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REPORT

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Development of a mechanism of action-reflective, dual target cell-based reporter bioassay for a bispecific monoclonal antibody targeting human CTLA-4 and PD-1

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ABSTRACT

T-cell-mediated immunotherapy has generated much excitement after the success of therapeutic biologics targeting immune checkpoint molecules. Bispecific antibodies (BsAbs) that recognize two antigen targets are a fast-growing class of biologics offering promising clinical benefits for cancer immunotherapy. Due to the complexity of the molecule structure and the potential mechanism of action (MOA) that involves more than one signaling pathway, it is critical to develop appropriate bioassays for measuring potency and characterizing the biological properties of BsAbs. Here, we present a dual target, cell-based reporter bioassay for a BsAb that binds human CTLA-4 and PD-1 and targets two subsequent signaling pathways that negatively regulate T-cell activation. This reporter bioassay is capable of measuring the potency of both antigen target arms in one assay, which would not be achievable using two single target bioassays. This dual target reporter bioassay demonstrates good performance characteristics suitable for lot release, stability testing, critical quality attribute assessment, and biological properties characterization of the CTLA-4/PD-1 BsAb. Furthermore, this assay can capture the synergistic effect of anti-CTLA-4 and anti-PD-1 activity of the BsAb. Compared to single target assays, this dual target bioassay could better reflect the potential MOA of BsAbs and could be used for evaluation of other bispecific biologics, as well as antibody combination therapies.

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Introduction

T cells play an essential role in many different types of immune responses that occur in cancer, infection, and autoimmune diseases. The specific interactions between T cell and antigen-presenting cells (APCs) direct T cell function, determine T cell fate, and regulate T cell anti-tumor response.[1,2] Basic research and clinical investigations have uncovered many immune regulatory molecules that regulate T cell functionality. Within such, immune checkpoint molecules play a central role in modulating T cell function through either activating signals (co-stimulatory molecules) or inhibiting signals (co-inhibitory molecules) on T cells. Immune checkpoint-based therapies targeting T cell functionality have generated much excitement after the success of antibody therapeutics, including pembrolizumab and durvalumab, which target programmed cell death protein 1 (PD-1) and the ligand PD-L1, respectively, and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA–4) ipilimumab in melanoma, metastatic urothelial carcinoma, bladder and lung cancer patients.[3–6]

Of the immune checkpoint molecules identified to date, the mechanisms of action (MOA) by which CTLA-4 inhibits T cell function is one of the best understood. CTLA-4, also called CD152, was the first immune checkpoint protein that was successfully targeted by ipilimumab in melanoma patients.[7,8] CTLA-4 is a transmembrane glycoprotein that binds CD80 (B7-1) and CD86 (B7-2) proteins on APCs. CTLA-4 acts as an "off" switch when bound to its ligands.[9,10]

In contrast to CTLA-4, PD-1, also known as CD279, predominantly regulates effector T cell activity within tissues and tumors as opposed to regulating T-cell activation in lymphoid organs.[11] PD-1 is a cell surface receptor that plays an important role in suppressing T cell activity through interaction with its ligands PD-L1 and PD–L2.[12–14] Similar to CTLA-4 signaling, PD-1 binding inhibits T-cell proliferation, and cytokine production such as interferon-γ (IFN-γ), tumor necrosis factor, and interleukin 2 (IL-2) production, and reduces T-cell survival.[15,16]

Despite remarkable success achieved by immune checkpoint blockades in cancer therapy, the majority of patients currently do not benefit from a single-target checkpoint blockade by conventional antibody-based therapeutics.[16] For example, although the PD-1 and CTLA-4 pathways have been translated to clinical applications for several cancer indications, most patients do not respond to a PD-1 or CTLA-4 blockade alone.[17–19] Therefore, antibody combination therapies that target more than one antigen are being recognized as a potential effective strategy for cancer therapies.[20,21] In preclinical animal studies, it has been shown that a combined blockade of CTLA-4 and PD-1 signaling pathways prolonged animal survival in a B16 melanoma model and in a metastatic

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osteosarcoma in K7M2 model, while the efficacy of monotherapy using antibodies that block either CTLA-4 or PD-1 alone is limited.[22,23] In clinical practice, combination treatment with anti-CTLA-4 and anti-PD-1 antibodies has resulted in a higher response rate for some tumors, although treatment-related autoimmune adverse events were also amplified in some patients.[24,25]

In addition to antibody combination therapies that use two single–target (monospecific) monoclonal antibodies (mAbs), bispecific antibodies (BsAbs) that interact with two distinct antigen targets simultaneously could have advantages in modulating two cellular signaling pathways, leading to reprogramming immune effector cells to enhance tumor cell killing.[26] More than 60 BsAbs are in clinical studies and more than 100 BsAbs formats have been reported in the literature.[27–29] Due to the complexity of the molecule structure and the technical problems associated with manufacturing BsAbs, such as stability, pairing, solubility, purity, titer and biological functionalities, it is more challenging to develop manufacturing processes for BsAbs compared to the conventional mAbs. To overcome these challenges, comprehensive analytical methods are needed to evaluate, control, and monitor critical quality attributes of the product to support process development, optimization, and validation. To measure biological activity in particular, a well-designed, robust and MOA-reflective potency assay is needed. In this study, we developed a cell-based dual target reporter bioassay that engages CTLA-4 and PD-1 simultaneously and the subsequent signaling pathways in a single assay. This CTLA-4/PD-1 dual target reporter bioassay captures the synergistic effect of anti-CTLA-4 and anti-PD-1 pathways with performance characteristics that are suitable for lot release and stability testing, as well as for biological property characterization of the CTLA-4- and PD-1-targeted BsAb (CTLA-4/PD-1 BsAb).

