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Review

Receptor Occupancy Assessment by Flow Cytometry as a Pharmacodynamic Biomarker in Biopharmaceutical Development

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Receptor occupancy (RO) assays are designed to quantify the binding of therapeutics to their targets on the cell surface and are frequently used to generate pharmacodynamic (PD) biomarker data in nonclinical and clinical studies of biopharmaceuticals. When combined with the pharmacokinetic (PK) profile, RO data can establish PKPD relationships, which are crucial for informing dose decisions. RO is commonly measured by flow cytometry on fresh blood specimens and is subject to numerous technical and logistical challenges. To ensure that reliable and high quality results are generated from RO assays, careful assay design, key reagent characterization, data normalization/reporting, and thorough planning for implementation are of critical importance during development. In this article, the authors share their experiences and perspectives in these areas and discuss challenges and potential solutions when developing and implementing a flow cytometry-based RO method in support of biopharmaceutical drug development. © 2015 The Authors Cytometry Part B: Clinical Cytometry Published by Wiley Periodicals, Inc.

Key terms: receptor occupancy; flow cytometry; pharmacodynamic biomarker; biopharmaceutical; drug development; PKPD relationship; dose selection; target binding; animal and human studies; clinical trial

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For the past few decades, the pharmaceutical industry has faced challenges of rising research and development costs as well as decreasing drug approval rates (1). To improve efficiency and reduce the costs associated with drug development, pharmacodynamic (PD) biomarkers have demonstrated promise in aiding in the rational design of clinical trials (2–6).

One critical component in the design of clinical trials is the selection of the optimal therapeutic dose. While starting dose decisions for first-in-human (FIH) studies based on the no adverse effect level (NOAEL) in nonclinical toxicity studies (7) have been successfully utilized for small and large molecules, the minimum anticipated biological effect level (MABEL) approach has been proposed for use in FIH trials of potentially high-risk products, where the toxicity is due to exaggerated pharmacology (8). In other cases, such as FIH studies in cancer patients, starting with a pharmacologically active dose (PAD) can be desirable if warranted by risk-benefit considerations. MABEL and PAD calculations are based on the analysis of the dose-response of the drug, and evaluation of pharmacokinetics (PK) and PD are important for characterizing this dose-response relationship. Both receptor occupancy (RO) and downstream signaling modulation can be appropriate measures for PD. Measurements of downstream modulation (9,10) are generally preferred as they provide information pertaining to drug effects on target receptor activation, especially in cases where receptor activation is not in linear relationship with RO (11–13). However, assays that measure receptor signaling and other effects downstream of target engagement may not always be feasible. In these

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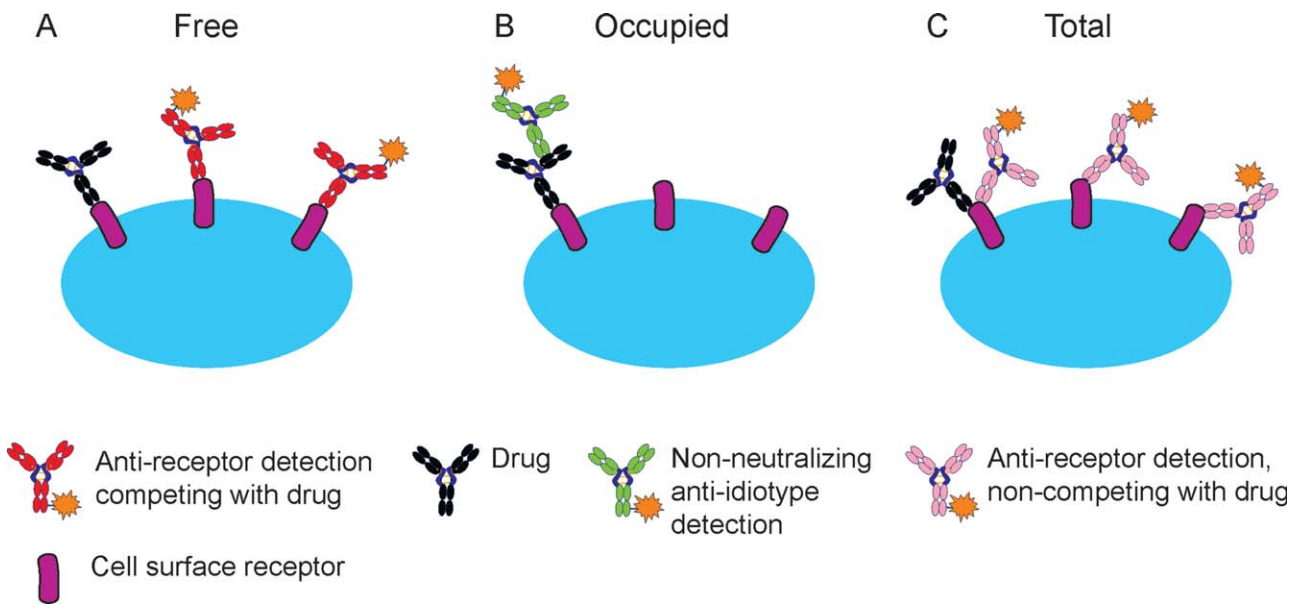

FIG. 1. The three basic RO assay formats. **A**: In the free receptor format, unbound receptors are detected using a detection reagent that competes with drug for receptor binding. **B**: The drug-occupied receptor format uses a non-neutralizing ADA as a detection reagent to measure drug-bound receptors. **C**: The total receptor assay format uses a non-competing anti-receptor antibody as a detection reagent.

cases, RO can be used as a PD biomarker to enable for the quantitative evaluation of PKPD relationships and can provide useful information for initial dose range finding in early phases of clinical trials. Ultimately, RO results must be linked to the clinical outcome, as clinical efficacy is critical for further refining human doses.

