Sensitivity and drug tolerance of antidrug antibody assays in relation to positive control characteristics

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Aim: Detection and characterization of antidrug antibodies (ADA) play a key role in understanding the relation of ADA with safety and efficacy. Positive controls (PCs) are used to estimate the sensitivity and assay sensitivity in the presence of drug (drug tolerance) of assays to detect ADA. We investigated a number of factors that may drive sensitivity and drug tolerance. **Results:** We found no correlation between affinity and sensitivity and sensitivity in the presence of drug in multiple assays with two antibody–drug conjugates. Multiple factors that influenced sensitivity and drug tolerance were observed, yet these had limited overall predictive value for all investigated assay formats, PCs and compounds. **Conclusion:** Assay sensitivity and drug tolerance as studied here, are likely driven by multiple factors such as cut point confidence level, stoichiometry of PC–drug complexes and presentation of epitopes.

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Risks and clinical impact associated with unwanted immunogenicity to therapeutics have been extensively described in literature and guidances [1–5]. Detection and characterization of anti drug antibodies (ADA) play a key role in understanding the relation of ADA with safety and efficacy [1–5]. In regulatory guidance, titer and neutralizing ability are mentioned as two important characteristics of an ADA response. Other characteristics such as affinity of ADA are mentioned as a parameter that may be characterized on a case-by-case basis [2,6]. However, formation and characteristics of immune complexes may dictate effects on safety and efficacy, although these could be difficult to study *in vivo*, as reviewed by Rojko *et al.* [7]. *In vivo*, complex formation of ADA or surrogate ADA plays an important role in clearance, where even megadalton complexes may be formed [8,9]. However, these immune complexes do not necessarily lead to immediate toxicological findings, such as hypersensitivity-related adverse events [7,10].

The complexities of ADA detection and dependency on formats have been described in various reviews, for example, by Gunn *et al.* [11] and Wadhwa *et al.* [12]. Often, drug hyperimmunized animal-derived positive controls (PCs) are used as surrogate ADA. These PCs are used to estimate the relative assay sensitivity of an assay. Sensitivity may be defined as concentration at which the PC is predicted to be detected with a statistically derived 95% certainty or as a (geometric) mean [13]. However, drug (drug-on-board [DOB]) still present in a sample can hinder ADA detection. Drug tolerance of an assay may be defined as the ability of the assay to detect ADA in the presence of a defined concentration of drug, however, calculation of drug tolerance is currently ill defined [14]. As sensitivity and drug tolerance are dependent on the PC used, they are both relative and may not be predictive for ADA in preclinical species and human subjects at all sampling time points.

It has been suggested in general terms in literature that the use of a high-affinity PC will produce a better sensitivity value than the use of a lower-affinity PC in the same assay [13,15–17]. Few studies have been published on the relation between affinity and sensitivity, for example, Li *et al.* [18] reported a trend, not a correlate, between

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PC affinity and assay sensitivity. Liang *et al.* [19] reported four PC antibodies where a decrease in affinity negatively impacted sensitivity. Furthermore, it has been suggested that the relation for drug tolerance and affinity is inverse [17]. Although in theory affinity may be a predictor for drug tolerance and sensitivity of a PC, more parameters may influence detection of PC. When characteristics of a PC could be linked to sensitivity and drug tolerance in an ADA assay, it could be used to predict which ADA would be detected in an assay and which would fail to do so.

Here we describe a study in which we investigated the correlation between PC characteristics potentially key in binding to a drug with assay drug tolerance and sensitivity. As antibody drug conjugates (ADCs) are multimodality therapeutics, they were considered as an attractive compound to which multiple different monoclonal and polyclonal PCs could be generated. In this study two model compounds (ADC1 and ADC2) were assessed in screening and confirmatory assays using several assay formats: in other words, a homogeneous bridging assay on the Meso Scale Diagnostics llc (MSD, MD, USA) platform, a homogenous bridging assay in solution using the AlphaLISA system and a sequential assay using surface plasmon resonance (SPR) as readout. It should be noted that both the SPR and AlphaLISA-based assays do not use a wash step prior to readout and as such should be able to detect low-affinity interactions. It is not the intent of this study to make a comparison between the platforms, but instead use them as model systems to study the relation between binding characteristics of PCs and relative sensitivity, both with and without DOB. ADC1 and multiple polyclonal as well as monoclonal PCs were evaluated in a bridging electrochemiluminescence-based assay (ECLIA), bridging AlphaLISA-based assay and direct SPR-based assay. Drug tolerance and sensitivity of ADC2 and multiple PCs were evaluated in a bridging AlphaLISA-based assay only.

Materials & methods

ADCs & PC

mAb1 and mAb2 are humanized monoclonal antibodies (mAb, Sython Biopharmaceuticals BV, Nijmegen, The Netherlands). The mAbs were conjugated with duocarmycin-based linker-drugs (payloads) and the resulting ADC1 and ADC2 were purified as described previously for other ADCs [20]. A Drug Antibody Ratio of 1 (DAR1) species from ADC1 was purified from other DAR species by hydrophobic interaction chromatography (purity >96%). PCs were obtained from immunized test animals, except for PC8 which was obtained from Biorad's (Puchheim, Germany) HuCAL library. PCs were affinity purified and found equal to greater than 95% pure as determined by SDS-PAGE (data not shown). Reactivity of PC was determined based on the immunogen and counter screen (where applicable: ADC, protein carrier conjugated payload, protein carrier, mAb and isotype control). Reactivity toward ADC as described in Table 1 indicates the potential for reactivity toward neo-epitopes formed after conjugation next to mAb and payload.