Results

Assessment of anti-CTLA-4 activity of CTLA-4/PD-1 BsAb using a CTLA-4 bioassay

To assess whether the anti-CTLA-4 arm of the CTLA-4/PD-1 BsAb has biological activity targeting CTLA-4, we developed a single target CTLA-4 cell-based assay and compared the activity with the conventional anti-CTLA-4 mAb (positive control for anti-CTLA-4 activity). A serial dilution of CTLA-4/PD-1 BsAb or anti-CTLA-4 mAb was incubated with Jurkat cells engineered to express CTLA-4 and a luciferase reporter under the control of NFκB promoter (Jurkat/CTLA-4/NFκB-Luc) in the presence of Raji cells that express B7 proteins (CD80/86) endogenously and anti-CD3 antibody. The anti-CTLA-4 arm of CTLA-4/PD-1 BsAb interacts with the CTLA-4 on Jurkat/CTLA-4/NFκB-Luc cells and blocks CTLA-4 from interacting with CD80 and CD86 on Raji cells. As shown in Figure 1a, the anti-CTLA-4 mAb blocked CTLA-4:CD80/86 interaction that resulted in BsAb or mAb concentration-dependent activation of the NFκB promoter and the luciferase protein expression. Compared to the anti-CTLA-4 mAb, however, the BsAb showed reduced activity with the curve shifting to the right. The EC_{50} values of anti-CTLA-4 mAb and CTLA-4/PD-1 BsAb were 1.007 ng/mL and 6.946 ng/mL, respectively. These results confirmed that CTLA-4/PD-1 BsAb possesses anti-CTLA-4 activity that is less potent than a conventional anti-CTLA-4 mAb.

Assessment of anti-PD-1 activity of CTLA-4/PD-1 BsAb using a PD-1 bioassay

To characterize whether the anti-PD-1 arm of the CTLA-4/PD-1 BsAb has biological activity targeting PD-1, we developed a single target PD-1 bioassay and compared the activity with the conventional anti-PD-1 mAb (positive control for anti-PD-1 activity). A serial dilution of CTLA-4/PD-1 BsAb or anti-PD-1 mAb was incubated with Jurkat cells that were engineered to express PD-1 and a luciferase reporter under the control of nuclear factor of activated T-cells (NFAT) (Jurkat/PD-1/NFAT-Luc) in the presence of Chinese hamster ovary (CHO) cells engineered to express PD-L1 and OKT single-chain variable fragment (scFv) (CHO/PD-L1/OKT3). The anti-PD-1 arm of CTLA-4/PD-1 BsAb or anti-PD-1 mAb blocks PD-1 binding to its target, PD-L1, on the CHO/PD-L1/OKT3 cells. The association of the anti-PD-1 arm of CTLA-4/PD-1 BsAb or anti-PD-1 mAb to PD-1 resulted in the blocking of inhibitory PD-1/PD-L1 pathway that leads to the activation of NFAT and the expression of luciferase protein in the Jurkat/PD-1/NFAT-Luc cell. As shown in Figure 1b, the CTLA-4/PD-1 BsAb showed reduced activity (curve shifted to the right) compared to the conventional anti-PD-1 mAb. The EC_{50} values of anti-

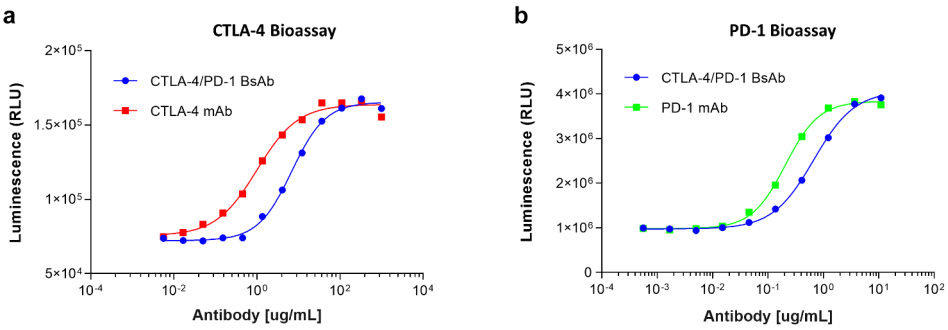

Figure 1. Anti-CTLA-4 activity and anti-PD-1 activity of CTLA-4/PD-1 bispecific antibody. (a) Anti-CTLA-4 activity of CTLA-4/PD-1 BsAb was evaluated in a CTLA-4 cell based assay. CTLA-4/PD-1 BsAb (blue circle) shows anti-CTLA-4 activity that is less potent than an anti-CTLA-4 mAb (red square) as curve shifts to the right. (b) Anti-PD-1 activity of CTLA-4/PD-1 BsAb was tested in a PD-1 cell based assay. CTLA-4/PD-1 BsAb (blue circle) shows anti-PD-1 activity that is less potent than an anti-PD-1 mAb (green square) as curve shifts to the right.

PD-1 mAb and CTLA-4/PD-1 BsAb were 0.206 and 0.674 ng/mL, respectively. These results confirmed that CTLA-4/PD-1 BsAb has anti-PD-1 activity that is less potent than an anti-PD-1 mAb.

Establishment of CTLA-4 and PD-1 co-expressing T cell line

To establish a potency assay that engages two antigen targets in one assay, a cell line that expresses both the target antigens and is responsive to each antigen-regulated signaling is needed. We developed a dual-antigen target-expressing cell line by transfecting the Jurkat cell line with constitutive human CTLA-4 and PD-1 together with a luciferase reporter gene under the control of an IL-2 promoter, which contains both NF-κB and NFAT elements. Figure 2a–c shows the fluorescence-activated cell sorting (FACS) analysis results of the engineered Jurkat cells that co-express CTLA-4 and PD-1 (Jurkat/CTLA-4/PD-1/IL2-Luc). Compared to negative control (parental cells), Jurkat/CTLA-4/PD-1/IL2-Luc cells expressed high levels of CTLA-4 and PD-1 (Figure 2).

Development of a dual target reporter bioassay for CTLA-4/PD-1 BsAb

To examine whether the engineered Jurkat/CTLA-4/PD-1/IL2-Luc cell line is responsive to both anti-CTLA-4 mAb and anti-PD-1 mAb, we designed a dual target reporter bioassay that uses two cell lines. As shown in Figure 2d, the assay uses Jurkat/CTLA-4/PD-1/IL2-Luc cells and CHO/PD-L1/CD80 cells that engages CTLA-4 with CD80, and PD-1 with PD-L1 in the same assay. The cells were incubated with serially diluted antibodies under four different conditions side by side: 1) anti-CTLA-4 mAb alone; 2) anti-PD-1 mAb alone; 3) a 1:1 mixture of anti-CTLA-4 mAb and anti-PD-1 mAb; and 4) CTLA-4/PD-1 BsAb. All test conditions had the same total antibody concentration. For target antigen binding sites (CTLA-4, PD-1), anti-CTLA-4 mAb and anti-PD-1 mAb contain the same binding epitopes as those in CTLA-4/PD-1 BsAb.