Frequently, mechanism-based PKPD models are constructed to describe the quantitative relationship between PK and RO (14). Incorporation of RO data from animal studies into a PKPD model can enable prediction of RO in humans for dose selection (15). RO assays have been developed and applied in both nonclinical and clinical studies to provide insight into PKPD relationships. For example, RO on circulating cells has been utilized as a PD biomarker for a number of therapeutic antibodies, such as anti-PD-1 (2), anti-PD-L1 (3), AMG479 (4), ATR-107 (5), and Etrolizumab (6).

The clinical study with the CD28 targeting immunomodulating agent, TGN1412, further exemplifies the importance of the use of RO in dose selection. Due to differences in TGN1412 pharmacology between nonhuman primates and humans, the NOAEL obtained from nonclinical safety studies was not relevant to humans. As a result, the administered starting dose of 0.1 mg/kg in the FIH trial for TGN1412 (calculated based on NOAEL) led to life-threatening cytokine release syndrome in healthy volunteers (16,17). Based on the in-vitro binding affinity of TGN1412 to its target receptor, the starting dose of 0.1 mg/kg would result in [mt]90% RO. If RO was assessed in nonclinical safety studies and a MABEL approach had been used based on targeting a 10% RO level, the recommended starting dose would have been significantly lower (30,000 times lower if RO is estimated based on in-vitro binding affinity) than that which was actually used (16,18).

RO and/or pharmacological effects should be routinely assessed in nonclinical safety studies to demonstrate not only the relevance of the animal species, but also adequate target binding and/or receptor activation for safety evaluations (18,19).

In this article, we describe RO assay formats, and discuss challenges and recommendations for developing and implementing flow cytometry-based RO methods in support of biopharmaceutical drug development. Although most examples are based on therapeutic antibodies, the same conceptual approaches can be readily applied to other types of biopharmaceuticals.

RO ASSAY FORMATS

The three basic formats for RO assays are free, drug-occupied, and total receptor measurements as shown in Figure 1. These formats have been used as PD measurements in drug development (2,20,21).

In the free receptor measurement (Fig. 1A), the proportion of receptors not bound by drug is quantified using a fluorescence-labeled detection reagent to the receptor. Common detection reagents include the drug itself, an antibody that binds to the same epitope as the drug (i.e., a competitive antibody), or the receptor ligand.

A second RO assay format measures drug-occupied receptor (Fig. 1B). In this format, the proportion of receptors bound by drug is determined by the measurement of the bound drug. A fluorescence-labeled antidrug antibody (ADA) that does not compete with drug binding is used for detection in this format. Commonly used

reagents include non-neutralizing anti-idiotypic antibodies or antibodies with specificity to the Fc of the drug.

A third format is the measurement of total receptor levels (Fig. 1C), including both free and drug-occupied receptor. In this format, the detection reagent is generally an anti-receptor antibody that binds to an epitope distinct from that of the drug (i.e., a non-competing antibody). An alternative approach is to detect drug-occupied receptors as described above following the incubation of the specimen with excess drug to saturate all receptors (2,3,22).

CONSIDERATIONS WHEN DEVELOPING FLOW CYTOMETRY-BASED RO ASSAYS

Selection of Assay Format

A critical component when planning to use an RO assay for PD assessments is determining the most appropriate assay format. The selection of assay format largely depends on the mechanism of action (MOA) of the drug and availability of reagents. The primary mode of action for antagonistic drugs is to block ligand binding to receptors and subsequent downstream signaling events. Since free receptor assays are designed to measure receptors available for ligand binding, they are the preferred format. When a free receptor assay is not feasible, one alternative is to develop an assay that measures drug-occupied receptor. Free receptor levels can be theoretically derived from the drug-occupied receptor dataset by PKPD modeling.

The use of an assay that measures total receptor or the number of receptor-expressing cells is ideal when the proposed MOA of the drug involves up or down-regulation of receptor (21), ablation of receptor-expressing cells (23–25), or mobilization of receptor-expressing cells from tissue into the circulation (26). In the case when target receptor expressing cells are ablated (e.g., by ADCC or ADC), free receptor measurements can identify the degree of RO that is necessary for cell killing. However, RO would normally be short-lived due to drug-induced cell ablation resulting in consequent difficulties in data interpretation.

