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Development of NanoLuc Bridging Immunoassay for Detection of Anti-Drug Antibodies

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ABSTRACT

Anti-drug antibodies (ADAs) are generated in-vivo as an immune response to therapeutic antibody drugs and can significantly affect the efficacy and safety of the drugs. Hence, detection of ADAs is recommended by regulatory agencies during drug development process. A widely accepted method for measuring ADAs is “bridging” immunoassay and is frequently performed using enzyme-linked immunosorbent assay (ELISA) or electrochemiluminescence (ECL) platform developed by Meso Scale Discovery (MSD). ELISA is preferable due to widely available reagents and instruments and broad familiarity with the technology; however, MSD platform has gained wide acceptability due to a simpler workflow, higher sensitivity, and a broad dynamic range but requires proprietary reagents and instruments. We describe the development of a new bridging immunoassay where a small (19kDa) but ultra-bright NanoLuc luciferase enzyme is used as an antibody label and signal is luminescence. The method combines the convenience of ELISA format with assay performance similar to that of the MSD platform. Advantages of the NanoLuc bridging immunoassay are highlighted by using Trastuzumab and Cetuximab as model drugs and developing assays for detection of anti-Trastuzumab antibodies (ATA) and anti-Cetuximab antibodies (ACA). During development of the assay several aspects of the method were optimized including: (a) two different approaches for labeling drugs with NanoLuc; (b) sensitivity and dynamic range; and (c) compatibility with the acid dissociation step for improved drug tolerance. Assays showed high sensitivity of at least 1.0 ng/mL, dynamic range of greater than four log orders, and drug tolerance of >500.

1. Introduction

The first antibody to be approved by FDA for therapeutic use was Muromonab [1], an anti CD3 mouse monoclonal antibody, for prevention of kidney transplant rejection. Being a mouse monoclonal antibody, it elicited a strong immune response in the patients including development of ADAs and resulted in severe side effects. Muromonab was followed by the approval of the first chimeric antibody (Abciximab) in 1995, the humanized antibody Daclizumab in 1999, and finally, the first fully human antibody (Adalimumab) in 2003. With improvements in antibody structure, the rate of adverse immune responses dropped from >70% for Muronomab to less than 10% for fully human antibodies. Nevertheless, the presence of even low concentrations of ADAs can have a serious impact on the efficacy, pharmacokinetics profile, and safety of the drugs. ADAs can form immune complexes with the drug and cause rapid clearance and reduced bioavailability. Neutralizing ADAs, which are a subset of total ADA response, can reduce the drug efficacy by preventing the drug from binding to the target. In light of such significant impact of ADAs on patient health, regulatory bodies such as the FDA and EMA require strict monitoring of immunogenicity during drug development, clinical testing, and post launch[2, 3].
A variety of formats for ADA detection have been proposed including the bridging immunoassay, affinity capture elution (ACE) assay, solid-phase extraction with acid dissociation (SPEED), precipitation and acid dissociation (Panda) assay, and antigen binding test (ABT) [4-12]. Among these, the bridging immunoassay is most frequently used and involves incubating samples containing ADAs with a ‘bridging mixture’, which is a mixture of drug labeled with a biotin for capture and the same drug labeled with a separate tag used for detection. ADAs make bridges with the two differently labeled drug molecules in solution and form bridging complexes. These complexes are subsequently captured on a streptavidin plate via the biotin tag, and are quantitated using the detection tag [11, 13-16]. Bridging immunoassays are however, susceptible to the presence of free drug in the sample, which will compete with the labeled drug for formation of bridging complexes and will reduce the signal or can even cause false negative results. Tolerance for free drug in such cases is improved by incorporating an acid-dissociation step to dissociate ADA-Drug immune complexes followed by neutralization in the presence of bridging mixture. During neutralization, some of the ADAs form bridge complexes and are captured on the streptavidin plates and detected.

Bridging immunoassays are frequently performed on a traditional ELISA platform or on electrochemiluminescence (ECL) platform developed by MSD. With the traditional ELISA format, high sensitivity is achieved by using a bridging mixture containing drug labeled with biotin along with Digoxigenin labeled drug for detection [17-19]. After capture on the Streptavidin plate, bridge complexes are detected by incubation with an anti-Digoxigenin antibody labeled with HRP. This approach has two different incubation and washing steps and requires a secondary antibody labeled with HRP. Direct labeling of drugs with HRP has been tried to simplify the workflow but the resulting assays were less sensitive [5, 20]. Unlike ELISA, the ECL platform involves direct labeling of drug with electrochemiluminescent labels (SULFO-TAG) for detection, therefore, eliminating the secondary antibody incubation step, and simplifying the protocol (one washing step). In addition, electrochemiluminescence signal results in a sensitive assay (around 1.0 ng/mL) with at least three-log order dynamic range, and a better drug tolerance [21, 22]. However, the ECL platform requires specialized and proprietary equipment and so there is a desire for alternative assay formats, which have the performance advantages and simple workflow of the ECL platform, but can be performed on widely available ELISA platforms.

In this paper, we describe the development of a bridging immunoassay based on a traditional ELISA format where NanoLuc luciferase is used as detection label. NanoLuc is a small (19kDa) monomeric enzyme, which produces a bright stable glow type light, 100 fold brighter than traditional luminescent reporters like Firefly luciferase and Renilla luciferase [23, 24]. In addition, NanoLuc can be genetically fused with variety of proteins including antibodies and as a result, several novel biological applications have been developed including bioluminescence resonant energy transfer (BRET) for protein-protein interactions and highly sensitive ELISAs [25-27]. We hypothesized that NanoLuc will bring unique capabilities to bridging immunoassays including; (a) drugs can be labeled recombinantly with NanoLuc, and may result in improved assay performances compared to the use of chemically labeled drug-HRP conjugate; (b) if drug labeled with NanoLuc can meet desired assay specification that will eliminate the use of labeled secondary antibody and simplify the workflow; and (c) the bright luminescence signal from NanoLuc will provide high sensitivity and broad dynamic range seen in the luminescence-based MSD platform. Although, recombinant labeling of drugs is a key advantage of the NanoLuc, a novel method for chemical labeling of drugs with NanoLuc was also developed and the assay performances of two differently labeled drugs were similar.