As shown in Figure 2e, all treatment conditions showed concentration-dependent drug responses. Compared to CTLA-4/PD-1 BsAb and co-administration treatment of anti-CTLA-4 mAb and anti-PD-1 mAb groups, however, the activity was moderate with either anti-CTLA-4 mAb or anti-PD-1 mAb alone. A strong drug response and synergistic effect was seen for CTLA-4/PD-1 BsAb and co-administration of anti-CTLA-4 mAb or anti-PD-1 mAb with an enhanced upper asymptote compared to the anti-CTLA-4 mAb or anti-PD-1 mAb alone (Figure 2d). The results indicate that the interactions between CTLA-4 and CD80, and PD-1 and PD-L1 were inhibited by either CTLA-4/PD-1 BsAb or co-administration of anti-CTLA-4 mAb and anti-PD-1 mAb. As differentiated from the CTLA-4/PD-1 BsAb-treated group, a steep slope and a hook effect in drug response were seen in the co-administration group (Figure 2e). These results demonstrate that the dual target-expressing cell line is responsive to both CTLA-4 and PD-1 blockage.

Optimization of CTLA-4/PD-1 dual target reporter bioassay

Effects of cell number and assay incubation time

We evaluated the effects of the cell number and the ratio of target cell (Jurkat/CTLA-4/PD-1/IL2-Luc cell) versus APC (CHO/PD-L1/CD80) on the dual target reporter assay. In the presence of 20,000 cells per well of CHO/PD-L1/CD80 cells in a 96-well plate, we tested a range of cell numbers for Jurkat/CTLA-4/PD-1/IL2-Luc cells from 10,000 cells per well to 80,000 cells per well. An increased drug response was seen when the Jurkat cell number increased from 10,000 cells per well to 40,000 cells per well, but no further increase in drug response

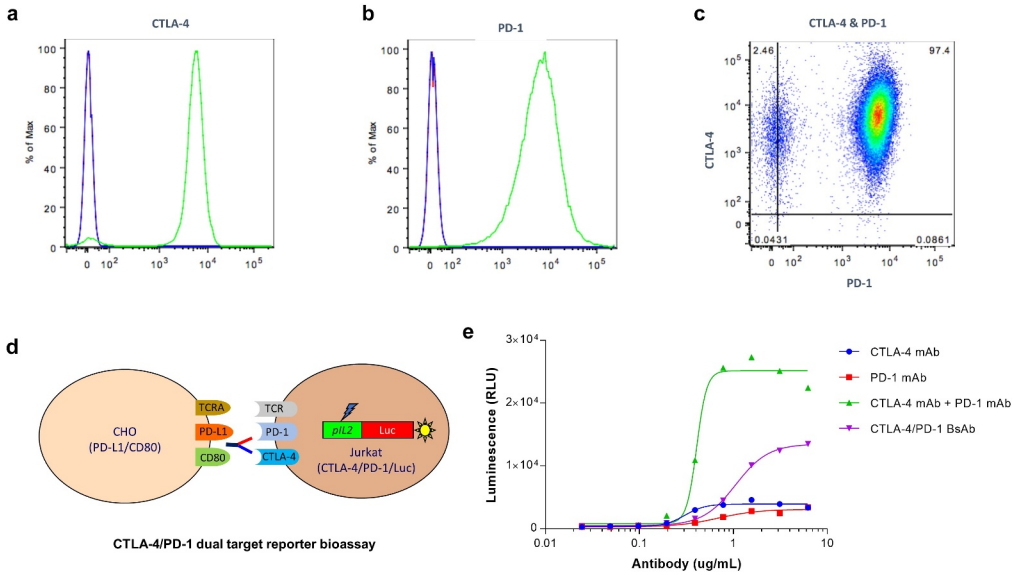

Figure 2. Design and development of dual target reporter bioassay for CTLA-4 and PD-1 blockage. (a) Co-expression of human CTLA-4 and PD-1 in an engineered Jurkat reporter cell line was analyzed by FACS to confirm the expression of human CTLA-4 (green line in a), expression of human PD-1 (green line in b) and co-expression of CTLA-4 and PD-1 on the same cell population (c). (d) Illustration of CTLA-4/PD-1 dual target reporter bioassay; (e) anti-CTLA-4 mAb, anti-PD-1 mAb, CTLA-4/PD-1 BsAb in CTLA-4/PD-1 dual target reporter bioassay. Strong drug responses were seen in CTLA-4/PD-1 BsAb and co-administration of anti-CTLA-4 mAb and anti-PD-1 mAb. A hook effect was seen in the co-administration group (anti-CTLA-4 mAb plus anti-PD-1 mAb). TCR = T cell receptor; TCRA = TCR activator

was seen when the number of target cells was at 80,000 cells/well (Figure 3a). To examine the influence of CHO/PD-L1/CD80 cell number on the assay performance, we tested a range of cell numbers for CHO/PD-L1/CD80 cells from 5,000 cells per well to 40,000 cells per well, in the presence of 40,000 Jurkat/CTLA-4/PD-1/IL2-Luc cells per well. As shown in Figure 3b, an increased drug response with a higher upper asymptote was observed when the number of CHO/PD-L1/CD80 cells were increased.

To evaluate the impact of assay incubation time, the number of Jurkat/CTLA-4/PD-1/IL2-Luc cells and CHO/PD-L1/CD80 cells were set at 80,000 cells per well and 20,000 cells per well, respectively. A concentration-dependent drug response was seen after 3 h of incubation at 37°C. A stronger response (higher upper asymptote) was observed when the incubation time was increased, but a hook effect was seen after a 5–6 h incubation period (Figure 3c).

Assay optimization using DoE

We further conducted multi-factorial design of experiments (DoE) during assay condition optimization. A total of 12 assay runs were conducted to evaluate the following factors: 1) CHO/PD-L1/CD80 cell seeding time; 2) CHO/PD-L1/CD80 cell number; 3) Jurkat/CTLA-4/PD-1/IL2-Luc cell number; and 4) assay incubation time. Each factor was evaluated at a high, medium and low level. As shown in Figure 4, at maximal desirability level for the accuracy across the assay range, signal to noise ratio, lower/upper asymptotes and slope, the optimal assay conditions were determined centered at: 1) 18 h cell seeding for CHO/PD-L1/CD80 cells; 2) 20,000 cells per well for CHO/PD-L1/CD80 cells; 3) 40,000 cells per well for Jurkat/CTLA-4/PD-1/IL2-Luc cells; and 4) 5.5 h assay incubation time.

