-cell proliferation reflects the extent of the CD28 receptor blockade. Although arguably the relative proportion of dendritic cells and T cells in the in vitro MLR assay may not entirely reflect the in vivo situation, the general agreement between the in vitro MLR EC_{50} and CD28 RO EC_{50} suggest that the in vitro MLR assay is a reasonable, sensitive way to evaluate the extent of the CD28 receptor blockade (i.e., RO) in vivo.

Another method to calculate the MABEL dose would be to convert the MABEL from animal efficacy studies to an HED based on the body surface area normalization (FDA, 2005; Muller et al., 2009; Muller and Brennan, 2009). In the mice treated with the anti-mCD28 dAb-001, a dose of 4 μg/kg (0.004 mg/kg) resulted in 41% and 0% RO at 24 and 72 hours, respectively, and did not have any effect on KLH-induced IgG responses measured on day 14. Therefore, the 4 μg/kg dose could be considered as the MABEL in mice. In the cynomolgus monkeys treated with the anti-hCD28 dAb-001, a dose of 50 μg/kg (0.05 mg/kg) still produced an 87% RO at 24 hour post dose, thus the MABEL cannot be defined from the study conducted. However, based on the PK/PD model established, one could estimate a hypothetical dose of 0.91 μg/kg that would result in a 10% RO at maximum plasma concentration with no meaningful biologic activities. Accordingly, based on a MABEL dose of 4 μg/kg in mice and a hypothetical MABEL dose of 0.91 μg/kg in monkeys, one could convert them into the HED of 0.32 μg/kg (based on mice) and 0.29 μg/kg (based on monkeys). For a 70-kg human subject, this would result in a dose of 23 and 21 μg based on the MABEL dose in mice and monkeys, respectively. The results are in line with the proposed MABEL dose of 10 μg (within ~2-fold).

In the case of the TeGenero superagonist incident, the dose of TGN1412 that resulted in serious side effects associated with a cytokine release syndrome in normal healthy volunteers was 0.1 mg/kg. Based on retrospective analyses from the Duff report (Expert Group on Phase One Clinical Trials, 2006) as well as others (Muller et al., 2009), it was estimated that, for TGN1412, 10% RO would have been achieved in humans at an approximate dose of 1.5 μg/kg (assuming a K_d of 1.88 nM and total target expression of 0.65 nM), which was more than 60 times lower than the actual starting dose that led to >90% RO and a massive cytokine release. More recently, TGN1412 was administered to human volunteers at very low doses (0.1 to 7 μg/kg) without a cytokine storm (Tabares et al., 2014), further supporting the MABEL concept.

Given the fact that CD28 is a high-risk target, the selection of the MABEL dose as the FIH safe staring dose for BMS-931699 was well justified. The proposed MABEL dose (10 μg) for BMS-931699 was over 1000-fold less than the projected human efficacious dose (14–23 mg for subcutaneous dosing or 21–34 mg for intravenous infusion; unpublished data). Furthermore, the exposure at the proposed starting intravenous dose was projected to be approximately 76,000-fold below that of the NOAEL (15 mg/kg/week i.v. for 5 doses) established in the monkey toxicity study (unpublished data). On the basis of the Food and Drug Administration (2005) guidance, the maximum recommended human starting dose derived from the NOAEL in monkeys, with a 10-fold safety factor applied, was 29 mg or 0.49 mg/kg. In this regard, the proposed starting dose based on the MABEL approach was significantly lower (2900-fold). Importantly, the Phase I studies with BMS-931699 revealed that a single dose comparable to 29 mg led to ≥80% CD28 RO in the clinic (unpublished data), indicating that the traditional toxicology-based NOAEL approach is not suitable to derive a FIH safe starting dose for this high-risk target.

However, the MABEL dose selection is not necessarily the lower, the better. Instead, the ultimate goal of the MABEL-based starting dose is to balance the risks and the benefits and improve the efficiency of clinical trial designs. To do so, the selection of the MABEL dose needs to be data, target, and mechanism driven. It is critical to demonstrate the relevance of in vitro cellular, binding, or RO data to in vivo target-engagement and downstream PD responses in animal species, with the goal of understanding human in vitro data and its in

TABLE 4
Comparison of various approaches in determining MABEL

Approaches	MABEL
	μg/70 kg
In vitro MLR EC_{10}[a]	10
In vitro K_d[b]	3.1
MABEL conversion from mice[c]	23
MABEL conversion from monkeys[c]	21

[a]Calculated as the MLR EC_{10} (0.32 nM) multiplied by the human plasma volume (0.04 l/kg).
[b]The plasma concentration achieving 10% RO was estimated using the equation described by Muller and Brennan (2009), with the in vitro K_d (0.41 nM) and the total CD28 expression (0.65 nM) as inputs. The estimated plasma concentration (0.1 nM) was then multiplied by the human plasma volume (0.04 l/kg) to derive the MABEL.
[c]Calculated based on the body surface area conversion from the MABEL estimated from mice (4 μg/kg) and monkeys (0.9 μg/kg), respectively.

vivo significance. Without sound understanding of the pharmacology of a novel target, simply calculating the MABEL dose based on an in vitro value is not desirable.