SPR-based ADA assay

Analysis were performed using a Biacore T200 instrument (GE healthcare, IL, USA), capture buffer was 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.4 with 150 mM NaCl, 3 mM EDTA and 0.005% Surfactant P20, running buffer was identical to capture buffer but contained 0.5 M NaCl. Antimouse IgG (GE Healthcare, BR-1008-38) was immobilized on one flow channel of a CM5 chip (GE Healthcare, 29149603) according to the manufacturer's instructions (amine coupling kit, GE Healthcare, IL, USA, BR-100050). In one cycle mouse antibiotin (10 µg/ml in capture buffer, 5 µl/min, 240 s) was captured, followed by capture of biotinylated ADC (20 µg/ml in capture buffer, 5 µl/min, 90 s). Sample (K2-EDTA plasma 1:10 diluted with running buffer) was injected (10 µl/min, 300 s) after which dissociation was followed for 20 s. The difference between baseline signal and signal after injection was used as sample response. Surface regeneration (30 µl/min) was performed using 10 mM glycine-HCl pH 1.7 (120 s), 25% ethylene glycol pH 11 (90 s) and running buffer (30 s). Needle wash was performed by dipping the needle in 20% isopropyl alcohol (IPA) in 5 mM NaOH. For the confirmatory assay the ADC spike was set at 30 µg/ml.

ECLIA-based ADA method

All incubation steps were carried out at ambient temperature in a sealed microtiter plate on a plate shaker (350 revolutions per minute [RPM]). Streptavidin gold plates (MSD, L15SA1) were blocked with 200 µl blocking solution (0.05 M HEPES, 150 mM NaCl, 2% Casein pH 7.4), incubated for 2 h and washed four-times with 300 µl phosphate-buffered saline/0.05% Tween 20. Samples (K2-EDTA plasma) underwent acid dissociation (30 µl 0.3 M acetic acid to 20 µl sample) for 20 min. Just prior to application in the assay, 900 µl master mix was added to 2100 µl Tris pH 8.5 of which 50 µl was added to each acidified sample (2 h incubation of the sample

Table 1. Screening assay sensitivity (without drug-on-board).

PC number	Clonality	Reactivity	SPR (Biacore)		ECLIA (MSD)		AlphaLISA (Envision)			
			ADC1		ADC1		ADC1		ADC2	
			Screening assay (ng/mL)	Confirmatory assay (ng/mL)	Screening assay (ng/mL)	Confirmatory assay (ng/mL)	Screening assay (ng/mL)	Confirmatory assay (ng/mL)	Screening assay (ng/mL)	Confirmatory assay (ng/mL)
PC1	Mono	Payload	2350	966	ND	ND	6108	11196	140	1261
PC2	Mono	Payload	170	114	ND	ND	27	35	23	53
PC3	Mono	Payload	ND	ND	ND	ND	4	7	ND	ND
PC4	Mono	Payload	415	393	1613	3014	3010	2213	35	79
PC5	Poly	ADC	658	186	ND	ND	18	30	NA	NA
PC6	Poly	ADC	456	146	164	285	14	8	NA	NA
PC7	Poly	mAb	1836	481	ND	ND	39	69	NA	NA
PC8	Mono	mAb	602	207	ND	ND	875	709	NA	NA
PC9	Mono	Payload	211	132	ND	ND	>10000 [†]	>10000 [†]	>10000 [†]	>10000 [†]
PC10	Poly	ADC	NA	NA	NA	NA	NA	NA	9	14
PC11	Poly	ADC	NA	NA	NA	NA	NA	NA	6	6
PC12	Poly	ADC	NA	NA	NA	NA	NA	NA	4	7
PC13	Poly	ADC	NA	NA	NA	NA	NA	NA	6	10
PC14	Poly	mAb	NA	NA	NA	NA	NA	NA	30	54
PC _{mix1}	Mix	mAb & payload	ND	ND	171	454	ND	ND	NA	NA
PC _{mix2}	Mix	mAb & payload	NA	NA	NA	NA	NA	NA	4	6
PC _{mix3}	Mix	mAb & payload	ND	ND	ND	ND	7	11	NA	NA
PC _{mix4}	Mix	mAb & payload	NA	NA	NA	NA	NA	NA	3	ND
PC15	Mono	Payload	NA	NA	NA	NA	NA	NA	295	ND
PC16	Mono	Payload	NA	NA	NA	NA	NA	NA	107	ND
PC17	Mono	mAb	NA	NA	NA	NA	NA	NA	1	ND

[†]Insufficient response to give a reportable result.

ADC: Antibody drug conjugate; ECLIA: Electrochemiluminescence-based assay; NA: Not applicable; ND: Not determined; mAb: Monoclonal antibody; MSD: Meso Scale Diagnostics LLC; PC: Positive control; PC_{mix1} = PC4 + PC6 (1:1); PC_{mix2} = PC2 + PC10 + PC11 + PC12 + PC13 (4:1:1:1:1); PC_{mix3} = PC2 + PC6 (1:1); PC_{mix4} = PC15:PC16:PC17 (1:1:1); SPR: Surface plasmon resonance.

mix). The master mix consisted of a 1:1 mixture of 6 µg/ml biotinylated and SulfoTag labeled ADC (confirmatory spike ADC final concentration of 25 µg/ml). The sample mix (50 µl) was transferred to the blocked streptavidin gold plate and incubated for 1 h. Subsequently the plate was washed with four-times 300 µl phosphate-buffered saline/0.05% Tween 20, 150 µl read buffer was added and the electrochemiluminescence signal was then measured on a Mesoscale Sector imager 2400 (MSD).