Qualification of CTLA-4/PD-1 dual target reporter bioassay

The qualification of the CTLA-4/PD–1 dual target reporter bioassay was performed according to the International Conference on Harmonization (ICH) Q2(R1) guidelines. The assay qualification results are summarized in Table 1. The results demonstrate that the assay has good linearity, precision, and accuracy across an assay range from 60–167% relative potency. Figure 5a shows representative concentration-dependent drug response curves obtained by testing CTLA-4/PD-1 BsAb samples at 60, 77, 100, 130, and 167% relative potency levels. A good linear relationship between expected relative potency values and the observed potency values of BsAb was observed with an R-squared value of 0.9994 (Figure 5b). The repeatability (n = 6) and intermediate precision (n = 6) were 5.7 and 7.3%, respectively. The accuracies of the assay across 5 potency levels (60, 77, 100, 130, and 167%) were 105.6 to 110.1% (Table 1).

The dual target bioassay is specific for CTLA-4/PD-1 BsAb. The assay specificity was assessed by testing formulation buffer and three functional irrelevant in-house antibodies side-by-side in the assay. Figure 5c shows that only CTLA-4/PD-1 BsAb demonstrated a concentration-dependent drug response in the dual target reporter bioassay. Neither the formulation buffer nor the three functional irrelevant antibodies made in-house showed drug response.

The ability of CTLA-4/PD-1 dual target reporter bioassay to detect potency changes in stressed CTLA-4/PD-1 BsAb samples was also tested. Using thermal- or ultraviolet (UV)-stressed samples, two independent assay runs were performed for these samples and one reportable potency value per sample was generated. The results show a decrease in relative potency for the thermally stressed and UV-stressed CTLA-4/PD-1 BsAb samples compared to the control (Figure 5d). The sample that was stressed at 60°C for 2 h showed an ~50% decrease in relative potency compared to the control group, suggesting that the CTLA-4/PD-1 dual target reporter bioassay is a stability indicating potency assay.

Characterization of CTLA-4/PD-1 dual target reporter bioassay

Stressed anti-CTLA-4 mAb in CTLA-4/PD-1 dual target reporter bioassay

Next, we examined the activity of anti-CTLA-4 mAb samples with or without thermal stress in the CTLA-4/PD-1 dual target reporter bioassay. Compared to CTLA-4/PD-1 BsAb (as positive control), both intact anti-CTLA-4 mAb and thermal-stressed anti-CTLA-4 mAb showed a dramatically decreased drug response with a low upper asymptote (Figure 6a). The thermal-stressed anti-CTLA-4 mAb showed weaker drug response than the intact anti-CTLA-4 mAb (inset in Figure 6a).

Comparison of low and high affinity anti-PD-1 mAbs in CTLA-4/PD-1 dual target reporter bioassay

We further evaluated the drug response of two in-house made anti-PD-1 mAbs with either low and high affinity binding to PD-1 in the dual target reporter bioassay (Figure 6b). Compared to CTLA-4/PD-1 BsAb, both anti-PD-1 mAbs

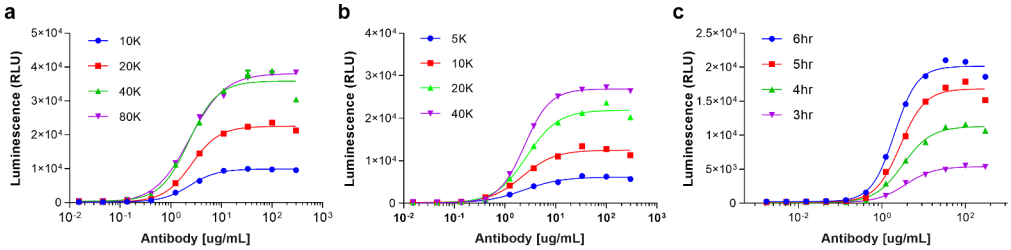

Figure 3. Effect of cell number and assay incubation time on dual target reporter bioassay. (a) Evaluation of Jurkat/CTLA-4/PD-1/IL2-Luc cells, (b) Evaluation of CHO/PD-L1/CD80 cells, (c) Evaluation of assay incubation time.