To test our hypothesis, we selected Trastuzumab and Cetuximab drugs as model drugs and evaluated NanoLuc bridging immunoassay for detection of anti-Trastuzumab antibodies (ATA) and anti-Cetuximab
antibodies (ACA). There were two reasons for selecting these model systems; first, sequences for Trastuzumab (anti HER2) and Cetuximab (anti EGFR) are available in the public domain, which allowed us to make recombinant fusions of drugs with NanoLuc and evaluate them as detection reagents. Second, ATA and ACA were commercially available for use as positive controls to optimize NanoLuc bridging immunoassays. With these two model systems, we demonstrate a sensitivity of 1.0 ng/mL and a broad dynamic range of four log orders for human serum samples spiked with ATAs and ACA. Furthermore, the assay was optimized for high drug tolerance and at FDA recommended assay sensitivity of 100 ng/mL, can tolerate >500 fold excess of free drug.

2. Materials and methods

2.1. Labeling of Trastuzumab and Cetuximab with biotin

Trastuzumab and Cetuximab were labeled with amine reactive long chain biotin (ThermoFisher Scientific) using the manufacturer’s suggested protocol. Briefly, drugs were dialyzed into 100 mM bicarbonate buffer (pH 9.6) and reacted with 20 molar excess of amine reactive biotin for 1h. Unreacted biotin was removed by Zeba desalting column (ThermoFisher Scientific). Concentrations of biotin labeled drugs were calculated by measuring absorbance at 280nm.

2.2. Recombinant drug-NanoLuc conjugates

Trastuzumab and Cetuximab fused with NanoLuc at the C-terminus of the heavy chain were custom ordered from Absolute Antibody (Oxford, UK). Fusions were made using publically available Trastuzumab and Cetuximab sequences with expression in HEK293. For purification, a HisTag was added after NanoLuc and purification was done using Ni²⁺ column. Protein A or G columns typically used for antibody purification were not used due to adverse effect of low pH elution on NanoLuc activity [26]. Non-denaturing SDS-PAGE gel was used to characterize the NanoLuc conjugated drugs. In rest of the paper, Trastuzumab and Cetuximab recombinantly labeled with NanoLuc are mentioned as Trastuzumab-rNanoLuc and Cetuximab-rNanoLuc respectively.

2.3. Chemical labeling of Trastuzumab and Cetuximab with NanoLuc

2.3.1. Expression and purification of HisTag-NanoLuc-HaloTag

Genetic fusion of NanoLuc with HaloTag was achieved by combining existing NanoLuc and HaloTag sequences (Promega Corp.) separated by a short Gly-Ser-Ser-Gly linker. Purification was facilitated by transferring the fusion using flanking Sgfl and Xba1 sites into a modified pF1K Flexi vector (Accession Number AY753577) which added an N terminal 6His purification tag with the sequence Met-Lys-His-His-His- His- Ala-Ile-Ala.

Glycerol stock of E. Coli, expressing HisTag-NanoLuc-HaloTag fusion protein was used to inoculate 50 mL starter cultures, which were grown overnight at 37 °C in LB media containing 25 µg/mL kanamycin. Starter cultures were diluted 1:100 into 500 mL fresh LB media, containing 25 µg/mL kanamycin, 0.12% glucose, and 0.2% rhamnose. Cultures were grown for 22-24 h at 25 °C. Cells were pelleted by centrifugation (10,000 rpm) for 30 min at 4 °C and re-suspended in 50 mL PBS. 1 mL protease inhibitor cocktail (Promega), 0.5 mL RQ1 DNase (Promega), and 0.5 mL of 10 mg/mL lysozyme (Sigma) were added, and the cell suspension was incubated on ice with mild agitation for 1 h. Cells were lysed by sonication at 15% power at 5 sec intervals for 1.5 min (3 min total) and subsequently centrifuged at 10,000 rpm for 30 min at 4 °C. Supernatant was collected and protein purified using HisTag columns.
(GE), following the manufacturer’s recommended protocol. Protein was eluted using 500 mM imidazole, dialyzed in PBS, characterized using SDS-PAGE gel and was >95% pure.

2.3.2. **Covalent attachment of HisTag-NanoLuc-HaloTag to Trastuzumab and Cetuximab**

Trastuzumab and Cetuximab were labeled with amine reactive HaloTag Succinimidy1 Ester (O4) Ligand (Promega), using protocol similar to that used for biotin labeling. Drugs activated with HaloTag ligand were incubated for at least 2 h with four molar excess of HisTag-NanoLuc-HaloTag fusion protein to allow covalent attachment. Non-denaturing SDS-PAGE gel was used to characterize the NanoLuc conjugated drugs. In rest of the paper, Trastuzumab and Cetuximab chemically labeled with HisTag - NanoLuc-HaloTag are mentioned as Trastuzumab-cNanoLuc and Cetuximab-cNanoLuc respectively.