Total receptor measurements are also useful for normalizing free receptor data from the same samples and to complement free receptor data for mechanistic PKPD modeling. This is especially useful when the receptor level or cell numbers change during the study. For example, receptors can be internalized and subsequently catabolized, leading to a decrease in receptor levels upon drug binding (21). Alternatively, positive feedback mechanisms following chronic drug administration can result in upregulation of the receptor (27). In such cases, normalization can allow for quantification of the receptor fraction that is free of drug, minimizing misinterpretation of drug effects on free receptor due to changes in total receptor levels (discussed in more detail below).

In some cases, cell surface RO measurements are not always feasible, and alternative assays must be explored. The extracellular domains of some receptors are released into circulation by proteolytic cleavage and can bind to the drug (28). Quantification of RO of a soluble receptor represents an alternative to cell surface RO. While this may be a tractable surrogate measurement for cell surface RO, the physiological relevance of soluble RO must be assessed. Another option is to quantify the accumulation of a ligand for the receptor in the circulation as an indirect measure of RO (29–32). However, the natural ligand for the receptor may be unknown or, if known, may bind to multiple receptors, in which case, the ligand levels may not be increased following administration of drugs. The anti-EGFR therapeutic cetuximab led to an increase in the EGFR ligand TGFα whereas EGF and other ligands did not significantly increase after cetuximab administration (31). Lastly, RO can be theoretically derived by mechanistic PKPD modeling from nonlinear PK profiles (33). Due to assumptions used when conducting PKPD modeling, however, theoretical RO calculation may potentially be inaccurate. As a result, RO measurements, if feasible, are always preferred over model-estimated values.

Receptor Expression Levels

To establish the PKPD relationship of a drug, RO is commonly assessed longitudinally using fresh whole blood specimens, as blood collection is a minimally invasive procedure and is amenable for repeat sampling. As such, the feasibility of performing an RO assay is dependent on target expression levels on circulating cells.

The ideal scenario is one in which target receptors are expressed at relatively high levels on an abundant circulating cell population. This would allow for a broad assay dynamic range on a detectable cell population. Often, however, the target receptor is expressed at low levels on circulating cells, leading to significant challenges. In cases of inherently low receptor expression, and thus low signal intensity, the assay dynamic range may not be adequate to accurately assess RO. This may be countered by the use of a high affinity detection antibody conjugated with a bright fluorophore or amplification of the signal with secondary detection reagents. In cases, where these approaches are not feasible, another possible means of enhancing assay signal intensity involves ex-vivo stimulation of whole blood specimens to induce receptor expression (34,35). However, this approach is not preferred since target saturation on cells with physiologically relevant expression levels might differ from what is observed after ex-vivo stimulation.

In certain cases, target receptor is present only on a rare cell population in the circulation, necessitating the analysis of several cell surface markers and multistep gating to define the population. In such a situation, it is imperative that the effect of low events on precision of RO values be assessed during assay development. This can be achieved by spiking blood specimens with reduced quantities of cells with occupied receptors and determining the lowest spiked cell quantity at which RO values do not deviate from prespecified precision acceptance criteria. Ideally, the gating strategy used to identify

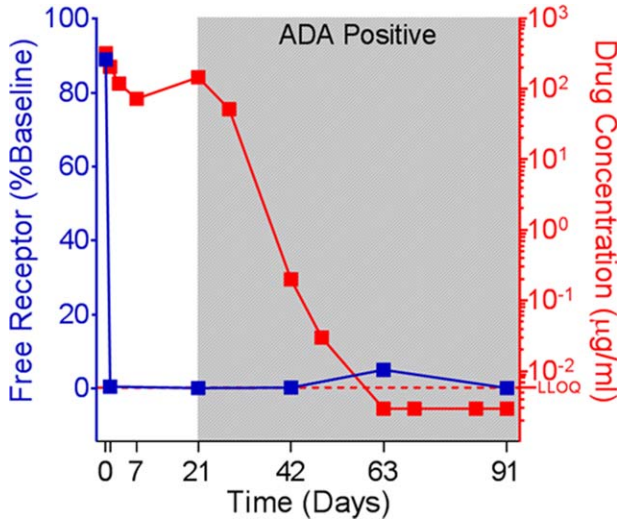

FIG. 2. Time profiles of free receptor and drug concentration in an individual monkey. The animal was dosed every 7 days. Free receptor was determined on CD3+ lymphocytes in whole blood with the labeled drug. The percent of free receptor at baseline (blue) plotted on the left Y-axis and the concentration of the drug (red) is plotted on the right Y-axis. Postdose results after the first dose and pre-dose result of all consecutive doses are shown. This animal became ADA-positive at Day 21, followed by a loss in exposure to the drug (in the gray shaded area). The free receptor appears to be fully occupied (0% free, in the gray shaded area) despite lack of exposure, an artifact of the RO assay.

the rare cell population in the clinical samples should be mimicked in the validation experiments. One liability to the performance of RO assays on rare cell populations is the need for increased sample volumes. Typically, this is not problematic in clinical settings as blood volumes are infrequently limiting. However, in nonclinical setting, animal welfare guidelines limit frequency and volumes of blood that can be drawn from an animal, which may preclude the ability to detect ample numbers of rare events for reliable RO quantification.