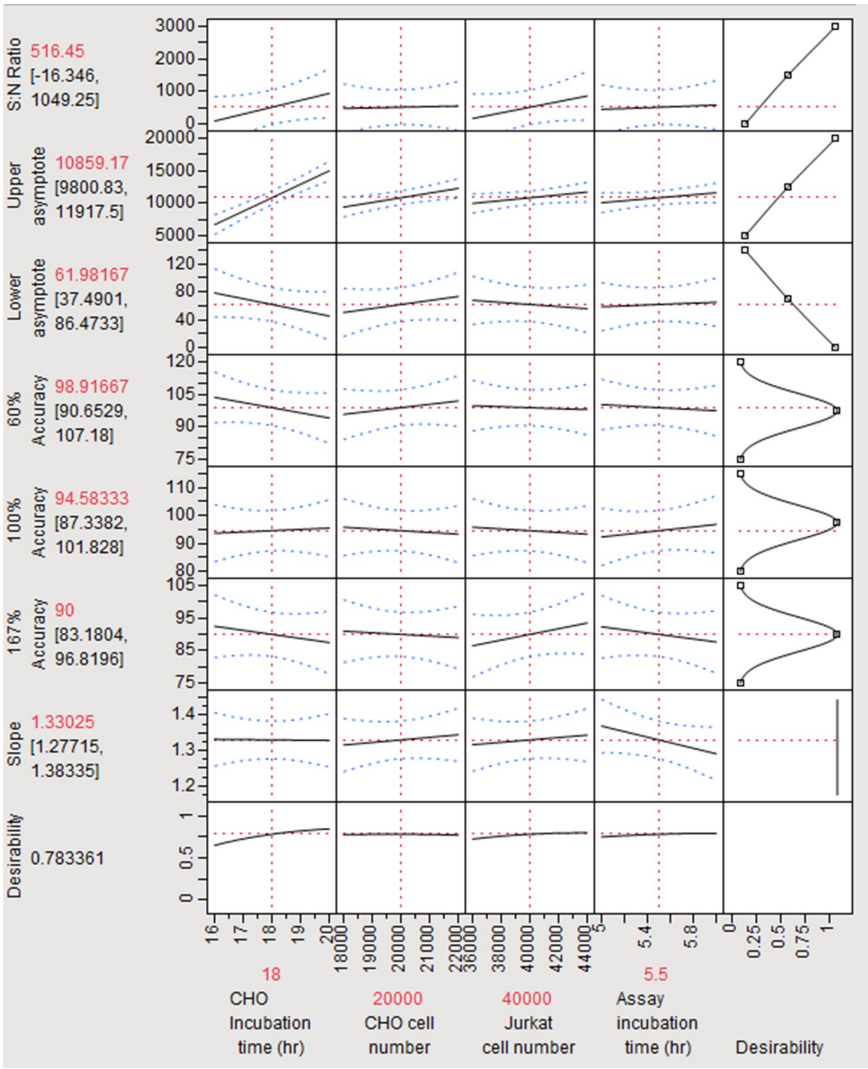

Figure 4. Factorial design of experiments for assay condition optimization. Four experimental factors were evaluated for the CTLA-4/PD-1 dual target reporter bioassay: (1) CHO/PD-L1/CD80 cell seeding time, (2) CHO/PD-L1/CD80 cell number per well, (3) Jurkat/CTLA-4/PD-1/IL2-Luc cell number per well, and (4) assay incubation time.

showed significant decreases in drug response. As expected, the low affinity anti-PD-1 mAb showed weaker response than the high affinity anti-PD-1 mAb (Figure 6b).

Table 1. Summary of method qualification results.

Qualification Parameters		Results
Precision	Repeatability	%GCV = 5.7
	Intermediate precision	%GCV = 7.3
Linearity and Range	R^2	0.9994
	Range	60–167%
Accuracy (5 levels)	60%RP	109.1%
	77%RP	110.1%
	100%RP	107.1%
	130%RP	108.2%
	167%RP	105.6%
Specificity		No response to formulation buffer or irrelevant antibodies
Stability Indicating Potential		Thermo- and UV-stressed samples demonstrated a decrease in potency

GCV = geometric coefficient of variation.

Modified BsAbs in CTLA-4/PD-1 dual target reporter bioassay

We further tested two modified BsAbs that contain either a modified anti-PD-1 antigen-binding fragment (Fab) arm (CTLA-4/dummy BsAb) or a modified anti-CTLA-4 Fab arm (PD-1/dummy BsAb) by replacing the anti-PD-1 Fab arm or the anti-CTLA-4 Fab arm with the Fab portion of R347, a control mAb. As shown in Figure 6c, no meaningful activity was observed in CTLA-4/dummy BsAb, and a strong activity was observed with the PD-1/dummy BsAb treatment. In contrast, intact CTLA-4/PD-1 BsAb showed the strongest activity among the three different treatments. The response with the intact CTLA-4/PD-1 BsAb showed a lower EC_{50} (curve shifts to the left) and somewhat higher upper asymptote compared with the PD-1/dummy BsAb-treated one (Figure 6c). We further compared: 1) co-administration of anti-CTLA-4 mAb plus anti-PD-1 mAb; 2) co-administration of CTLA-4/dummy BsAb plus

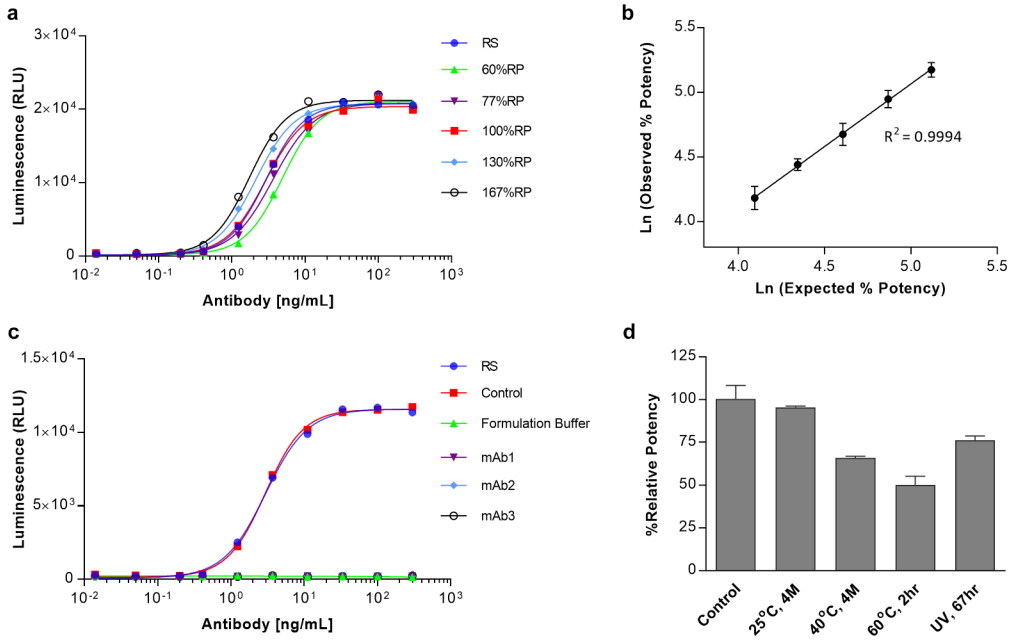

Figure 5. Linearity, specificity, and stability indicating potential of CTLA-4/PD-1 dual target reporter bioassay. (a) Concentration-response of PD-1/CTLA–4 BsAb at different potency levels, (b) linear regression line across assay range 60–167% relative potency, (c) specificity testing, and (d) relative potency of thermal- or UV-stressed PD-1/CTLA4 BsAb samples.