2.4. **Antigen down ELISA for comparing activity of unlabeled and labeled drugs**

Recombinant HER2 and EGFR (Acrobiosystems) proteins were diluted to 2.0 µg/mL in 100 mM bicarbonate buffer (pH 9.6) and 50 µl was added to the wells of white high binding 96 well plates (Corning). The plates were incubated for 1-2 h at room temperature (RT) with mild agitation to allow proteins to adsorb to the plate. The plates were subsequently blocked by 1 h incubation with Super Block (Thermo Fisher Scientific). Labeled and unlabeled Trastuzumab or Cetuximab were serially diluted into Super Block and added to HER2 or EGFR coated plates (50 µl/well) respectively. Plates were incubated for 1 h at RT with mild agitation. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST), then incubated for 1 h with anti-Human-IgG (H + L)-HRP (horseradish peroxidase) conjugate (Southern Biotech) diluted 1:5000-fold in Super Block. Plates were washed three times with PBST, then once with PBS and Super Signal ELISA Pico Chemiluminescent HRP substrate (Thermo Fisher Scientific) was added and plates read on a Tecan Genios Pro plate reader.

2.5. **Affinity measurements of ATAs and ACA using Octet**

Affinity measurements for three ATAs and one ACA were performed by Neoclone (Madison, WI) on the Octet QK (Pall, CA) using streptavidin biosensor. Biotinylated drugs-Trastuzumab or Cetuximab-were immobilized on the streptavidin Biosensor by incubating sensors with 15 µg/mL of antibodies for 20 min at 1000 rpm. Typical immobilization levels were 1.2 nm for Cetuximab and 2.8 nm for Trastuzumab. Affinity measurements for ATA or ACA were determined by running association and dissociation at seven concentrations between 60 and 0.9 μg/mL. Sample dilutions were made in PBS pH 7.4. Association was measured for 900 sec and dissociation phase was for 3600 sec. Data were fitted to a 1:1 interaction model using ForteBio data analysis software 6.4.1.2. ATAs and ACA used in these experiments were: (a) ATA1 (Abnova# MAB11130); (b) ATA2 (BIO-RAD# HCA168); (c) ATA3 (BIORAD# HCA166); (d) ACA1 (Abnova# MAB11129).

2.6. **NanoLuc bridging immunoassay in absence of free drug**

Human serum samples spiked with ATA or ACA were added (25 µL/well) to a 96-well non-binding plate (Corning). Human serum used in these experiments was pooled human male serum (Sigma# 4522). A stock solution of bridging mixture containing equal concentration of biotin and NanoLuc labeled drug was prepared in phosphate buffer saline (PBS). For example, 5.0 µg/mL of bridging mixture has 5.0 µg/mL each of biotin labeled drug and NanoLuc labeled drug. Bridging mixture was added (25 µL/well) to the samples and plates incubated for 1-2 h at room temperature in order for bridge complexes to form. Samples were subsequently transferred to a white 96 well high capacity streptavidin plate.
(Thermo Fisher Scientific) and further incubated for 2 h to capture the bridge complexes. Wells were washed three times with PBST (PBS containing 0.05% Tween20) followed by addition of NanoLuc Glo reagent to detect captured bridge complexes. Luminescence signal was read on a Tecan GeniosPro instrument.

2.7. NanoLuc bridging immunoassay with acid dissociation for detection of ATA and ACA in presence of excess drug

Human serum samples spiked with ATA or ACA at 100 ng/mL and increasing concentrations of unlabeled Trastuzumab or Cetuximab, respectively (0-100 µg/mL) were incubated for 2 h at room temperature. This step allows formation of immune complexes similar to one present in real samples where free drugs are present along with ADAs. Samples were subsequently acidified by adding one volume of human serum sample and four volumes of 300 mM acetic acid and incubating for 1 h to dissociate immune complexes formed in previous step. In a separate 96 well non-binding plate (Corning), equal volumes of acidified sample, 1M Tris (pH 8.5) neutralization buffer, and bridging mixture were added and incubated for 2 h to simultaneously neutralize the acidified samples and form the bridge complexes with labeled drug present in bridging mixture. Mixture was subsequently transferred to high capacity streptavidin plates and processed as described in previous section.

2.8. Data analysis

To calculate lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) of NanoLuc bridging immunoassay, plots were fitted to a 4-parameter equation with 1/Y^2 weighing function (GraphPad Prism 7). Original concentrations of the positive controls used in the assay were compared to the values obtained by interpolating from the fitted graphs. LLOQ and ULOQ were lowest and the highest concentrations respectively where back fitted values were within 20% of the actual value and %CVs were less than 20% of the replicate readings. Limit of detection (LOD) was calculated as concentration that gave signal above the mean plus three standard deviation of the signal from a negative sample. In addition, LOD of the negative sample was also used for purposes of calculating drug tolerance. Drug tolerance is defined as a ratio of drug to ADA concentration, which gives signal above LOD.

For this technology demonstration work, a single pooled human serum sample was used as negative control, and mean and standard deviation of analytical replicates were used for LOD and drug tolerance determination. However, for a validated immunogenicity assays, negative serum samples from 20-50 naïve human subjects, and use mean and the standard deviation from these biological replicates will be needed to establish cut point for the purposes of drug tolerances [2, 28].

Results

The goal of this study was to evaluate the use of NanoLuc luciferase for ADA detection using the bridging immunoassay format shown in Figure 1. Trastuzumab and Cetuximab were used as model drugs and ADA immunoassays were optimized for detection of three different ATAs and one ACA. Briefly, drugs were labeled with biotin and with NanoLuc and mixed in equal amount to make a bridging mixture. Human serum samples containing ATA or ACA are mixed with respective bridging mixture to form bridge complexes, which are captured on a white streptavidin plate, washed, and quantitated using NanoLuc luminescent signal. Key steps during the study were: (a) labeling and characterization of Trastuzumab...
and Cetuximab with biotin and NanoLuc; (b) characterization of three different monoclonal ATAs and one monoclonal ACA used as positive controls for assay optimization; (c) optimization of bridging mixture to obtain maximum sensitivity and dynamic range; and (d) determining the tolerance of the assay to the presence of free drug.