When target receptor is absent on all subsets of circulating blood cells, RO can be determined in an alternative tissue if receptor expression in that tissue is known. For example, the percent of EGFR saturation by panitumumab was determined in dissociated tumor tissue from xenograft models (36). In fact, RO in the tissue relevant to drug action may be more appropriate than assessment in blood, particularly when drug partitioning to the tissue is low. However, serial tissue biopsies are inconvenient and sometimes not feasible. As a result, blood RO can still be used as a guide to dosing, with recognition that higher doses might be needed than the doses that produce maximum RO in blood.

Reagent Considerations

As with many assays, reagents are a major determinant of RO assay performance. It is recommended that reagent requirements for RO assessment be considered in the early stages of a project and, if necessary, that reagent generation be incorporated into the timeline for RO assay development.

When developing flow cytometry-based assays, use of high affinity detection antibodies conjugated with a bright fluorophore, such as Brilliant Violet™(37), or biotin labeled anti-receptor antibodies in conjunction with a secondary fluorophore-conjugated streptavidin can increase both overall assay signal intensity and dynamic range. Aside from these general reagent properties, specific reagent considerations unique for each RO assay format and associated challenges are further discussed in this section.

Fluorophore-labeled drug is commonly used as a detection reagent in free receptor assays since it is well-characterized in terms of quality and stability, available in sufficient amounts throughout development, and is unequivocally competitive for target binding with the administered drug. However, the use of a labeled drug as a detection reagent can lead to misleading results in the presence of ADA (29). Figure 2 shows the PK and free receptor profiles for an individual monkey that developed an ADA response following weekly drug administration. For RO assessment in this example, free receptor was measured using labeled drug. Prior to ADA development on Day 21, circulating drug was maintained at high levels while free receptor was fully blocked as expected. After ADA became positive on day 21, the drug was cleared rapidly, however free receptor levels appeared to remain maximally blocked even when drug levels were undetectable. This represents an assay artifact, likely due to inhibition of the detection reagent by neutralizing ADA, and, thus, preventing the accurate detection of free receptors (Fig. 3A). When RO data are interpreted in isolation from PK and ADA data, the assay artifact leads to an underestimation of free receptor and thus the false conclusion that receptor is fully occupied, even though there is no drug present as determined by PK. In addition to the effects of neutralizing ADA, the presence of non-neutralizing ADA can result in an overestimation of free receptor due to the formation of complexes of multiple molecules of detection reagent with either the free receptor or the drug-occupied receptor (Fig. 3B). As a result, labeled drugs may not be appropriate for use as detection reagents when a high incidence of ADA is expected, such as in multidose animal studies.

ADA interference in RO assays can be avoided by the use of detection reagents that compete with the drug for binding to the receptor and that have no sequence similarity with the drug and thus would not be affected by ADA. Use of the receptor ligand is particularly valid for detecting functionally active receptors; however, the ligand affinity for the receptor is often too low to be useful for robust RO assays. Alternatively, antibodies that compete with the drug can be successfully used for detection of free receptors when available, provided that the ADAs do not cross react with regions of sequence similarity of the detection antibodies.

While the use of a nontherapeutic detection reagent may overcome ADA interference, other factors could still