AlphaLISA-based ADA assay

All incubation steps were carried out at ambient temperature in a sealed microtiter plate on a plate shaker (300 RPM). Assay buffer consisted of 0.025 M HEPES, 1% Dextran T500, 0.5% Triton X100, 0.05% Proclin 300 and 1% Casein in MilliQ, pH 7.4. Exposure to white light was avoided in all steps with donor beads (green filters on light fixtures). Samples (K2-EDTA plasma) were acidified by adding 90 µl 300 mM acetic acid to 10 µl sample (30 min incubation). Master mix buffer was prepared just prior to use and consisted of 1 M Tris pH 8.5 and assay buffer in a of ratio 7:3. The master mix consisted of 0.225 µg/ml biotin-ADC, 30 µg/ml ADC conjugated acceptor beads (PerkinElmer, Rotterdam, The Netherlands, 6772002) and, when applicable 5 µg/ml confirmatory ADC drug spike. Thereafter, 10 µl of the sample mix with 20 µl master mix was transferred to a 384-well plate (PerkinElmer, 6005350) and incubated for 1 h. Streptavidin donor beads 30 µg/ml (PerkinElmer, 6760002) were added to each well (20 µl in assay buffer) and incubated for 1 h. Plates were transferred to a PerkinElmer Envision multilabel reader and read after 10 min.

Composition gradient multiangle light scattering

Composition gradients (CGs) were created using a Calypso II system (Wyatt, Dernbach, Germany) and measured in an online UV-Vis and a miniDAWN TREOS multiangle light scattering (MALS) detector.

ADC and PC samples were prefiltered with a 0.1 μm filter and diluted to a concentration of 0.02 mg/ml in a HEPES buffer at pH 7.4 with 150 mM NaCl. Sample injection was controlled by pump 1 for ADC and by pump 2 for PC samples. Analysis was carried out at ambient temperature. Each analysis started with 20 priming steps, injecting 0.1 ml of both samples separately, followed by five plateaus ranging from 0 to 80% of pump 1 (injecting 0.8 ml with a 120 s delay) to determine self-association of ADC. Thereafter, 15 single crossover steps were performed ranging from 100 to 0% of pump 1 and 0 to 100% pump 2, using plateau times of 10 min up to 16 h, depending on the time to equilibrium for each interaction pair. Thereafter, five plateaus ranging from 80 to 0% of pump 2 were measured (injecting 0.8 ml with a 120 s delay) to determine self-association of PC.

Calculations and modeling were performed using Wyatt's Calypso software. In the ADC1-DAR1 experiments data of the crossover gradient were fitted using the reversible association + cross-virial coefficients (CVC) model. Stoichiometries of 1:1 and 2:1 were allowed to be fitted. In all other CG-MALS experiments, the light scattering data were used to determine the time to equilibrium at each single plateau, the maximum Mw at equilibrium at each plateau and the maximum Mw in the complete crossover gradient, including the composition of ADC and PC at this plateau for further evaluation. In all experiments, the five plateaus of self-association at start and end of the gradient were used to verify that self-association of compounds was negligible.

AF4-UV-MALS-RI

AF4 analyses were performed using a Shimadzu auto-sampler and pump connected to a Wyatt Eclipse 3+ separation system. Detection was performed using a Shimadzu single wavelength UV detector at a wavelength of 214 nm, a Wyatt miniDAWN TREOS MALS detector and a Wyatt Optilab T-rEX differential refractive index detector. The separation was performed using a Millipore 10 kDa Mw cut-off regenerated cellulose membrane in a short channel with a length of 145 mm and a wide spacer with a thickness of 350 μm . The tests were carried out at ambient temperatures using a 10 mM HEPES buffer at pH 7.4 with 150 mM NaCl as the mobile phase. All tests were performed using a focus flow of 1.5 ml/min for 2 min. Separation was performed using a constant cross flow of 3.0 ml/min for 5 min, followed by a decrease of the cross flow from 3.0 to 0.0 ml/min in 15 min and a 10 min elution at 0.0 ml/min for another 10 min. The channel flow was kept constant at 1.0 ml/min. The PC:ADC complexes were analyzed by injecting 10 μl of pre-incubated mixtures containing 80 $\mu\text{g}/\text{ml}$ of both the PC and the ADC diluted in the mobile phase. Molar mass calculations were performed using Wyatt's Astra 6 software. The light scattering data were fitted to the Zimm formalism and the UV signal was used as the concentration signal. A specific refractive index (RI) increment (dn/dc) of 0.185 ml/g and a UV extinction coefficient of 14.3 ml/(mg cm) was applied. The UV extinction coefficient was calculated from the RI signal of four different antibodies and PCs preparations. The calculated UV extinction coefficient of each was $14.3 \pm 6\%$ ml/(mg cm).

SPR-based affinity

ADC was immobilized on a C1 chip (GE Life Sciences, Chicago, IL, USA) surface by injecting 1 $\mu\text{g}/\text{ml}$ ADC in 10 mM sodium acetate pH 6.0 for 300 s in an EDC(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)/NHS(N-hydroxysuccinimide)-activated flow channel at a flow rate of 10 $\mu\text{l}/\text{min}$. Subsequently, reactive groups on the chip surface were blocked with 1 M ethanolamine pH 8.0 according to manufacturers' protocol. Affinity analysis was performed by single cycle kinetics analysis on a Biacore T200 instrument (GE life Sciences). Baseline stabilization was set at 1 min where upon five increasing concentrations of PC in running buffer (10 mM HEPES buffer at pH 7.4 with 150 mM NaCl, 3 mM EDTA and 0.005% v/v surfactant P20) were injected. For each step an association time of 180 s was set, followed by a dissociation time of 600 s, all at a flow rate of 10 $\mu\text{l}/\text{min}$. Regeneration was performed with 10 mM glycine pH 1.5 (60 s with flow rate of 30 $\mu\text{l}/\text{min}$). Double blank subtraction was performed on the observed sensorgrams using a non-ADC (blank) immobilized reference flow channel and running buffer. Sensorgrams were fitted with a 1:1 Langmuir model for ADC2 and a bivalent analyte model for ADC1 (affinities for ADC1 and ADC2 may not be directly compared). The equilibrium dissociation constants (KD) were calculated using the Biacore T200 evaluation software (v3.1).