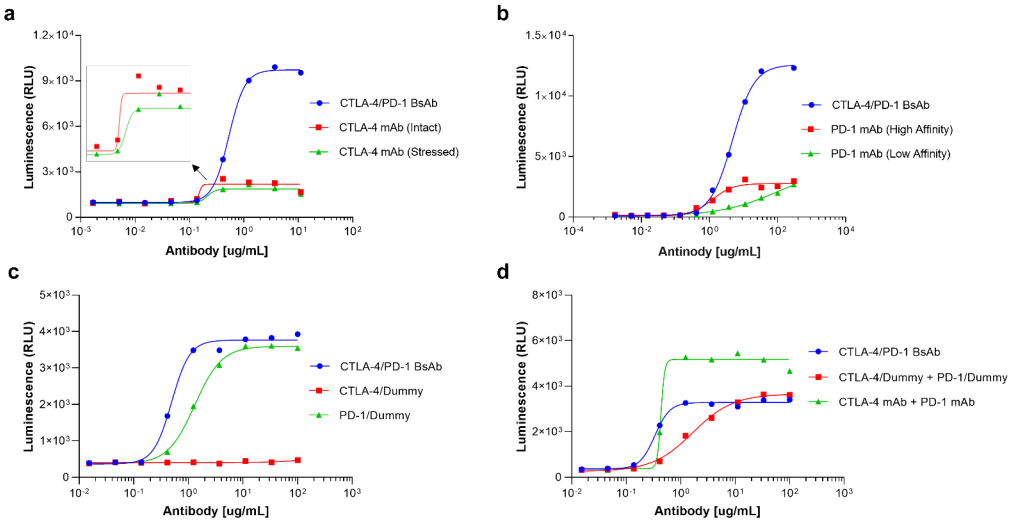

Figure 6. Comparison of monospecific antibodies, intact BsAb and modified BsAb in CTLA-4/PD-1 dual target reporter bioassay. (a) Intact anti-CTLA-4 mAb and thermal-stressed anti-CTLA-4 mAb were tested side-by-side with CTLA-4/PD-1 BsAb in CTLA-4/PD-1 dual target reporter bioassay; (b) High affinity anti-PD-1 mAb and low affinity anti-PD-1 mAb were tested side-by-side with CTLA-4/PD-1 BsAb in CTLA-4/PD-1 dual target reporter bioassay; (c) Modified BsAbs were tested in CTLA-4/PD-1 dual target reporter bioassay; (d) Co-administration of modified BsAbs was tested in CTLA-4/PD-1 dual target reporter bioassay.

PD-1/dummy BsAb; and 3) intact BsAb. The results demonstrated a weaker response with co-administration of CTLA-4/dummy BsAb plus PD-1/dummy BsAb compared to intact BsAb (higher EC_{50}) or co-administration of anti-CTLA-4 mAb plus anti-PD-1 mAb (higher EC_{50} and reduced upper asymptote) (Figure 6d). These results suggest that the CTLA-4/PD-1 dual target reporter assay can distinguish different formats of Fab arms (anti-CTLA-4, anti-PD-1) in both separate and co-administration settings.

Mis-pair/half-hole samples in CTLA-4/PD-1 dual target reporter bioassay

During protein engineering and manufacturing process development, one of the main technical challenges for the BsAb format is to achieve the correctly paired assembly of four unique polypeptide chains.[30,31] To overcome this challenge, sensitive analytical tools are needed. The BsAb used in this study is an asymmetric bispecific antibody. We examined whether the CTLA-4/PD-1 dual target reporter bioassay is capable of detecting mis-paired species. Figure 7 shows the

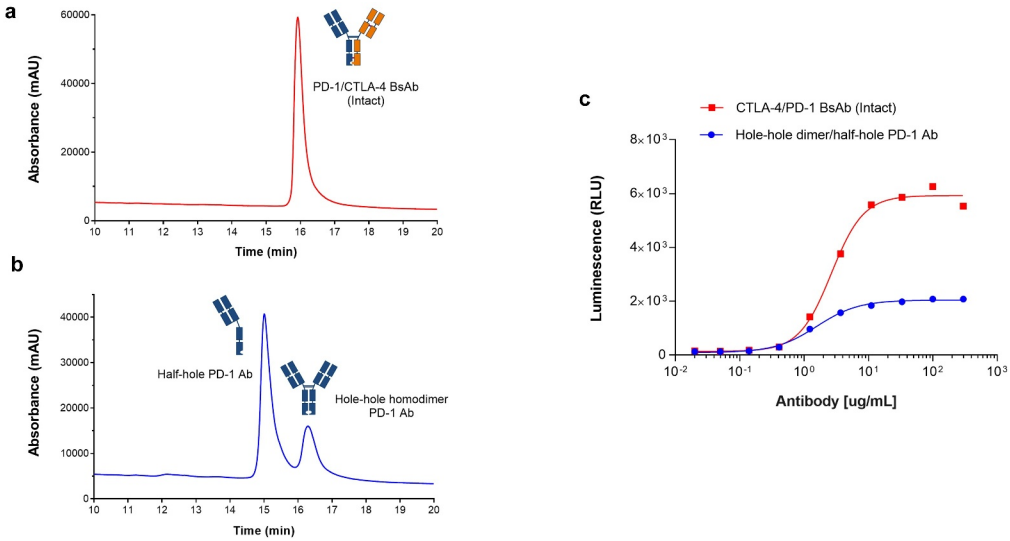

Figure 7. LC-MS analysis and comparison of PD-1/CTLA4 BsAb and mis-pair samples in CTLA-4/PD-1 dual target reporter bioassay. (a) Subunits generated from correctly paired CTLA-4/PD-1 BsAb; (b) Subunits generated from LC swap mis-pair/half-hole species; (c) Activity test results of intact and mis-paired/half-hole CTLA-4/PD-1 BsAb in CTLA-4/PD–1 dual target reporter bioassay.

results of LC-MS analysis for the intact CTLA-4/PD-1 BsAb, the PD-1 half-hole antibody as the main species and a mis-paired sample that contains the PD-1 hole-hole dimer antibody as a minor species. We tested both the intact BsAb sample and the mis-paired/half-hole anti-PD-1 antibody sample in the CTLA-4/PD-1 dual target reporter bioassay side-by-side. As expected, the mis-pair sample (hole-hole dimer)/half-hole anti-PD-1 antibody showed a decreased drug response with a dramatically reduced upper asymptote compared to the intact BsAb sample. These results suggest that the CTLA-4/PD-1 dual target reporter assay requires the engagement of both functional antigen target arms (CTLA-4, PD-1) in the same assay. The results indicate that the dual target reporter bioassay can be used for analyzing the integrity of the molecule and characterizing mis-paired species in CTLA-4/PD-1 BsAb samples.