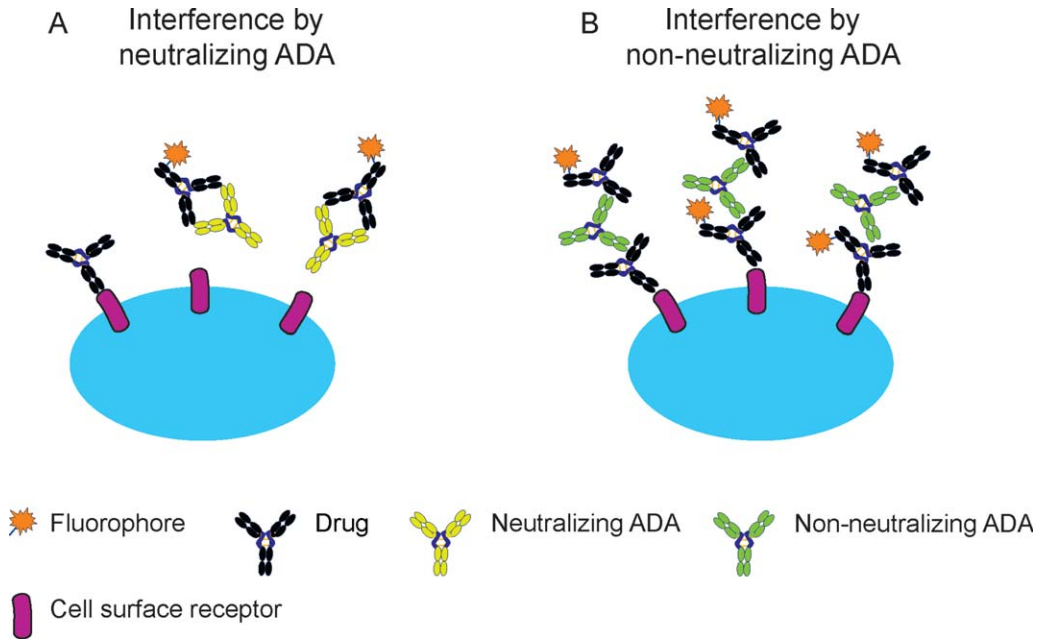

FIG. 3. Potential free receptor assay artifacts resulting from ADA when drug is used as a detection reagent. **A**: In the presence of neutralizing ADA, labeled drug used as detection reagent is blocked from binding to free receptor thereby resulting in the underestimation of free receptors. **B**: Non-neutralizing ADA can result in an overestimation of free receptor by bridging of detection reagent to drug bound to the receptor or complex formation of multiple detection reagents on free receptor.

influence the accuracy of free receptor measurements and should be evaluated. For example, the detection reagent may bind to an epitope distinct from that of the drug resulting in partial binding to drug-occupied receptor in addition to free receptors. This could lead to an overestimation of free receptor levels, especially when the target is abundantly expressed.

Another factor that may result in inaccuracy of free receptor measurements is the perturbation of the dynamic equilibrium between free and drug-occupied receptors during assay incubation steps (29). A high affinity detection reagent could out-compete the drug bound to the receptor, leading to an overestimation of free receptors especially in assays requiring long incubation times or high detection reagent concentrations. As such, an evaluation of the relative binding affinities of the detection reagent compared to drug should be part of the selection criteria.

For assessments of drug-occupied receptors, the detection reagent is used to detect drug bound to the receptor. A fluorescence-labeled, non-neutralizing anti-idiotypic antibody or an antibody with specificity for Fc (2,3,22) or a modified Fc region (38–40) can be used for detection. Selection of specific, high affinity reagents is essential for robust detection sensitivity. It is crucial to demonstrate lack of inhibition of drug binding to its target receptor by the detection reagent.

For total receptor assessments, the detection reagent is generally an anti-receptor antibody that binds to an epitope distinct from that of the drug. The anti-receptor antibody must have sufficient affinity for the receptor to ensure detection sensitivity and must not compete with the drug for receptor binding. Another option is to add excess drug and then use ADA as detection reagent (2,3,22). When a drug-occupied receptor method with addition of excess drug is used to assess total receptor, the concentration of drugs added to the assay should be optimized to ensure full occupancy of the receptor and the dissociation of drug from receptor should be minimized during washing steps of staining procedures.

When developing and validating a RO assay using a multiplex approach to measure more than one receptor (or multiple receptor forms, e.g., free and total receptors) in a single assay, the interactions between detection reagents for each receptor and the drug should be characterized. Generally, a detection reagent with comparable affinity to the target receptor as the drug should be used if available as the affinities of the detection reagent relative to the drug for each receptor may potentially influence the accuracy of RO determination.

Sample Considerations

As part of the RO method design, a number of factors involving sample stability should be considered. Certain types of sample processing methods (i.e., PBMC preparation and/or washing procedure) will likely alter the dynamic equilibrium between drug-occupied and free receptors and should be minimized. Anticoagulants could affect receptor binding, stability of receptor expression or cellular metabolism and thus should be assessed early in method development. For example, sample stability may be extended by collecting blood

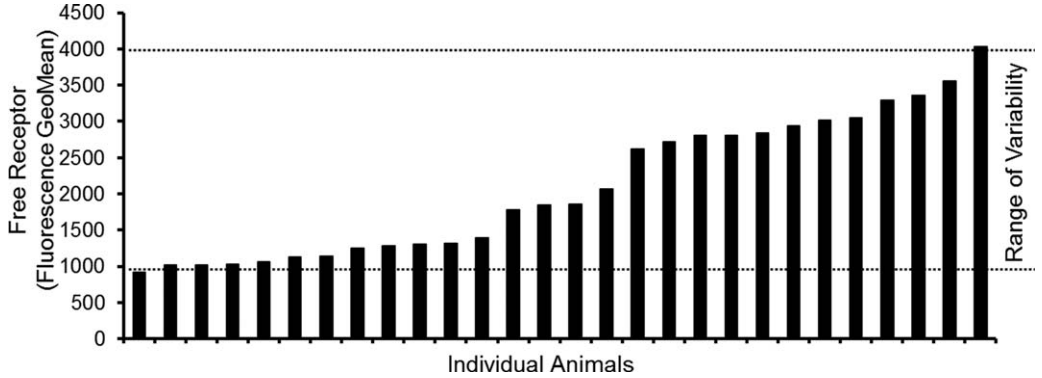

FIG. 4. Variance in receptor levels between individual monkeys necessitates data normalization. Baseline measurement of free receptor levels by flow cytometry across 28 individual monkeys shows 3.5 fold variance in fluorescence intensity.

specimens in commercially available stabilizing reagent (e.g., Cyto-Chex® from Streck or TransFix® from Cytomark), although fixation effects should be characterized (41,42). The study population is another critical consideration as receptor expression levels could differ across animals of different origins or across patient populations. In general, RO assay development should be conducted in animals of the same origin as the study population. For clinical studies, RO assay development is generally carried out with blood from healthy subjects, but should be evaluated in diseased samples prior to implementation, if possible.