Statistical analysis

All analyses were performed using SAS[®] 9.4 (SAS, Huizen, The Netherlands) unless stated otherwise. P-values smaller than 0.05 were considered significant.

3D model of the relation between response & concentration DOB & PC

Iterative fits of the models described in the results section to the response data were performed in R version 3.0.2 (The R Foundation for Statistical Computing).

Calculation cut points & sensitivity

Cut point (CP) factors or CPs were calculated for each assay in line with commonly accepted statistical approaches [13]. For the screening assays CP factors (CPFs) were determined to be able to calculate a run specific CP. Fixed CPs were calculated for the confirmation assays. To determine the CPFs or CPs of the ECLIA-based and AlphaLISA-based ADA assays, two technicians analyzed 51 individual plasma samples on 3 days for a total of six runs (three NC samples per plate, all samples in duplicate). In contrast, for the SPR-based ADA assay two technicians executed two runs on separate days on two instruments for a total of eight runs (samples in singlet, nine NC samples distributed evenly in the run). The calculated CPs were interpolated on PC curves (with and without DOB) and the (geometric) mean PC concentration was determined per run. All PCs were evaluated in six runs in all ADA assays with the exception of PC4 and PC6 (single run) in the MSD-based ADA assay, PC15–17 and PC_{mix4} in the AlphaLISA-based ADA assay (three runs) and a head-to-head comparison of DOB in the SPR-based assay (single run). The mean sensitivity and the corresponding 95% upper limit were calculated for both the screening assays and the confirmation assays.

Calculation hill slope for drug tolerance assessment

The sensitivities (per DOB concentration) of the screening assay and the confirmatory assay are plotted against DOB, overlaid in one plot. The relation between the sensitivity and DOB (both in natural logarithmic scale) are estimated by assuming a four-parameter logistic model:

$$f(x) = D + \frac{(A - D)}{1 + e^{B \cdot (C - \ln \text{conc DOB})}}$$

A is the lower asymptote, D is the upper asymptote, C is the point of inflection (point on the S-shaped curve halfway between A and D) and B is the slope parameter of the curve. ‘lnconcDOB’ is natural logarithm of the concentration of DOB. In calculation of the hill slope to describe the relation between sensitivity and DOB, the linear part of the curve was used to fit linear equation model. The slope of the linear equation was used as a measure for the drug tolerance.

Parallelism hill slope for drug tolerance assessment

For the screening assay and the confirmatory assay, it was investigated if the four-parameter dose response models of both assays were parallel. Therefore, full models and reduced models were fitted. In the full model, a four-parameter model was fitted for the screening assay and the confirmatory assay separately. In the reduced model, the lower asymptote (A) and upper asymptote (D) were estimated separately for the screening assay and confirmatory assay, but the slope (B) and point of intersection (C) were kept the same for the screening assay and confirmatory assay. A log likelihood ratio test (LRT-test) was performed to investigate if the full model and reduced model differ significantly. In case the LRT-test was not significant ($p > 0.05$), the curves of the screening assay and the confirmatory assay are considered parallel.

Correlation affinity with sensitivity & hill slope

Affinity and coefficient of variation (CV%) were estimated using a general linear model. The analysis was performed on the natural logarithmic transformed observed KD (KD_{obs}). The least squared mean affinity (log scale) was back transformed to give the geometric mean affinity. The estimated residual variation (log scale) was back transformed to give the CV% per PC. The calculated (mean) affinity was plotted against the sensitivity or hill slope for drug tolerance, both in natural logarithmic scale. The Pearson correlation coefficients were calculated and correlation coefficients with a p-value smaller than 0.05 were considered significant.

Correlation CG-MALS complex formation parameters with sensitivity & hill slope (drug tolerance)

Pearson correlation was assessed in Minitab® version 17.2.1 (Minitab, Inc). Maximum average MW at complex equilibrium, the time to reach equilibrium and the ratio between ADC and PC which gave the maximum light scattering signal (ratio ADC:PC in complex) were plotted against sensitivity (natural log scale) and hill slope (drug tolerance).

Factor analysis

A factor analysis (varimax rotation) was performed for the affinity parameters KD_{obs} off-rate an on-rate and the complex-binding parameters maximum observed MW (maxMW), time to equilibrium at maxMW and ratio PC to ADC at maxMW. The correlation was determined (Pearson correlation coefficient) between the first three principal components with the hill slope and the mean sensitivity (log-scale).

