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Research paper

Pharmacokinetic immunoassay methods in the presence of soluble target

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ABSTRACT

Soluble targets represent a special challenge when employing ligand binding assays to support pharmacokinetic analysis of monoclonal therapeutics. Target-engaged antibody is not available for binding in immunoassays employing anti-idiotype-specific antibodies or target for capture. We investigated several formats of total antibody assays that show reduced interference of soluble targets: direct target capture, indirect target capture and acid dissociation. While indirect target capture worked well for a regular affinity antibody against DKK1, a high affinity antibody against PCSK9 required an additional acid dissociation step. The choice of a suitable format was antibody and target dependent. Our results offer several choices to approach immunoassay development for soluble targets.

1. Introduction

Recent years have seen a rise in biologics-based medications. Bioanalytical methods for pharmacokinetics (PK) of antibodies and large proteins (>20 kD) mostly rely on immunoassays. As these assays generally use unpurified serum or plasma, the assay design needs to take the presence of the compound's targets into consideration. Soluble targets represent a specific challenge: At low drug-target ratios, very few binding sites of the drug may be available to engage immobilized target in the immunoassay whereas at high drug-target ratios the reverse effect can be observed. These effects can impact the measured PK profile. One example is tumor necrosis factor alpha (TNFα which is targeted by several drugs on the market [Etanercept, Infliximab, Adalimumab, Golimumab (Weinberg and Buchholz, 2006). Assays measuring Remicade were reported to exhibit TNFα interference (Ternant et al., 2006). Other similarly problematic targets are Dickkopf-related Protein 1 (DKK1, associated with bone resorption and cancer) (Pinzone et al., 2009; Qian et al., 2007; Sheng et al., 2009) and Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9, associated with Cholesterol-receptor regulation) (Hedrick, 2009; Lopez, 2008a,b). Soluble ligands may also arise from shedding of membrane-associated molecules such as in the case of erb2/Her2 (Carney et al., 2003; Luftner et al., 2003; Maple et al., 2004).

Sometimes free and total assays are used in parallel to gather a more complete pharmacokinetic picture in the presence of soluble targets. Free compound assays preferably measure molecules that are not associated with the target whereas total assays preferably measure molecules that are target-engaged. Different approaches may be used to develop

Abbreviations: TNFα, Tumor necrosis factor alpha;DKK1, Dickkopf-related Protein 1;PCSK9, proprotein Convertase Subtilisin/Kexin type 9; ECD, extracellular domain.

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a total compound immunoassay with minimal soluble target interference. Besides dissociation steps (Patton et al., 2005; Roch et al., 1990; Thomas et al., 1990), an indirect capture assay to address potential interference of shed Her2/neu receptor in a Herceptin PK assay has been reported (Maple et al., 2004). Recently, Doucet et al. used enzymatic digestion to degrade interfering targets (Doucet and Avrameas, 2010). Through careful optimization of pepsin digestion conditions, they were able to digest soluble target in samples while retaining the activity of the antibody of interest.

We investigated the effect of soluble targets in PK assays for antibodies against TNFα, DKK1, PCSK9 and Her2/neu. We present several assay formats to measure total compound.

2. Methods

Several assay conditions were common between the different assay formats: The assay diluent consisted of 0.2% I-block (Tropix) + 3% bovine serum albumin + 0.05% Tween20 and 10 μg/mL of Immunoblock (ANP Technologies). Assay steps were separated by 6 washes with phosphate buffered saline (PBS) supplemented with 0.05% Tween20. Plates were incubated without shaking unless noted otherwise below. Human serum samples, calibrators and quality controls (QCs) were minimally diluted five-fold in assay diluent resulting in 20% of serum being applied onto the assay plates in duplicates of 200 μl sample volume per well. All other steps were carried out with 100 μl per well. Plates were blocked with 250 μl per well. All assays presented used a four-parameter logistic curve fit to calculate sample concentrations from the calibrator curve. Interference from potential patient sera was simulated by spiking the antibody analyte in triplicate at concentrations around the lower limit of quantitation into individual serum donor lots. Each spike sample was tested in duplicate in the respective assays. Samples were incubated at room temperature for 1 h, frozen over night and tested in the respective assays after thawing. The analytical recovery of was compared between individual serum lots and a control spike into a normal pooled serum lot. Similarly, samples to test tolerance to soluble target were prepared by spiking QCs with recombinant target protein in independent duplicates. Spike samples were incubated at room temperature for 1 h prior to freezing. After thawing, each duplicate spike sample was tested in the respective assays in duplicate replicates. The analytical recovery of target-spiked QCs was compared to that of unspiked QCs. Pre-incubation of tolerance and interference samples allowed for binding events of soluble targets and potentially interfering substances to occur prior to application of samples in the assays. Human sera from normal and disease populations were obtained from Bioreclamation.