Another important sample consideration is the possibility of receptor shedding or internalization following specimen collection. To limit enzymatic cleavage and receptor internalization, RO assays may be performed at 4°C as lower temperatures reduce these activities. Other approaches, such as prior treatment of cells with sodium azide (36,43,44) to abolish ATP-depended receptor internalization or the use of protease inhibitors to inhibit rapid receptor shedding (45), may be effective means in overcoming such challenges.

Data Normalization and Reporting

When collecting flow cytometry-based RO data, there is inevitable variability that arises from instrumental, experimental, and sample-specific sources. As such, it is critical to implement proper procedures and controls during data acquisition and analysis for assessment of non-specific signals. The most common means for monitoring instrument-based variability is to compare fluorescence values over time and across instruments using common QC materials such as calibrated fluorescent beads or stabilized cells during the course of the study (46). When coupled with the use of standardized daily instrument calibration and operation procedures, this enables for the robust characterization of flow cytometer-based variability. Experimental controls used in RO assays to determine nonspecific binding typically include isotype, fluorescence minus one (FMO) or non-specific background controls (47). The nonspecific background control is commonly generated by the addition of saturating levels of either the drug itself (for free receptor assays) or unlabeled detection reagent (for either free or total receptor assays) to an aliquot of the sample prior to staining with the labeled detection reagent. The specific signal for receptor quantification is often derived by subtracting this non-specific signal from the total signal acquired without addition of the prior saturating reagent. In some cases where the non-specific and specific signals vary proportionally, the ratio of total signal over nonspecific signal can also be used for receptor calculation (48). Subtraction or ratiometric approaches should be evaluated during method development and selected to fit the intended purpose of the method.

Even with well-characterized assays and carefully controlled experiments, high subject-to-subject variability can be a confounding factor. As an example shown in Figure 4, free receptor levels were highly variable among 28 untreated monkeys (CV = 46%). In the absence of a means to account for this high level of variability, it would be difficult to compare free receptor levels post-treatment across individual monkeys, potentially leading to data misinterpretation. Normalization using pre-treatment receptor levels has proven to be an effective approach to interpret treatment-modulated RO effects (49). Expressing each post treatment sample as a percentage relative to its corresponding pre-treatment sample can reduce variability and facilitate data interpretation. In addition, the collection of multiple pre-treatment baseline values over multiple time points allows for the assessment of longitudinal variability of receptor levels and decreases the likelihood of using an aberrant denominator across the entire subject time profile.

An example of the use of data normalization steps to improve the ability to interpret RO data is shown in Figure 5. During the longitudinal study presented, a high day-to-day variability in raw data generated more than anticipated RO variability between monkeys within the same dose group, and a less than anticipated difference in duration of maximal inhibition of free receptor between dose groups (Fig. 5A). Therefore, raw data

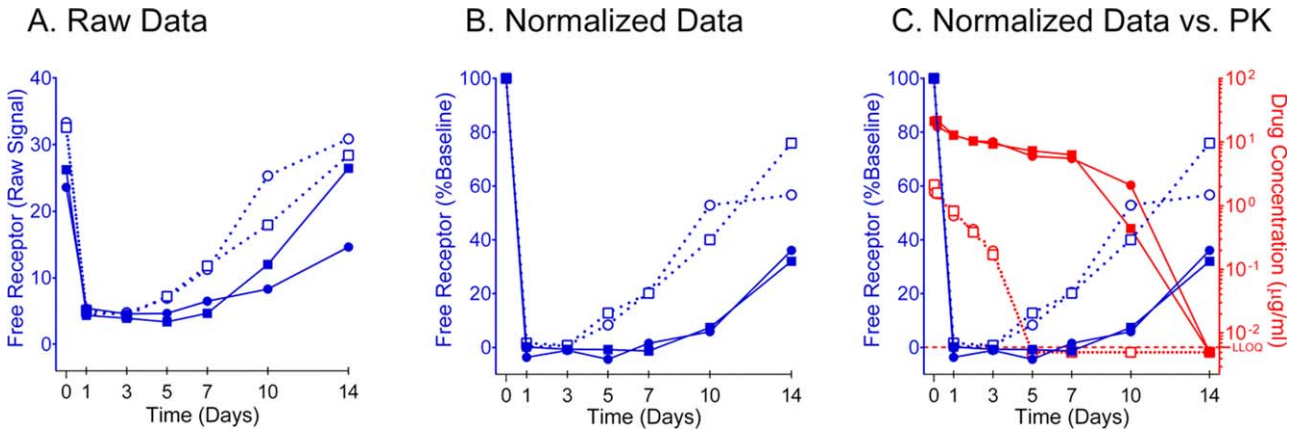

FIG. 5. Normalization improves data quality. Groups of two monkeys received either 0.1 mg/kg (dotted lines) or 1 mg/kg (solid lines) of a receptor targeting drug. Free receptor was measured by a competitive antibody to the drug. **A**: Raw free receptor signal. **B**: Normalized free receptor signal. Percent of free receptor was calculated by subtracting the background signal from raw data, calculating fold change over background signal, normalizing to the predose baseline signal. **C**: Time profiles of serum drug concentration (red) and percent free receptor (blue).