Discussion

T cell co-signaling receptors play an important role in T cell functionalities, such as cell proliferation, subset differentiation, effector function and survival.[1,32] Immunomodulatory BsAbs targeting T cell co-signaling pathways could be crucial for developing effective tumor immunotherapy. To date, a large number of BsAb drug candidates that use diverse approaches to target T cells co-stimulatory and co-inhibitory signaling pathways have been reported in the literature.[28,29] This diversity is the result of BsAbs that contain multiple functional components in the antibody molecule, such as Fab, Fc domains, scFv, and receptor ligands.[29,33] Due to the complexity of molecule structure, it has been highly challenging to establish reproducible and MOA-reflective bioassays for measuring the potency and characterizing biological properties of BsAb. Here we report a quality control suitable dual target bioassay for measuring the potency of CTLA-4/PD-1 BsAb. The results demonstrate the ability of this dual target bioassay to assess anti-CTLA-4 and anti-PD-1 activity, as well as to capture the synergistic effect of the PD-1 and CTLA-4 blockades by either CTLA-4/PD-1 BsAb or co-administration of anti-CTLA-4 mAb and anti-PD-1 mAb.

Mechanisms of T cell co-inhibition are of considerable importance for cancer immunotherapy.[34,35] There is a strong scientific rationale for concurrent CTLA-4 and PD-1/PD-L1 blockades due to the distinct roles of CTLA-4 and PD-1 in immune regulation, as the two pathways are nonredundant immune checkpoints.[36,37] Through developing two single target bioassays, the CTLA-4 bioassay and the PD-1 bioassay, we confirmed that the CTLA4/PD-1 BsAb used in this study possesses both anti-CTLA-4 and anti-PD -1 activities. Although these conventional single target bioassays could be used for assessing CTLA-4 and PD-1 blockage activity of CTLA-4/PD-1 BsAb, these single target assays are not capable of evaluating the potential interaction (such as synergistic effect) when both antigen targets are blocked simultaneously. The data shown in this study indicate the importance of engaging both antigen targets, CTLA-4 and PD-1, in the same assay. The results suggest that simultaneous blockade of both CTLA-4 and PD-1 pathways, either by CTLA4/PD-1 BsAb or co-administration of anti-CTLA-4 mAb and anti-PD-1 mAb, leads to enhanced T-cell activation compared to CTLA-4 or PD-1 inhibition by anti-CTLA-4 mAb or anti-PD-1 mAb alone.

It is not fully understood yet why co-administration has higher potency on the dual target cell-based assay than CTLA4/PD-1 BsAb. One possible explanation is that, compared to CTLA-4/PD-1 BsAb in which there is only one arm each binding to PD-1 and CTLA-4, bivalent single-target antibodies contain two arms in each antibody that bind the same antigen (PD-1, or CTLA-4) at the same time. Association between the target antigens (PD-1, CTLA-4) with two identical arms each in bivalent single-target antibodies could lead to faster cellular internalization of PD-1 and CTLA-4 than those of BsAb in which the two arms bind two different antigens. As both CTLA-4 and PD-1 are inhibitory receptors, a faster cellular internalization of single-target antibody-antigen complex

in co-administration could result in stronger blockades of inhibitory signal from PD-1 and CTLA-4, leading to a stronger activation of target cells and ultimately to higher potency. Compared to the conventional single target CTLA-4 and PD-1 bioassays, the dual target bioassay shown in this study is more reflective of the potential MOA of CTLA-4/PD-1 BsAb. Our results provide additional evidence of the separate roles of CTLA-4 and PD-1 immune checkpoints in regulating T cell functionality, which could contribute to anti-tumor immune responses.[36] Although the underlying mechanism is not fully understood, the combinatorial T cell modulation through CTLA-4 and PD-1 signaling pathways could enhance the motility and migration, as well as the activation state, of T cells into and within tumors.[38]

While the single target CTLA-4 and PD-1 cell-based assays we developed could be used for monitoring the anti-CTLA-4 and anti-PD-1 activity of CTLA-4/PD-1 BsAb, having one dual target cell-based potency assay (CTLA-4/PD-1 dual target bioassay) instead of two separate single–antigen target potency bioassays in the quality control system is more economical when considering the resources for critical reagents, method validation, assay transfer, and life-cycle management. The assay qualification results indicate that this dual target potency assay is a robust assay with good linearity, precision, and accuracy over the assay range 60–167% relative potency. The assay has stability-indicating potential, as a dramatic potency drop was observed in thermal and UV stressed test samples (Figure 6d). Deceases in potency due to thermal- or UV-stress are expected. These stress conditions induce deamidation and oxidation in the complementarity-determining regions, as well as conformation changes of the CTLA-4/PD-1 BsAb. After heat-stress treatment at 60°C for 2 h, for example, the protein would have been unfolded, resulting in the potency loss, as the differential scanning calorimetry T_{onset} is ~ 55°C for this molecule. The assay trends observed confirm that this assay has consistent performance, with less than 5% failure rate (data not shown).

In addition to potency measurement, the CTLA-4/PD-1 dual target potency assay is useful for assessing the molecule integrity and critical quality attributes of CTLA-4/PD-1 BsAb. The assay is able to detect the changes of either antigen target arm, as shown in this study using thermal-stressed anti-CTLA-4 mAb samples, anti-PD-1 mAb samples that have different PD-1 binding affinities, and modified and mis-paired BsAbs. Compared to either CTLA-4/PD-1 BsAb or co-administration of modified BsAbs with dummy Fab arms, co-administration with anti-CTLA-4 mAb and anti-PD-1 mAb showed the strongest drug response (Figure 7d). However, a steep slope and a hook effect in response curve were seen in co-administration with anti-CTLA-4 mAb and anti-PD-1 mAb. These results suggest that such combination treatment may require a unique dosing strategy to achieve an optimal therapeutic window with appropriate drug response.

In contrast to the CTLA-4/PD-1 dual target potency assay that is currently in use for lot release and stability testing, the two single target bioassays shown in this study (CTLA-4 bioassay, PD-1 bioassay) could be useful as characterization assays for assessing the change of each target binding domain (anti-CTLA-4 Fab, anti-PD-1 Fab) during BsAb product and manufacturing process development. Thus, this study is a demonstration of one dual target potency assay for a dual target BsAb, which could be potentially applicable to other bispecific antibodies or the combination therapy using two monospecific mAbs.