Results & discussion**Sensitivity**

Relative sensitivity (without DOB) has been defined as the concentration of the PC at which the assay result is expected to be above the CP at least 50% of the time, but preferably 95% of the time [13], when reporting sensitivity in validation. However, a geometric mean (or arithmetic mean) may be more robustly estimated than a confidence limit, especially when the number of observations is limited, therefore, mean sensitivity (natural log scale) is used in further analysis on correlations with PC–drug binding characteristics. PCs (PC1–PC14) used in these studies, with the associated reactivity and clonality, are listed in Table 1. The PCs used in this study are from both monoclonal as well as polyclonal origin and bind to different epitopes on the ADCs, in other words, the payload, the mAb or ADC. The polyclonal PC to the ADC may encompass reactivity to the payload, mAb and/or neo-epitopes formed after conjugation. Taken together, this set of PC formed a representative sample for the investigations conducted in this study. In a similar fashion sensitivity of an assay in presence of DOB may be calculated, even though, formally the term sensitivity in ADA assay validation usually refers to relative sensitivity without DOB. As of here, the term sensitivity will be used both in presence or absence of DOB, as indicated in each instance. The MSD-based ECLIA is a commonly used platform in the industry. The assay for ADC1 was established after thorough optimization (buffer composition, ratio-labeled drugs, labeling conditions). However, limited data were gathered using the ECLIA, as surprisingly, unacceptable differences in sensitivity PC curves were found between batches of streptavidin gold coated plates for ADC1, which had not been observed previously with other compounds (data not shown). In spite of the limited data gathered on the platform, the authors included it as a point of reference for the SPR and AlphaLISA-based assays.

Sensitivity (without DOB) was determined for multiple PC (PC1–PC14); some of which could be evaluated for both ADC1 and ADC2. Clear differences in sensitivity for PCs as listed in Table 1 could be observed in each assay. Of note, the ranking in sensitivity of the three assays differs between PCs, for example, as observed for PC4 and PC6. Interestingly, when mixing PC, the resulting PC mix showed similar sensitivity as for the most sensitive PC in the mix (see Table 1), thereby demonstrating that sensitivity, as investigated here, is not governed by simple additive processes.

Overall, in the AlphaLISA and ECLIA bridging assay, the confirmatory assay was less sensitive compared with the screening assay. This may not be surprising as the CP for the confirmatory assay is based on a 99% confidence level and the CPF for the screening assay is at a 95% confidence level. This difference in confidence has an impact on the sensitivity, as illustrated in Supplementary Figure 1, showing that sensitivities are more similar overall if the same 95% confidence limit is applied in both assays (assuming PC response curve characteristics are similar for both the screening as well as the confirmatory assay). However, in the SPR-based assay the confirmatory assay appeared more sensitive than the screening assay. Upon closer inspection this may be attributed to the greater variance and thus broader distribution in response found for samples of individuals in the screening assay compared with the confirmatory assay. The spiked and nonspiked responses closely correlated (with a systematic difference, Pearson correlation of 0.99, data not shown) and thereby the resulting response (difference on the natural log scale between spiked and nonspiked results) had a narrow distribution, resulting in a more sensitive assay. This was not the case for the other assays.

A comparison between PC1 and PC4 in the same assay format (AlphaLISA) showed dissimilar sensitivities for ADC1 and ADC2 (Table 1), yet PC2 showed similar sensitivity. PC1 and PC4 recognize a similar epitope on the

payload, which differs from the epitope recognized by PC2. Likely, presentation of an epitope may impact the reported sensitivity of a PC.

Drug tolerance

In general, increasing levels of DOB will limit the amount of ADA that can be detected. The relation between DOB and observed sensitivity is often not linear in nature, as may be expected for protein–protein interactions. It should be noted that in this case the term sensitivity is also used to describe assay sensitivity in the presence of drug. Below we describe a number of approaches with which the relation between response and the concentration of PC and DOB may be described. We investigated each of them and provide justification for the selected approach.

3D model of the relation between response & concentration DOB & PC

The relation between assay response and the concentration DOB and PC may be described by a 3D model. In Supplementary Figure 2, an example of the relation between PC concentration and DOB concentration to response is given. As a proof of principle, we created a function describing the screening assay response for a PC (mix of PC2 and PC6) and different concentrations DOB (ADC1). In the model, a parameter termed (1) $x.mid(Drug)$ was calculated using a separate model (2), whereas drug represents the concentration DOB. The fitted values of the response for the asymptotic upper and lower limits (A, B) and scale were constant in model (1). The parameter $x.mid$ from the response model (2) depends on the concentration drug and was best described by a three-parameter logistic curve. The parameter is used to model the effect of DOB on response.

$$\ln(response) = A + (B - A) / (1 + \exp(\frac{x.mid(Drug) - \ln.PC}{scale})) \quad (1)$$

$$x.mid(Drug) = Asym \times (1 - \exp(-Drug \times R_0)) + lrc \quad (2)$$

Two independent datasets were used with the same PC and ADC; one to establish the model, the second (data not shown) to verify validity of the model. Function coefficient values were fitted and were listed in Supplementary Table 3. Overall, the fitted coefficients were similar, thereby validating the approach. In general, the advantage of this approach was that, using the assay CP value, at any given drug concentration the concentration of PC that may be detected could be estimated, or vice versa, irrespective of the DOB and PC levels used to establish the model (Supplementary Table 3). A disadvantage is that modeling would require an extensive dataset at a range of DOB and PC concentrations. In addition, the required statistical capabilities may not always be available in bioanalytical labs. Furthermore, it assumed limited variance between runs, which will not be the case for most screening assays (run-specific CP). Hence, this approach may be of limited practical use in defining relative assay drug tolerance.