Fig. 1. Schematic of NanoLuc bridging immunoassay.

3.1. Preparation and characterization of labeled drugs

Two critical components for a sensitive NanoLuc bridging immunoassay are biotin labeled drug for capturing bridge complexes and NanoLuc labeled drug for detection. Biotinylation of antibodies has been extensively reported and the standard protocol was followed in this study for labeling two drug molecules with biotin. For labeling of drugs with NanoLuc, both the recombinant and chemical approaches were used. For chemical labeling, a novel approach using HaloTag technology [29, 30] was utilized for a covalent and oriented attachment of NanoLuc to the drugs (Fig. 2A). This is a two-step process in which the drug is first chemically activated with small HaloTag ligand (M.wt: 509) using well-established amine chemistry similar to that used for biotinylation. In the second step, purified His-NanoLuc-HaloTag fusion protein is added to the activated drug for oriented and covalent attachment. During optimization, various ratios of His-NanoLuc-HaloTag to drug were tested and a ratio of four gave the best results. Labeled drugs were characterized using a non-denaturing SDS-PAGE gel (Fig. 2C) where unlabeled Trastuzumab and Cetuximab (Lane 1 & 2) give a single band around 150 kDa and upon chemical labeling (Trastuzumab-cNanoLuc and Cetuximab-cNanoLuc), multiple high molecular weight bands appear; corresponding to drug molecules with varying number of His-NanoLuc-HaloTag molecules. Most of the drug is converted into NanoLuc conjugates, indicating a high efficiency of labeling method. A small amount of His-NanoLuc-HaloTag (~50 kDa band) remains unreacted (estimated to be less than 20% of the starting amount) based on which we estimated an average of three His-NanoLuc-HaloTag molecules per drug molecule and an average molecular weight of 300kDa.

Recombinant drug-NanoLuc fusions (Trastuzumab-rNanoLuc and Cetuximab-rNanoLuc) were also generated (Fig. 2B). Both, expressed very well and could be easily purified at >95% purity as confirmed by non-denaturing SDS-PAGE (Figure 2C, Lane 5 and 6). Moreover, a single band at higher molecular weight indicates a homogeneous population of two NanoLuc per drug molecule (m.wt: ~186 kDa) highlighting a key benefit of the recombinant approach over the chemical conjugation approach.
Fig. 2. Schematic of drug labeled chemically (A) and recombinantly (B) with NanoLuc. (C) Non-denaturing SDS-PAGE gel of unlabeled Trastuzumab and Cetuximab (Lanes 1 and 2), Trastuzumab, and Cetuximab labeled chemically with NanoLuc (Lane 3 and 4) and recombinantly with NanoLuc (Lanes 5 and 6).

Functionality of the drug may be affected after labeling if the labels are present close to the antigen binding site, or the drug becomes structurally unstable due to the presence of the labels [31]. Any such impact on Trastuzumab and Cetuximab after labeling with biotin or NanoLuc (chemically or recombinantly) was investigated using antigen down ELISA method (Fig. 3A). HER2 or EGFR are coated on the plate, followed by addition of increasing concentration of Trastuzumab and Cetuximab (labeled and unlabeled) respectively. The amount of captured Trastuzumab and Cetuximab are detected using HRP labeled anti-Human antibody. For Trastuzumab, the binding affinities of unlabeled and biotin labeled drug were similar but a decrease in the relative affinities of NanoLuc labeled antibodies (recombinant as well as chemical) were seen by the rightward shift in dose-response curves (Fig. 3B). For Cetuximab the binding affinities of unlabeled, biotin labeled, and NanoLuc labeled drug were very similar (Fig 3C). An assumption in the assay is that the detection antibody will bind equally well to unlabeled drug and drug labeled with various tags. In a previous study [32] we saw that Trastuzumab was more sensitive to labeling than Cetuximab but this had minimal impact on downstream assays, so no further optimization was performed.
Fig. 3. (A) Schematic of antigen down ELISA used for comparing activities of unlabeled drugs and drugs labeled with biotin and NanoLuc. Dose response curves for (B) unlabeled Trastuzumab and Trastuzumab labeled with biotin or NanoLuc (C) unlabeled Cetuximab and Cetuximab labeled with biotin or NanoLuc. Each data point is the average and standard deviation of three replicate readings. Standard bars are too small and not visible in the plot. Data were fitted to a 4-parameter logistic regression equation.

3.2. Characterization of positive control ADA antibodies

In patients, ADA response is polyclonal in nature with varying amounts of antibodies of different specificities and affinities. As a result, it is not possible to have a reference material for developing quantitative ADA immunoassays. The FDA suggests the use of either monoclonal or polyclonal antibodies, developed in-house or available commercially, as positive controls for establishing assay sensitivity, dynamic range and other performance parameters. It is known that the performance of the ADA immunoassay will depend on the affinities and binding site of the positive controls [5, 33]. Therefore, a variety of ADAs, three different ATAs and one ACA, were used as positive controls. In
addition, $K_D$ values of all four positive controls were measured on Octet platform to correlate affinities and assay performances (Table 1). ATA2 and ATA3 are anti-idiotype human antibodies which were generated from Fab fragments using HuCAL platform [34]. Affinities for both fragments are in sub nanomolar range (as reported by manufacturer) and Fab fragment used for ATA2 is a strong binder compared to that used for ATA3 (by a factor of 20 fold). $K_D$ values of full length ATA2 and ATA3 have higher affinities compared to Fab fragment by a factor of 80 and 280 fold respectively with a similar 20 fold difference. Higher affinities of full length IgG format is not surprising, and in fact, fold increase of up to ~4000 have been reported [35]. Unlike ATA2 and ATA3, ATA1 and ACA1 are mouse monoclonal antibodies generated using traditional approaches using $F(\text{ab})_2$ as antigen. Binding sites for these antibodies are not known and they also had lower affinities compared to anti-idiotype antibodies.