analysis could lead to misinterpretation of RO results. In an effort to account for high variability, raw data were first normalized to the background non-specific signal control (determined by addition of saturating levels of drug to fully block the free receptor) and, subsequently normalized by animal-specific pre-treatment baseline signal. Normalized results were reported as the percent of free receptor at baseline (Fig. 5B), demonstrating reduced animal-to-animal variability, and more distinct differences between 2 dose groups. Moreover, the normalized results demonstrated full free receptor blockade following administration of the drug at 0.1 and 1 mg/kg doses and the duration of receptor blockade was dose-dependent, consistent with the PK profile (Fig. 5C).

In the event that total receptor numbers change between samples as a result of either receptor up or down-modulation or change of receptor-expressing cells, it is recommended to normalize using total receptor values and report free receptor as a percent or fraction of the total receptor to minimize variability (44). Since total and free receptor results are often generated by two separate assays, normalized results should only be considered relative.

Challenges for Implementing RO in Nonclinical and Clinical Studies

Following the design and development of a RO assay, the practicality of implementing the assay to support nonclinical or clinical studies should be considered as numerous factors could affect assay performance during the course of study.

Many potential challenges in RO assay implementation can be assessed by careful design and execution of pre-study validation experiments. Typically, flow cytometry-based assay validation procedures are "fit-for-purpose" in that the goal is to assess assay performance primarily in conditions applicable to the study (50). However, there is also a standard set of parameters characterized for most flow cytometry-based RO assays including assessments of intra- and inter-assay precision and sample stability (51). Precision measures are designed to assess the relative reproducibility of a given result within and between assays performed on the same specimen(s). Assay imprecision is often the result of reagent or sample instability or methodological errors, which can often be ameliorated by analyst training. If an RO assay is to be transferred to an external nonclinical or clinical study site for testing, adequate time should be allotted for assay transfer and validation, as well as a pilot experiment (nonclinical) with animals of the same origin as the study animals. This practice ensures equivalent performance of the assay at external sites and allows adjustments of assay parameters if needed, following transfer.

Sample stability is an important consideration in RO assays since the time from sample collection to analysis may affect actual RO levels if sample stability is not well controlled. Sample stability can be optimized by using different types of anticoagulants or a commercially available stabilizing reagent as described above. Staining and fixation of samples immediately following collection at the study site are options that may be explored when stability timeframes are not adequate and if the procedure(s) can be standardized between sites (MedImmune unpublished data).

Signal stability of the RO assay must be monitored throughout the course of the study by use of positive control specimens. Stabilized blood specimens may provide valuable information on assay performance (52,53). When stabilized blood is not suitable due to either deleterious effects on target receptor expression or loss of receptor-expressing cells, other control specimens should be considered. These may include target receptor-expressing cell lines (genetically engineered or otherwise) or aliquots of cryopreserved peripheral blood

mononuclear cells (PBMC) containing a population expressing target receptors. The latter option may be especially useful as large lots of cryopreserved PBMC are readily available from multiple commercial sources, and the use of these materials may allow for uniformity throughout the course of the study. Like all positive controls, any lot-specific variation should be assessed.

RO assays require a minimum blood volume of ~100 μl that may be difficult to obtain in the desired frequency especially in preclinical studies in which samples are collected for many different assessments. Therefore, and because of practical consideration, it may be necessary to consider a multiplexing approach in which the RO assay is paired with other flow cytometry-based read-outs (e.g., immunophenotyping) in the same specimen. Furthermore, multiplexing is also attractive if multiple RO assays are necessary due to targeting multiple receptors either by a single drug or by combination therapy (54). While multiplexing would result in a more complex assay necessitating additional optimization and validation steps, it is a possible solution to sample volume limitations and may be more cost-effective.

Consideration of potential logistic issues that may arise during implementation of RO assays supporting clinical or nonclinical studies is critical. The availability of contract laboratories in the geographic locale of the study is an important consideration since sample shipment across borders can present numerous logistical problems, the net result being poor sample quality due to exceeding stability limitations. Careful assessment of a contract research organization's global footprint is essential, and the performance of each local laboratory should be assessed directly as part of the prestudy CRO selection process (55).

The application of RO assays to support nonclinical safety studies requires special considerations. The high sensitivity of RO assays coupled with the typically high doses used for safety studies generally precludes the ability to detect a dose-response effect and therefore limits the utility of RO results for PKPD modeling. Although, safety studies can be designed in such a way as to generate data that may inform dose selection strategies. For example, the addition of one or two low-dose cohorts to the study may enable the detection of dose-dependent changes in RO. Analysis of the wash-out period may also allow for the assessment of PKPD relationships. In addition to informing dose decision, RO could also be used to confirm target engagement during the study and help to interpret effects of ADA on PKPD.