Fixed DOB or PC concentration

Another approach that may be used is to define drug tolerance for a fixed concentration of PC in a similar fashion as for relative sensitivity. This may be done by taking a concentration range of drug spiked to a fixed concentration of PC. Drug tolerance may then be defined as the (geometric) mean or an appropriate confidence limit of the concentration of drug (DOB) where the PC (fixed concentration) is still found positive. For example, in the ECLIA 500 ng/ml PC (mix of PC4 and PC6) was still confirmed positive in presence of 3 µg/ml DOB (geometric mean, data not shown). Alternatively, a PC concentration range spiked to a fixed concentration of drug can be assessed, in other words, the sensitivity at a given level of DOB. The advantage is that calculation of drug tolerance is relatively straightforward; however it provides little information on drug tolerance at different concentrations of PC and drug.

Relative sensitivity as function of DOB

When relative sensitivity is plotted against DOB, a nonlinear model may be adequate to describe the relation. However, within a restricted range of DOB the relation may be approximated by a linear model (deviation from linearity may be observed at DOB concentrations close to zero). An example of the relation between relative

Figure 1. Example of the relation between sensitivity and drug-on-board. Example of the relation between sensitivity in presence of DOB (ng/ml) for PC13 with ADC2 in the AlphaLISA-based assay. DOB: Drug-on-board.

sensitivity and DOB is given in Figure 1. The linear relation between sensitivity and concentration DOB (both in natural log-scale) at a fixed concentration of the PC may be represented by the hill slope (Figure 1). A steeper hill slope (greater number) translated to a greater influence of DOB on detection of PC (low drug tolerance) was compared with a more shallow hill slope (high drug tolerance). The disadvantage of this approach is that it would require a dataset consisting of multiple DOB and PC concentrations. The advantage was that the calculation is relatively straightforward and it captures the drug tolerance of the assay over a broad range of DOB concentrations. Therefore, for our assessments into drug tolerance we have selected the hill slope of the linear part of the function describing the relation between the concentration DOB and the associated relative sensitivity, as parameter for further analysis. The homogeneous bridging assay with AlphaLISA readout was chosen as a model system to fully characterize the drug tolerance of different PCs for ADC1 and ADC2. In addition drug tolerance of the SPR-based method was determined in a more limited approach for the screening and confirmatory assay, due to assay throughput. PC curves with 0 and 1000 ng/ml DOB were assessed head-to-head. The reduction in sensitivity was expressed as a ratio, in other words, the difference in sensitivity divided by the difference in DOB. Although this ratio may also be considered a hill slope as these ratios could not be compared directly to the hill slopes determined in the AlphaLISA-based method as linearity cannot be assessed, however, they may be considered reflective of the impact of DOB.

Drug tolerance, expressed as the hill slope of the linear relation between sensitivity and concentration DOB was determined for multiple PCs (PC1–PC14) in the AlphaLISA-based assay. Clear differences (up to fourfold) in hill slope between PCs can be observed in each assay (Table 2). No significant differences between hill slopes (AlphaLISA-based assay) were found between the screening and confirmatory assay for PC–ADC combinations when tested for parallelism (LRT-test, $p > 0.15$ was found for each comparison, data not shown). Hence, even though absolute sensitivity levels may differ between the PC, the impact of DOB seems similar in the screening and confirmatory AlphaLISA-based assays. Differences (uptil and greater than tenfold) in the impact of DOB between PC were also observed in the SPR-based method (Table 2).

Table 2. Drug tolerance expressed as the hill slope of sensitivity and drug-on-board.

PC number	SPR (Biacore)		AlphaLISA (Envision)			
	ADC1		ADC1		ADC2	
	Screening assay ratio	Confirmatory assay ratio	Screening assay hill slope	Confirmatory assay hill slope	Screening assay hill slope	Confirmatory assay hill slope
PC1	0.56	0.38	0.30	-0.02 [†]	0.25	0.28
PC2	0.06	0.31	1.17	1.44	1.02	0.96
PC3	ND	ND	0.69	0.75	ND	ND
PC4	0.16	0.33	0.35	0.32	0.79	0.72
PC5	1.06	1.19	0.97	1.11	NA	NA
PC6	1.87	1.96	0.96	0.98	NA	NA
PC7	6.05	2.31	0.81	0.88	NA	NA
PC8	0.59	0.78	0.53	0.55	NA	NA
PC9	0.01	0.05	ND	ND	ND	ND
PC10	NA	NA	NA	NA	1.01	1.02
PC11	NA	NA	NA	NA	0.78	0.80
PC12	NA	NA	NA	NA	0.89	0.88
PC13	NA	NA	NA	NA	0.85	0.95
PC14	NA	NA	NA	NA	0.77	0.67

[†]Hill slope found to be not deviating from zero.

ADC: Antibody drug conjugate; NA: Not applicable; ND: Not determined.; PC: Positive control; SPR: Surface plasmon resonance.