Materials and methods

Materials

Recombinant CTLA-4/PD-1 BsAb, modified BsAb materials, anti-CTLA-4 mAb, high affinity anti-PD-1 mAb and low affinity anti-PD-1 mAb were generated using CHO mammalian cells at AstraZeneca (Gaithersburg, MD). UV-stressed sample was generated following ICH Guideline Q1B by exposing CTLA-4/PD-1 BsAb to UV (320– 400 nm) plus cool white light (400–800 nm) for 67 h. Various cell culture reagents were from Thermo Fisher Scientific (Madison, WI). Fetal bovine serum (FBS) was from Hyclone. Steady-Glo™Luciferase Assay System was from Promega (Madison, WI).

Cell lines and cell culture

CHO/PD-L1/OKT3 was engineered at AstraZeneca. Cells were cultured in DMEM with 10% FBS supplemented with Non-essential Amino Acids, 0.5 mg/mL Geneticin and 0.4 mg/mL Hygromycin B. Jurkat/NFAT-Luc/PD-1 (Promega, Madison, WI) were cultured in RPMI1640 with 10% FBS supplemented with 0.5 mg/mL Geneticin and 0.1 mg/mL Hygromycin B. Raji cells (ATCC, Rockville, MD) were cultured in RPMI 1640 with 10% FBS. Jurkat/NFkB-Luc/CTLA-4 cell line was engineered at AstraZeneca. Cells were cultured in RPMI1640 with 10% FBS supplemented with sodium pyruvate and 2 µg/mL puromycin.

CHO/PD-L1/CD80 (Promega, Madison, WI) were cultured in F12(Ham's) nutrient supplemented with 0.25 mg/mL Geneticin, 0.05 mg/mL Hygromycin B, 0.01 mg/mL Blasticidin.

Generation of dual target cell line expressing CTLA-4/PD-1/IL2-luciferase reporter

Jurkat/IL2-Luc/CTLA-4 cells were transduced with lentivirus vector encoding for human PD-1 gene. The stable pool was maintained in complete cell culture medium (RPMI 1640 supplemented with 10% FBS) with sodium pyruvate, MEM non-essential amino acids, sodium pyruvate, 0.25 mg/mL geneticin and 0.05 mg/mL Hygromycin B and 0.1 µg/mL puromycin.

FACS analysis

CTLA-4/PD-1/IL2-Luc Jurkat cells were incubated with 0.5 µg/mL Alexa Fluor 647 anti-human CTLA-4 antibody (Cat# 349919, Biolegend, San Diego, CA) and with 0.25 µg/mL PE anti-human PD-1 antibody (Cat# 329906, Biolegend, San Diego, CA) for 30 min at 4°C in FACS buffer (DPBS with 2% FBS). Isotype controls were used as negative controls to differentiate nonspecific background signal from specific antibody

signal. All of the data were acquired using LSRII Flow Cytometry Analyzer (BD biosciences, Franklin lake, NJ) and were analyzed using FlowJo software.

Single target reporter assay for measuring anti-CTLA-4 activity

This assay uses two cell lines: a Raji cell line that endogenously express B7 proteins (CD80, CD86) and a Jurkat cell line that was engineered to express CTLA-4 and a luciferase reporter gene under the control of NFkB promoter (Jurkat CTLA4/NFkB-Luc). In the presence of anti-CD3 antibody as co-stimulator, the association of antibody and CTLA-4 on Jurkat CTLA4/NFkB-Luc cells results in concentration-dependent activation of the NFkB transcription factor and the expression of luciferase protein. The amount of luminescence that is proportional to Jurkat cell activation was quantified in an Envision plate reader (PerkinElmer, Waltham, MA) after reaction with Steady-Glo luciferase substrate.

Single target reporter assay for measuring anti-PD-1 activity

This assay uses a CHO cell line carrying human PD-L1 (CHO/PD-L1) and a Jurkat line that was engineered to express human PD-1 and luciferase gene under the control of NFAT (Jurkat PD-1/NFAT-Luc). The association of antibody test sample, CHO/PD-L1 cells and Jurkat PD-1/NFAT-Luc cells results in concentration-dependent expression of the luciferase protein (Jurkat cell activation). The amount of luminescence was quantified in an Envision plate reader after reaction with Steady-Glo luciferase substrate.

CTLA-4/PD-1 dual target reporter bioassay

The CTLA-4/PD-1 dual target cell-based reporter bioassay for CTLA-4/PD-1 BsAb was developed using two cell lines: a CHO cell line that was engineered to express human CD80 and PD-L1 (CHO/CD80/PD-L1) and a Jurkat cell line that was engineered to express human CTLA-4 and PD-1 (Jurkat/CTLA-4/PD-1/IL2-Luc). After cells were incubated with a serial dilution of antibodies, binding of antibodies to the respective antigen targets (CTLA-4/PD-1) on the Jurkat/CTLA-4/PD-1/IL2-Luc cells resulted in concentration-dependent expression of the luciferase protein. The amount of luminescence was quantified in an Envision plate reader after reaction with Steady-Glo luciferase substrate.

Liquid chromatography–mass spectrometry

Liquid chromatography–mass spectrometry (LC-MS) measurements of the mass of intact antibodies were acquired by using an ultra–high-performance LC system and a Synapt G2 mass spectrometer (Waters, Milford, MA). A PLRP-S column (4,000 Å, 8 μm, 150 × 2.1 mm; Agilent, Santa Clara, CA) was used for reverse–phase separation. Mobile phases A and B were 0.05% trifluoroacetic in water and acetonitrile, respectively. Samples were eluted using a linear gradient. The mass spectrometer instrument was operated using the following parameters: ESI positive ionization, sensitivity mode, capillary voltage 3.2 KV, source temperature 140□, sampling cone 40 KV, extraction cone 4 KV, desolvation temperature 350°C. Mass spectra were collected at an m/z range of 850–4,000. Molecular mass was obtained through deconvolution of the mass data using the MaxEnt I software package.

Data analysis

The dose response curve was generated using a four parameter semi-logistical curve model through SoftMax Pro software (Molecular Device, San Jose, CA). EC_{50} values represent the concentration of antibody at which half-maximal activation was observed. After assessing similarity between reference standard and test samples, the percent relative potencies of the test samples were assessed by dividing the EC_{50} value of the reference standard by the EC_{50} value of each sample and multiplying by 100.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. All authors are or were employees (or intern) of AstraZeneca at the time this work was performed.

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Abbreviations

BsAb	Bispecific antibody
mAb	Monoclonal antibody
MOA	Mechanism of action
APC	Antigen-presenting cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
PD-1	Programmed cell death protein 1

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