Although RO assessments are sufficiently sensitive to monitor target binding and, thus, very useful in drug development, factors potentially influencing interpretation and utility of RO data should be evaluated. For example, the magnitude of clinical response is generally proportional to the amount of receptor bound. However, depending on the MOA of the drug, therapeutic effects may occur at different RO levels in that a dose resulting in saturating RO may be different from that required for a therapeutic effect. As such, it is critical to understand the relationship between RO and down-stream PD/clinical effects when dose prediction is based on RO. For antagonistic drugs that block cell surface receptors without depletion of cells, high level of RO is required for maximum blockade of down-stream receptor signaling (4,5,13) and the RO level is usually proportional to therapeutic effects. As such, RO is highly relevant as a PD. However, in cases where the MOA involves depletion of cells (23–25), high RO is usually not necessary and RO assessments are only possible in a short timeframe before most target cells are depleted. In such cases, monitoring target cell number is a more relevant and probably also more sensitive PD. Agonistic drugs may elicit maximum signals at low RO (11,12), in which case downstream receptor signaling may be more relevant PD and RO can be useful only when signaling measurements are not technically feasible or too variable for quantitation. Additionally, RO is commonly measured on blood cells from the circulation. If the target population is present in the circulation, then RO on this cell population is highly relevant. However, in many cases, drugs target distinct cell populations in tissues, in which case, RO as measured on circulating cells is utilized as a surrogate for tissue RO. Many RO assays fall into this category. It must be kept in mind that the drug is typically present at a lower concentration in tissue than in the central compartment. For example, antibody concentrations in blood have been reported to be ~10-fold higher than in epithelial lining fluid of lung, 1000-fold higher than in cerebrospinal fluid, and fivefold higher than in synovial fluid (56). As a result, free receptor blockade in blood is expected to be higher than in many other tissues. Therefore, a tissue-based correction factor may be needed when dose selection is based on RO from surrogate specimens. Alternatively, RO measured directly in tissues may be more relevant (36).

FUTURE PERSPECTIVES

Owing to their high sensitivity and relative ease of development, RO assays have utility in assessing PD effects in both nonclinical and clinical settings. However, there remain multiple areas upon which RO assays might be improved, including the inability to directly quantify occupied receptor numbers, lack of effective approaches to stabilize whole blood, inefficient data analysis and the lack of effective methodologies to perform RO assays on tissue specimens.

Currently, most RO assays are semiquantitative in that free or occupied receptors are often reported as percentage of pre-treatment baseline levels or as a percentage of total receptors. The longitudinal change of RO relative to pre-dose baseline levels following drug administration is usually sufficient for PD assessment and receptor density is not quantified. However, receptor density provides value if a quantitative relationship between receptor numbers and biological activity can be established, for example, if activation of a minimum number of receptor/cell is necessary for the cell to signal. Furthermore, the receptor density is generally useful

for mechanistic PK/PD models and, in absence of measurement, this parameter is estimated. Efforts to move from relative to absolute receptor quantification (= receptor density) have typically focused on the use of highly characterized detection reagents and fluorescent calibration beads (57,58).

Another point of improvement in implementation of RO assays is enhancement of sample stability. RO assays are typically performed on fresh blood specimens in order to minimize the probability of drug dissociation from target receptors during sample processing. Current efforts to stabilize blood with traditional paraformaldehyde-based fixatives often lead to unacceptable levels of signal loss or increased background staining levels (59). Ultimately, the ability to more effectively stabilize blood specimens immediately following collection would likely reduce variability and enable for a more quantitative assessment of RO.

The data management and quality control to enable cross-experimental normalization for large studies run at multiple sites during clinical trials are unique challenges for flow cytometry-based analyses. To this end, new normalization algorithms have been under evaluation to reduce assay-to-assay variability and to enhance the cross-evaluation of studies performed in large clinical trials at multiple sites (60,61).

Evaluating RO for receptors on solid tissues can be difficult if not impossible. A great level of variability resulting from the cellular homogenization process prior to antibody staining for flow cytometry is often observed. Currently, a number of approaches have been attempted. While all show promise, they each come with major limitations. Tumor biopsy evaluation with fine-needle aspirations or tissue dissections is confounded by intracellular and nonspecific staining, and is limited by low quantitative capability of immunohistochemistry (IHC). Alternative strategies, at least in animal studies, include performing surrogate RO on encapsulated cells implanted subcutaneously (62,63) or direct bioluminescent tissue imaging using intravenous injected fluorescence-labeled, receptor-specific antibodies in live animals (64,65). In addition, the most plausible route to tissue-based RO assays may be based on advances in the coupling of immunocytochemical methods with high-resolution laser ablation linked mass cytometry, such as CyTOF mass cytometry imaging (66).

Thus far, RO measurements have been primarily used for guiding dose selection. With improvements in quantification accuracy and sample stability, receptor quantification in disease tissues (such as tumor biopsies) could potentially be an alternative method to IHC, broadening the utility of RO in drug development.

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