### Table 1. Affinities of three ATAs and one ACA used for optimization of NanoLuc bridging immunoassay

| ATAs | $K_D$ (M) reported | $K_D$ (M) | $K_D$ (M) |
|------|------------------|----------|----------|
| Anti-Trastuzumab antibody (ATA) | ATA1 Mouse mAb generated using $F(\text{ab})_2$ fragment of Trastuzumab as antigen (Abnova; Clone# 5A4) | 6.36 x 10^{-11} | |
| | ATA2 Anti-idiotypic Human IgG1 (Bio-Rad; Clone# AbD18018) | 0.02 x 10^{-9}* | 2.16 x 10^{-12} |
| | ATA3 Anti-idiotypic Human IgG1 (Bio-Rad Clone# 16712) | 0.4 x 10^{-9}** | 1.43 x 10^{-12} |
| Anti-Cetuximab antibody (ACA) | ACA1 Mouse mAb generated using $F(\text{ab})_2$ fragment of Cetuximab (Abnova; Clone# 6D4) | 1.02 x 10^{-10} | |

* Affinities reported are for monovalent Fab format before they were converted to full length IgG.

### 3.3. Optimization of NanoLuc bridging immunoassay

After characterizing the individual assay components, the next step was to optimize the assay workflow. Key steps in the assay as depicted in Figure 1 were, (a) choosing optimum amount of bridging mixture; (b) choice of streptavidin plate for the capture of bridge complexes; and (c) incubation time for the formation and capture of bridge complexes. High binding capacity white Streptavidin plate (Pierce) and 1-2 h of incubation time for formation and capture of bridge complexes were used in this study based on earlier reports [18]. The amount of bridging mixture was optimized to obtain a wide dynamic range, high sensitivity, and high drug tolerance.

#### 3.3.1. Influence of bridging mixture on assay performance

For the two model drugs (Trastuzumab and Cetuximab), bridging mixtures containing labeled drugs at 1.0, 5.0 and 10.0 µg/mL were prepared. For this study, drugs labeled recombinantly with NanoLuc were used and molar ratio of biotin to NanoLuc labeled antibody was (1:0.8). ATA1 and ACA1 were spiked into undiluted normal human serum (at concentrations ranging from 0.5 ng/mL to 20 µg/mL) and mixed with the respective bridging mixture at a 1:1 ratio (25 µl of each) in 96 well non-binding plates. Samples were incubated for 1-2 h to allow formation of bridge complexes and then transferred to high capacity streptavidin plates to capture the bridge complexes followed by detection using NanoLuc tag. A linear relationship between concentration and signal was observed for both ATA1 and ACA1 (Fig. 4A and 4C) over a wide concentration range. Interestingly, for both ATA1 and ACA1 detection, a significant hook
effect (signal decreases upon increase in analyte concentration) is observed with 1.0 µg/mL of bridging mixture and happens because the excess analyte saturates the labeled drug and prevents bridge formation. Hook effect with 5.0, and 10 µg/mL of bridging mixture is not apparent because the highest concentration of ATA1 and ACA1 used in these experiments were 20 µg/mL and not high enough to trigger decrease in signal. Signal over background (S/B) plots of the same data (Fig 4B and 4D) reveals significant differences between two systems. For Trastuzumab, S/B ratio drops with increase in master mix concentration because, increase in non-specific background signal is higher compared to increase in specific signal. For Cetuximab, S/B ratio is not only significantly higher than Trastuzumab but also shows limited influence of master mix composition due to relatively low background even at higher master mix concentration. To calculate various assay parameters, data for 5.0 µg/mL of bridging reagent were fitted to a four parametric equation and a LLOQ of less than 1.0 ng/mL and a broad dynamic range of almost four log orders of magnitude were obtained (Table 2).

Similar results (Table 2) were obtained, when experiments were repeated using bridging mixtures containing drugs chemically labeled with NanoLuc (molar ratio of biotin to NanoLuc antibodies = 1.0:0.5) This is not surprising because the activities of drugs labeled with NanoLuc using two different methods were similar when tested using antigen down ELISA (Fig. 3). Although, HaloTag based labeling overcomes some of the problems of traditional chemical labeling such as random attachment and inactivation of reporter enzyme it still has the limitation of yielding a heterogeneous mixture of labeled drugs and random placement of labels on the drug, which may result in batch-to-batch variations. Therefore, subsequent assays were performed using drugs labeled recombinantly with NanoLuc.