In summary, a PK/PD modeling-based approach was employed to leverage all the available in vitro and in vivo preclinical data to determine the MABEL as the safe starting dose of BMS-931699 in a FIH trial. The relevance of the in vitro MLR assay to the whole blood CD28 RO assessment, as well as the relationship between the extent of the CD28 RO and the in vivo PD response (inhibition of KLH-induced IgG response) in mice and cynomolgus monkeys, was demonstrated through an integrated PK/PD analysis. As a result, the MABEL dose for BMS-931699 was calculated to be 10 μg (0.01 mg), with a CD28 RO predicted to be $\leq$10%. This dose was subsequently used as the FIH starting dose of BMS-931699 successfully.

Acknowledgments

The authors thank Yongmi An, James Tamura, and James Bryson of Bristol-Myers Squibb Protein Science and Neil Jaffe, Brian O'Mara, Chris Yonan, and Siegfried Rieble of Bristol-Myers Squibb Biologics Process Development for generating the mouse and human dAb materials used in the work described. The authors also acknowledge Virna Borowski, Xiaoxia Yang, Selena Kansal, and Luisa Salter-Cid at Bristol-Myers Squibb Immunology Discovery for their efforts in the design and conduct of the in vivo mouse studies. In addition, the authors thank Vito Sasseville, Jacintha Shenton, Helen Haggerty, Deborah DeVona, Tammy Bigwarf, Bonnie Wang, and Michael Graziano of Bristol-Myers Squibb Discovery Toxicology and Drug Safety Evaluation for contributions to the in vivo monkey experiments, as well as Murli Krishna from Bristol-Myers Squibb Bioanalytical Sciences for efforts in developing the mouse and monkey dAb assays.

Authorship Contributions

Participated in research design: Yang, Wang, Salcedo, Suchard, Zhang, Rodrigues, and Nadler,

Conducted experiments: Salcedo, Suchard, Xie, Schneeweis, Fleener, and Calore.

Contributed new reagents or analytic tools: Fleener and Calore.

Performed data analysis: Yang and Wang.

Wrote or contributed to the writing of the manuscript: Yang, Wang, Salcedo, Suchard, Shi, Rodrigues, Car, Marathe, and Nadler.

References

European Medicines Agency (2007) Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products. EMEA/CHMP/SWP/28367/07. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002988.pdf

Expert Group on Phase One Clinical Trials (2006). *Expert group on phase one clinical trials: final report*. TSO (The Stationary Office)

Food and Drug Administration (2005) Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers.US Department of Health and Human Services, Bethesda, MD.

Lenschow DJ, Walunas TL, and Bluestone JA (1996) CD28/B7 system of T cell costimulation. *Annu Rev Immunol* **14**:233–258.

Muller PY and Brennan FR (2009) Safety assessment and dose selection for first-in-human clinical trials with immunomodulatory monoclonal antibodies. *Clin Pharmacol Ther* **85**:247–258.

Muller PY, Milton M, Lloyd P, Sims J, and Brennan FR (2009) The minimum anticipated biological effect level (MABEL) for selection of first human dose in clinical trials with monoclonal antibodies. *Curr Opin Biotechnol* **20**:722–729.

Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, Tillou X, Wu G, Reneaudin K, and Hervouet J, et al. (2010) Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med* **2**:17ra10.

Sharpe AH and Freeman GJ (2002) The B7-CD28 superfamily. *Nat Rev Immunol* **2**: 116–126.

Suchard SJ, Davis PM, Kansal S, Stetsko DK, Brosius R, Tamura J, Schneeweis L, Bryson J, Salcedo T, and Wang H, et al. (2013) A monovalent anti-human CD28 domain antibody antagonist: preclinical efficacy and safety. *J Immunol* **191**: 4599–4610.

Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, and Panoskaltsis N (2006) Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* **355**:1018–1028.

Tabares P, Berr S, Römer PS, Chuvpilo S, Matskevich AA, Tyrsin D, Fedotov Y, Einsele H, Tony HP, and Hünig T (2014) Human regulatory T cells are selectively activated by low-dose application of the CD28 superagonist TGN1412/TAB08. *Eur J Immunol* **44**:1225–1236.

Address correspondence to: Zheng Yang, Route 206 & Province Line Rd., Bristol-Myers Squibb Company, Princeton, NJ 08543-4000. E-mail: zheng.yang@bms.com