2.1. Anti-TNFα antibody

Microtiter plates were coated with recombinant human TNFα (Invitrogen, cat # PHC3016) at 1 μg/mL in PBS at 4 °C for 12–16 h. After a wash step, plates were blocked with assay diluent for 1 h before adding samples for 2 h at RT. Calibrators and QC samples were prepared by spiking anti-TNFα antibody into pooled normal human serum and frozen at −70 °C until use. Captured compound was detected with mouse anti-idiotype specific antibody conjugated to horseradish peroxidase (HRP) (Merck, clone 18L17-2) at 30 ng/mL. Plates were developed with tetramethylbenzidine (TMB) (KPL, cat #05-76-00). To investigate assay tolerance to soluble target, QCs were spiked with recombinant human TNFα at 0.01, 0.1, 1 and 10 ng/mL (i.e. 0.4, 4, 40 and 400 pmol/L) in addition to the endogenous serum TNFα. Interference samples were prepared by spiking human sera from individual rheumatoid arthritis patients with anti-TNFα antibody at 60 ng/mL in triplicate.

2.2. Anti-DKK1 antibody

For a direct target-mediated capture assay, microtiter plates were coated with recombinant human DKK1 (Sino Biologicals) at 4 μg/mL in PBS at 4 °C for 12–16 h. Subsequently, plates were washed and blocked with assay diluent at 4 °C until use. For indirect target capture assays, plates were coated with a monoclonal mouse anti-human DKK1 antibody (R&D Systems cat #MAB1096) at 10 μg/mL. After a blocking step for 1 h at ambient temperature, recombinant human DKK1 was applied at 4 μg/mL. Pre-coated, dried plates also contained added stabilizer (ANP Technologies). Anti-DKK1 analyte antibody calibrators and QC samples were prepared in pooled normal human serum and frozen at −70 °C until use. Serum samples were applied to coated plates for 2 h at RT. Bound antibody was detected using mouse anti-human IgG2-specific monoclonal antibody at 290 ng/mL (Meridian Biosciences, cat #Z86002M) conjugated to Biotin. After a Streptavidin-HRP step (Jackson Immunoresearch, cat #016-030-084, 100 ng/mL) for 30 min at room temperature, plates were developed with TMB. To investigate assay tolerance to soluble target, QCs were spiked with recombinant human DKK1 at 0.01, 0.1, 1 and 10 μg/mL (i.e. 0.35, 3.5, 35 and 350 pmol/L) in addition to the endogenous serum DKK1. Each spike was prepared in duplicate and tested in the assay. Interference samples were prepared by spiking human sera from individual post-menopausal patients with anti-DKK1 analyte antibody at 200 ng/mL in duplicate.

2.3. Anti-PCSK9 antibody

For a direct target-mediated capture assays, microtiter plates were coated with recombinant human PCSK9 (Bluesky Technologies) at 5 μg/mL in PBS at 4 °C for 12–16 h. Subsequently, plates were washed and blocked with assay diluent at 4 °C until use. For indirect target capture assays, plates were coated with a mouse anti-human PCSK9-specific monoclonal antibody (R&D Systems, cat #MAB38888) at 5 μg/mL at 4 °C for 12–16 h. After a blocking step for 0.5 hs at ambient temperature, recombinant, human PCSK9 was applied at 2 μg/mL for 2 h at room temperature. Subsequently, coated plates were stabilized (ANP Technologies) and stored dry until use. Anti-PCSK9 analyte antibody calibrators and QC samples were prepared in pooled normal human serum and frozen at −70 °C until use. Serum samples were applied to coated plates for 2 h at RT. Bound antibody was detected using a rabbit anti-idiotype-specific monoclonal antibody (clone 242-14) at 160 ng/mL) for 2 h followed by Streptavidin-HRP (100 ng/mL, 30 min) and a TMB substrate step. An acid dissociation step was added in some cases as