Table 2. Summary of NanoLuc bridging immunoassays for detection of ATA1 and ACA1 antibodies using 5.0 µg/ml of bridging reagents

| Assay Parameter | Trastuzumab labeled with NanoLuc | Cetuximab labeled with NanoLuc |
|-----------------|----------------------------------|-------------------------------|
|                 | Recombinantly (Trastuzumab-rNanoLuc) | Chemically (Trastuzumab-cNanoLuc) | Recombinantly (Cetuximab-rNanoLuc) | Chemically (Cetuximab-cNanoLuc) |
| LOD             | 0.6 ng/mL                        | 0.6 ng/mL                     | 0.3 ng/mL                          | 0.6 ng/mL                      |
| LLOQ            | 0.6 ng/mL                        | 1.2 ng/mL                     | 0.6 ng/mL                          | 0.6 ng/mL                      |
| ULOQ            | 5.0 µg/mL                        | 5.0 µg/mL                     | 10.0 µg/mL                         | 10.0 µg/mL                     |
Fig. 4. Dose response curve for ATA1 and ACA1 spiked into human serum. Assay was performed using bridging mixture at 1.0, 5.0, and 10.0 µg/mL. (A) and (C) are the luminescence value for ATA and ACA respectively and each data point is the average and standard deviation of three replicate readings. Standard bars are too small and not visible in the plot. (B) and (D) are the signal over background ratio (S/B) for the ATA and ACA respectively. Data obtained using 5.0 µg/mL of bridging reagent were fitted to a 4-parameter logistic regression equation to calculate various assay parameters shown in Table 2.

In the previous experiment, similar sensitivities and dynamic ranges for both ATA1 and ACA1 were obtained because both were mouse monoclonal antibodies raised against F(ab)_2, and had similar affinities (Table 1). To understand if antibodies with different specificities and affinities would impact assay performances, we focused on three different anti Trastuzumab antibodies (Table 1) and generated dose response curves using 5.0 µg/mL of bridging mixture (Fig. 5 and Table 3). Both ATA2 and ATA3 are anti-idiotype antibodies but ATA2 is a stronger binder which likely results in higher sensitivity (0.6 ng/ml) compared to ATA2 (4.9 ng/ml). On the other hand, ATA3 has a higher ULOQ of at least 20 µg/ml compared to ~5.0 ng/mL for ATA2. Unfortunately, the relationship between the K_D of the positive control and assay performance does not hold true when comparing ATA1 and ATA2, which have similar assay performances even though their K_D values are significantly different. Our results seem to indicate that the sensitivity and the dynamic range of the bridging immunoassay is a complex interplay of affinity.
and binding site and cannot be predicted a-priori. Previous reports have alluded to this limitation and the FDA guidelines also acknowledge the limitation that positive standards are different from real samples and values reported for real samples using a specific control is relative and not absolute [2, 33].

**Table 3.** Assay performance of NanoLuc bridging immunoassay for detection of ATA1, ATA2, and ATA3 using 5.0 µg/mL of bridging reagents

| Assay Parameter | ATA1 | ATA2 | ATA3 |
|-----------------|------|------|------|
| LOD             | 0.6 ng/mL | 0.6 ng/mL | 4.9 ng/mL |
| LLOQ            | 0.6 ng/mL | 0.6 ng/mL | 4.9 ng/mL |
| ULOQ            | 5.0 µg/mL | 5.0 µg/mL | 20.0 µg/mL |

**3.3.2. Drug tolerance of NanoLuc bridging immunoassay**

Assays sensitivities were so far calculated by spiking ATAs and ACA into the drug free human serum samples. However, biologic drugs are dosed at very high concentrations (1-10 mg/kg of body weight) and have very long half-life (several weeks), and will be present in the samples collected for ADA analysis. Drug in the samples will interfere with the assay by complexing with ADAs; therefore, reducing the assay sensitivity and even causing false negative results. Assay drug tolerance is improved by introducing an acid dissociation step to dissociate immune complexes between drug and ADAs. Labeled drugs in a high pH buffer are subsequently added to simultaneously neutralize the sample and compete with free drugs to form bridging complexes. A key concern with use of acid dissociation step in NanoLuc bridging immunoassay was possible loss of NanoLuc activity during brief exposure to low pH solution during neutralization. To address this issue, NanoLuc luminescence signal in acidified serum sample was compared with serum that was never acidified and no significant difference was seen (data not shown), indicating that brief exposure of NanoLuc to acidic pH is not a concern. The drug tolerances of NanoLuc bridging immunoassay for three ATAs at 100 ng/mL were obtained in presence of excess Trastuzumab. Concentration of 100 ng/mL was selected, as this is the FDA recommended sensitivity for ADA detection.

 Samples of pooled Human serum spiked with 100 ng/mL of ATA and increasing amount of Trastuzumab (0-100 µg/mL) were incubated to form immune complexes. Samples were subsequently acidified,
neutralized in presence of 5.0 µg/mL of bridging mixture, and detected. Pooled serum containing no ATA but all other components was used as negative control and used to determine drug tolerance as described in Section 2.8. As expected, luminescence signal falls for all three ATAs with increasing amount of free drug as the bridging mixture competes with the free drug in the solution to form bridge complexes (Fig 6). It is worth noting that absolute RLU values in these experiments were lower than observed in Figure 5 because of sample dilutions due to acidification and neutralization. Inspite of different absolute signal, signal-to-background ratio (S/B) for three different ATAs were similar (Fig 6, inset) and 100 ng/mL of all three ATAs could be detected in presence of upto 50 µg/mL free drug, giving a drug tolerance of 500.