Correlations of binding characteristics to sensitivity & drug tolerance

Observed affinity

It was not unexpected that PCs behaved differently in ADA assays. As it has been suggested that affinity correlates to relative sensitivity [13,15–18], we wanted to investigate this for our model systems. Furthermore, it was investigated whether or not a correlation between drug tolerance and affinity could be found. For PC5–7 and PC10–14 affinity cannot be determined as these are polyclonal in nature. Furthermore, even though the affinity of an interaction may be determined well using various techniques and instruments [21,22], there may be intrinsic differences between methods or experimental setups leading to markedly different reported values [23,24]. However, an observed affinity (KD_{obs}) for polyclonal PCs may be determined in the same fashion as affinity for the monoclonal PCs using multiple analytical runs. When methodology and experimental setup are kept the same, it would allow for a head-to-head comparison of the observed affinity and sensitivity of the different PC. It must be noted that both ADC1 and ADC2 (ligand) likely contain two or more epitopes for each bivalent PC (analyte). Preferably, analysis conditions should have allowed a 1:1 Langmuir model fit to the sensorgram data; however, given the potential multivalent nature of the ligand–analyte interaction, this was difficult. Optimization of the analysis conditions allowed for ADC2 sensorgrams to be fitted well to the 1:1 Langmuir model. We were not successful in finding conditions where this would be the case for all ADC1–PC interactions (data not shown). Therefore, sensorgram data for ADC1 were fitted to the bivalent analyte model and the first order KD was reported as KD_{obs} (see Supplementary Figure 4 for an example sensorgram and fit). A direct comparison between KD_{obs} for ADC1 and ADC2 may therefore not have been valid; however, the KD_{obs} could be used for comparisons for each ADC individually. The observed affinities of PCs (PC1–PC14) to ADC1 and ADC2, respectively were found to range from approximately 10^{-8} to 10^{-11} M and were summarized in Table 3. Analysis of correlation between affinity and sensitivity was performed for both the screening and confirmatory assay. In Figure 2, the natural log of the sensitivity and KD_{obs} is plotted against each other for the AlphaLISA-based and SPR-based methods. The ECLIA-based method was excluded as only two PCs were assessed. Interestingly, no significant correlations between sensitivity and observed affinity were found for screening and confirmatory assays (Pearson correlation coefficients; $p > 0.47$). Furthermore, no significant correlations between sensitivity and drug tolerance, expressed as hill slope, were found (Figure 2, Pearson correlation coefficients; $p > 0.45$). It should be noted that affinity values found for ADC2 were relatively closely grouped ($\sim$ within 1 log unit) and could therefore obscure potential correlations when PCs with a wider range of affinities were available. No distinction between polyclonal and monoclonal PCs were made given that data would be too limited in number and range to draw sound conclusions. An exception was ADC1 and analysis using mAbs

Table 3. Geometric (least squares) mean of observed affinities.

PC	ADC1	N	ADC2	N	CV (%)
PC1	1.7E-10	7	>1.7E-10 [†]	NA	25
PC2	3.5E-10	10	3.8E-10	5	36
PC3	1.7E-09	7	ND	NA	10
PC4	7.3E-09	12	5.5E-10	5	18
PC5	1.6E-09	13	NA	NA	25
PC6	1.2E-09	9	NA	NA	5
PC7	7.4E-09	8	NA	NA	88
PC8	8.2E-11 [‡]	4	NA	NA	162
PC9	3.1E-08	9	ND	NA	1
PC10	NA	NA	2.2E-10	4	52
PC11	NA	NA	2.2E-10	5	62
PC12	NA	NA	2.5E-10	15	33
PC13	NA	NA	2.7E-10	5	57
PC14	NA	NA	7.5E-10	5	77

[†]Affinity was close to the instrument limits and could not be reliably determined mainly due to the slow off-rate. The affinity as observed for ADC1 was taken as an estimate for the affinity.

[‡]Kinetic rate constants close to the instrument limits.

ADC: Antibody drug conjugate; CV: Coefficient of variation; NA: N: Number; Not applicable; ND: Not determined; PC: Positive control.

only (insufficient polyclonal antibody results); yet no significant correlation or trend with affinity was observed (data not shown). Furthermore any generalized statement on correlation should hold true for both polyclonal and mAbs as both are used by bioanalytical scientists.

Stoichiometry of complexes

Affinity of PCs in itself appeared a poor predictor for establishing ranking of PCs with respect to relative sensitivity and drug tolerance in this study. Therefore, other factors were investigated that may steer behavior of PCs in ADA assays. Bridging assays require multivalency of an ADA or PC to result in a signal. IgG4 will show antibody arm exchange resulting in an apparent monovalent antibody, hence bridging assays intrinsically lack the ability to detect IgG4 ADA when antibody arm exchange has occurred [25,26]. Even though no monovalent PCs were used, some PCs or ADA may be less capable of bridging between drugs.

Determination of the stoichiometry of multivalent ligand–antibody complexes may be challenging, even more so for polyclonal antibodies. Therefore, the ability of monoclonal PCs to bridge two molecules was investigated first. In theory the ADCs possess two epitopes to which a monoclonal PC to the mAb may bind, and in case of the PCs against the toxin, they may even have more epitopes per molecule available. To look at the intrinsic ability to bridge two molecules, a variant of ADC1 that putatively had a single linker-drug attached was produced (ADC1-DAR1, 96.4% pure as assessed by HIC, 3% DAR2 impurity). We investigated complex formation of ADC1-DAR1 and PC1, PC2, PC4 and PC9. CG-MALS allows estimation of average MW of molecules and complexes formed in solution [27]. As only monovalent 1:1 or 2:1 (ADC1-DAR1: PC) complexes may putatively be formed, modeling may be relatively straightforward. The modeled CG-MALS data show that PC9 may have difficulty bridging two ADC1-DAR1 molecules, resulting in the predominantly monovalent behavior of PC9 (see Figure 3D). This would suggest a putative low sensitivity in bridging assays. In our models, PC1, PC2 and PC4 clearly show the ability to bind two ADC1-DAR1 molecules (Figure 3E, F & G). Although PC1, PC2 and PC4 complex formation with ADC1-DAR1 did not fit to a 1:1 model (Figure 3A, B & C), complex formation of PC9 with ADC1-DAR1 may be well described by a 1:1 model (Figure 3D). For illustrative purposes, the 2:1 model of PC9 with ADC1-DAR1 is shown in Figure 3H, which as expected with a more elaborate model also fits well, attributing a minor fraction to 2:1 binding (please note that ADC1-DAR1 still contains 3% DAR2 impurity). An advantage of CG-MALS is that complex formation may be followed in real time (an example is shown in Supplementary Figure 5), without a separation step that may potentially influence the observed complexes. However, the drawback is that the lack of a separation on MW will result in information on the mean MW only. In order to confirm the stoichiometry results by CG-MALS, ADC1-DAR1 complexes with PC1, PC2 and PC9 were analyzed using AF4-UV-MALS-RI. In all chromatograms a peak at monomers PC and ADC MW was observed. For PC1, peaks at approximately 300 and