![Fig. 6. Drug tolerance of NanoLuc bridging immunoassay for detection of three ATAs. ATA1, ATA2, and ATA3 are shown on separate plots for clarity. Human serum samples spiked with 100 ng/mL of ATA and different amount of drug were acidified and neutralized in presence of 5.0 µg/mL of bridging mixture and subsequently detected on streptavidin plates. Inset shows the same graph as signal to background (S/B) ratio. Each data point is the average and standard deviation of four replicates. Data were fitted to 4-parameter logistic regression equation.](image)

The extent of drug tolerance in the bridging ELISA is driven by competition between amount of bridging mixture and free drug present in the sample. Hence, it should be possible to improve the drug tolerance of the assay by increasing the amount of bridging mixture, which is exactly what we observed (Fig. 7) with ATA1. Drug tolerances of 1000, 500, and 250 were observed with 10.0, 5.0 and 1.0 µg/mL of bridging mixture. It is worth mentioning that drug tolerance cannot be improved indefinitely by increasing the amount of bridging mixture because of increase in signal from non-specific binding and lower signal over background ratios as is evident in Fig 7 and seen before (Fig 4).
Fig. 7. Relationship between bridging mixture and drug tolerance in NanoLuc bridging immunoassay. Human serum samples spiked with 100 ng/mL of ATA and different amount of drug were acidified and neutralized in presence of 1.0, 5.0 and 10.0 µg/mL of bridging mixture and subsequently detected on streptavidin plates. Signal to background ratio (S/B) is plotted against the free drug concentration. Each data point is the average and standard deviation of four replicates. Data were fitted to 4-parameter logistic regression equation.

4. Discussion

Immunogenic response to drugs can have major impact on drug’s safety and efficacy; hence, various regulatory bodies require testing of ADA responses during drug development process. The most common assay format for ADA testing is the bridging immunoassay with acid dissociation and have been implemented on variety of technology platforms such as ELISA, Meso Scale Discovery (MSD), and GYROS among others [36]. The MSD platform has become an industry standard possibly due to high sensitivity, wide dynamic range and a simplified protocol involving a single washing step. However, few reports have raised concerns about the dependence on proprietary reagents and instrumentation for assays that may be used over long periods. As a result, several improvement in ELISA based methods have been proposed to achieve performances similar to that of the MSD platform, but the protocols still involve multiple incubation/washing steps and use secondary antibodies labeled with HRP for detection [17, 19, 28]. Multiple incubation and washing steps make for longer assays and use of secondary antibody introduces the need for additional quality controlled reagents such as polyclonal antibodies and processes like HRP conjugation. Direct conjugation of drugs with HRP will simplify the method [5, 20] but the approach has been reported to have insufficient assay sensitivity and is rarely used [19, 20]. Although specific reasons for that loss have not been described, a probable reason may be inefficient labeling of drug or loss of drug activity after HRP labeling. Although, it is possible to make genetic fusions of HRP and AP with proteins [37-39] these methods have not been widely used in immunoassays.

In this study, use of NanoLuc as a reporter in bridging immunoassays for detection of ADA was evaluated. We hypothesized that extremely bright NanoLuc reporter will allow us to simplify and use ELISA workflow while maintaining the sensitivity and drug tolerance required of ADA immunoassays. We were encouraged to pursue this approach by a recent publication on the use of NanoLuc for the development of robust and highly sensitive assay for antibody screening [26]. The study attributed the advantages of NanoLuc for immunoassay to several factors including (a) ability to make genetic fusion of
antibodies and ScFv with a small NanoLuc tag; (b) extremely bright light intensity; and (c) long half-life (80 min) of the signal, which allows stacking of plates for high throughput studies.

To evaluate the use of NanoLuc for ADA detection, anti-Trastuzumab and anti-Cetuximab monoclonal antibodies were selected as model systems. For detection, NanoLuc fused chemically or recombinantly were both tested. Recombinant fusions of drug-NanoLuc could be easily obtained with high purity and homogeneous labeling of two NanoLuc per drug molecule. Recombinant fusions will have the advantage of lot-to-lot reproducibility (although we did not test multiple batches in our assays) which is an extremely useful attribute for validated assays. It should be noted that drug molecule in the recombinant fusion might not be identical to the original drug due to differences in expression systems.

Making genetic fusions of antibodies is becoming easier but still may not be possible for every researcher, therefore, a novel chemical method based on HaloTag was developed for labeling antibodies with NanoLuc. Traditional approaches where enzymes are chemically modified before conjugating to the antibody led to significant decrease in NanoLuc activity (data not shown), whereas the use of HaloTag-NanoLuc fusion maintained the NanoLuc activity while allowing covalent attachment. Another advantage of our approach was high labeling efficiency, which eliminates the need for having additional size exclusion or affinity chromatography step for removing unconjugated enzyme. In fact, due to the difficulties involved in separating unconjugated enzymes, many commercial antibody-enzyme conjugates are not purified and may cause high background in the assays [40, 41]. A drawback of our method is the addition of a 32 kDa HaloTag protein possibly resulting in steric inhibition in some cases but the combined molecular weight of HaloTag-NanoLuc fusion protein (50kDa) is in the range of HRP (42kDa) and much smaller than AP (~140kDa).

Labeling with NanoLuc slightly reduces the activity of Trastuzumab but no such change is seen with Cetuximab. Moreover, when combined with the fact that loss in Trastuzumab activity was similar for both recombinant as well as chemical approach, it seems that loss in activity may be due to structural perturbation rather than steric inhibition of antigen binding from NanoLuc and is drug specific. However, it is worth noting that bridging immunoassays using Trastuzumab and Cetuximab labeled with NanoLuc met specific assay requirements of sensitivity and dynamic range. Our results are in line with well-documented observations that labeling may affect antibody activity but still can result in reproducible downstream applications as long as labeling protocol is optimized and stringently controlled [31, 41]. A systematic long-term shelf life stability studies of NanoLuc labeled drugs were not done in this study but in general, antibody NanoLuc conjugates have been reported to be stable [26] and maintained their activity during multiple freeze thaw cycles (internal data).

NanoLuc bridging immunoassays for detection of ATAs and ACA are sensitive, with LODs in low nanogram/mL range, and compares favorably with other reported ADA assays [16, 19, 34, 42]. More importantly, high sensitivity was obtained using a standard ELISA format with a single washing step and without the use of expensive instrumentation. Assays also had a broad dynamic range of four log orders, which is important as ADA concentrations in serum may display a wide range of three log order or more even for fully human monoclonal antibodies [43]. Surprisingly, not much attention has been paid to the assay dynamic range in the literature even though excess ADA in the sample will result in a hook effect and an underestimation of ADA. Having a broad dynamic range is a clear advantage of the NanoLuc bridging immunoassay and is probably due to luminescence-based detection. Moreover, both the sensitivity and dynamic range were tunable by optimizing the amount of bridging mixture used in the
assay. Even though same assay format was used for detection of ATA and ACA, the S/B ratio for Cetuximab was significantly higher (Fig 4) and was primarily due to lower background signal from non-specific binding. Some antibodies are inherently ‘sticky’, prone to aggregation, have lower solubility or stability, and would result in unpredictable non-specific binding. Biophysical characterization of drugs is routinely done during drug ‘developability’ studies [44, 45] and similar studies may be necessary during assay specific optimization for maximizing S/B ratio.

In our assays equal amount of NanoLuc and biotin labeled drugs were used, which translates into the molar ratio of biotin to NanoLuc labeled drug of 1:0.8 and 1:0.5 for recombinant and chemically conjugated NanoLuc respectively. During initial optimization, different molar ratios of labeled drugs were tested (data not shown), which indicated possibility of further changes in assay performance depending on the specific assay as has been shown by others [8]. Finally, use of 100% serum sample in the assay will simplify the workflow, avoid unnecessary sample dilutions, and offer higher sensitivity [6].

Immune response to a drug is polyclonal, unique to a patient, and depends on the dosage and frequency of administration; therefore, a true positive control for optimization of assays to detect ADA is not available. Instead, either monoclonal antibodies or affinity purified polyclonal antibodies are often used as positive controls and are accepted by regulatory agencies (FDA/EMA). The choice between polyclonal and monoclonal positive control is typically left to the assay developer but recently use of monoclonal antibodies has been proposed as preferable because they can be consistently produced and are better suited as universal calibrator [33, 46]. NanoLuc bridging immunoassay was tested with small set of monoclonal antibodies against the two drugs, and although differences in assay performances were observed, all the antibodies could be easily detected at concentrations several fold lower than the 100 ng/mL cutoff recommended by FDA. It can be argued that our assay did not account for low affinity antibodies that may be present in a real polyclonal serum. Lack of an ideal positive control representative of a real patient sample is well understood in the field and FDA has detailed guidelines [2] on generating and use of positive controls in reporting results from real samples.

Antibody drugs have long half-life and are typically present in patient sample along with ADA, which results in formation of immune complexes with possibly false negative results. One approach to address this problem is to collect samples several weeks after dosing of drug, so that drug has been cleared from the system or is present at extremely low concentration. This approach ignores the possible impact of immunogenicity in the days and weeks immediately after the patient is exposed to the drug. Another approach to minimize drug interference is acid dissociation of immune complex followed by neutralization in presence of bridging mixture. Typically drug tolerances of 50-400 have been reported using bridging immunoassay with acid dissociation [8, 19, 28, 47], and recently a new method termed as precipitation and acid dissociation (PandA) is able to detect 27-67 ng/mL of ADA in presence of 250 µg/mL of free drug [6]. The PandA method however, is a multi-step protocol involving several centrifugation, washing, and incubation steps including one overnight incubation. NanoLuc bridging immunoassay was able to detect ADA at 100 ng/mL in the presence of 100 µg/mL of free drug (drug tolerance of 1000) with a simple workflow. For some context, mean peak concentrations for Trastuzumab and Cetuximab in serum are 123 µg/mL and 184 µg/mL respectively with half-life of 5.89 and 4.75 days [48, 49].

Drug tolerance was however tunable and could be further optimized by choosing an appropriate bridging mixture. We believe that high drug tolerance of the assay is a result of the bright luminescence
signal of NanoLuc along with low non-specific binding of NanoLuc labeled drugs. Bright luminescence signal is required for sensitive detection because, in the presence of large excess of free drug, only a very small fraction of ADA will form a bridge with labeled drug, and will be captured on the plate. Higher amount of labeled drug can increase the amount of bridge complexes and improve sensitivity but also increases the background signal as discussed earlier. We did not elucidate the individual contribution of bright signal and low non-specific binding on improved drug tolerance in this study and may investigate it in future studies. Although not done here, an interesting study to simplify the workflow will be to determine drug tolerance in the absence of acid dissociation, and instead leverage long incubation time and temperature to induce dissociation. Finally, for this technology demonstration study, technical replicates from a single pooled serum were used to determine drug tolerance whereas biological replicates from a large pool of naïve human serum would have to be used for development of a validated assay.

Conclusion

In conclusion, the feasibility of using NanoLuc bridging immunoassays for detection of ADA with high sensitivity, wide dynamic range, and high drug tolerance was demonstrated with Trastuzumab and Cetuximab as model drugs. Sensitivity and drug tolerance compared well with the published results generated using multi step ELISAs or expensive platforms like MSD. Additional advantages are a simplified workflow involving a single washing step and no need for secondary antibodies. Additional work is needed to (a) benchmark the NanoLuc bridging immunoassay with other platforms like MSD and ELISA under controlled conditions using identical reagents and matrix lots, and (b) determine accuracy, precision, matrix interference, and other assay parameters recommended by FDA. However, we believe that our approach will, not only find application in immunogenicity assays but immunoassay applications beyond ADA detection will also benefit with use of antibody labeled with NanoLuc.

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