Figure 2. Correlation between sensitivity and drug tolerance with affinity. Plots of affinity to (A) sensitivity of the SPR-based screening assay (ADC1), (B) sensitivity of the SPR-based confirmatory assay (ADC1), (C) sensitivity of the AlphaLISA-based screening assay (ADC1), (D) sensitivity of the AlphaLISA-based confirmatory assay (ADC1), (E) sensitivity of the AlphaLISA-based screening assay (ADC2), (F) sensitivity of the AlphaLISA-based confirmatory assay (ADC2), (G) drug tolerance (ratio) of the SPR-based screening assay (ADC1), (H) drug tolerance (ratio) of the SPR-based confirmatory assay (ADC1), (I) drug tolerance (hill slope) of the AlphaLISA-based screening assay (ADC1), (J) drug tolerance (hill slope) of the AlphaLISA-based confirmatory assay (ADC1), (K) drug tolerance (hill slope) of the AlphaLISA-based screening assay (ADC2), (L) drug tolerance (hill slope) of the AlphaLISA-based confirmatory assay (ADC2). ADC: Antibody drug conjugate; SPR: Surface plasmon resonance.

450 kDa were found, in other words, 1:1 and 2:1 complexes (ADC1-DAR1:PC, see Supplementary Figure 5C). For PC2, a complex of predominantly 450 kDa was observed (Supplementary Figure 6, up to megadalton complexes are observed when incubated with ADC1, data not shown). Both are in agreement with the stoichiometry data found with CG-MALS. However, no evidence for the presence of 2:1 complexes for PC9 with ADC1-DAR1 was found. CG-MALS data suggested predominant 1:1 complexes for PC9, however, identification of the formed complexes with AF4 showed to be less unambiguous. Next to the monomer peak of approximately 150 kDa a complex was observed with a mass of approximately 190–210 kDa. Such a complex is not expected based on the mass of both the PC and ADC1-DAR1. Furthermore, as the retention time at which the complexes elute is related to its hydrodynamic volume, the relation of mass versus hydrodynamic volume seems to be different for PC9 compared with PC1 and PC2 complexes as found by UV signal. This may suggest the presence of another mechanism not accounted for in the analysis such as changes in complex equilibrium during elution. The AlphaLISA-based bridging assay showed a sensitivity of > 10,000 ng/ml for PC9, yet for an SPR-based direct assay, where 1:1 stoichiometry is already sufficient for detection, sensitivity was approximately 250 ng/ml. Sample incubation times can play a role in the observed relative sensitivity [14]. Increasing sample incubation time (up to 4 h) for PC9 in the AlphaLISA assay did not result in any increase in response above background. Together these data suggest that PC9 shows an apparent monovalent behavior and as such has difficulty bridging two ADC molecules.

Figure 2. Correlation between sensitivity and drug tolerance with affinity (cont.). Plots of affinity to (A) sensitivity of the SPR-based screening assay (ADC1), (B) sensitivity of the SPR-based confirmatory assay (ADC1), (C) sensitivity of the AlphaLISA-based screening assay (ADC1), (D) sensitivity of the AlphaLISA-based confirmatory assay (ADC1), (E) sensitivity of the AlphaLISA-based screening assay (ADC2), (F) sensitivity of the AlphaLISA-based confirmatory assay (ADC2), (G) drug tolerance (ratio) of the SPR-based screening assay (ADC1), (H) drug tolerance (ratio) of the SPR-based confirmatory assay (ADC1), (I) drug tolerance (hill slope) of the AlphaLISA-based screening assay (ADC1), (J) drug tolerance (hill slope) of the AlphaLISA-based confirmatory assay (ADC1), (K) drug tolerance (hill slope) of the AlphaLISA-based screening assay (ADC2), (L) drug tolerance (hill slope) of the AlphaLISA-based confirmatory assay (ADC2). ADC: Antibody drug conjugate; SPR: Surface plasmon resonance.

Complex formation

Complex formation of PCs and ADCs may be driven by multiple processes. Factors such as complex formation kinetics, ratio between components constituting a complex and size of the complex may play a role in the response of a PC in an assay. Although it may play an important role, it was not possible to study complex formation in assays directly. Hence, we investigated ADC–PC complex formation characteristics by a number of parameters as determined by CG-MALS. CG-MALS may be used to determine the maximum average MW at complex equilibrium and the time to reach equilibrium. As modeling of the interaction was not feasible, given the potential number of different complexes formed, the ratio between ADC and PC which gave the maximum light scattering signal (ratio ADC:PC in complex) was determined as a measure of complex composition. Numbers smaller than one reflected reaction mixtures where more PC is present compared with ADC, where the hypothesis was that more PC is incorporated in complexes compared with ADC. It must be noted that these are surrogate parameters as they may not have captured multivalent binding in the investigated assays in all its complexity.

Sufficient material of PC1, PC2, PC4, PC6, PC8 and PC9 was available to assess these selected complex formation parameters with ADC1 and results are listed in Supplementary Table 7. Subsequently, correlation of these parameters to sensitivity and drug tolerance was investigated for the AlphaLISA-based and SPR-based ADA methods. No correlations were found for the SPR-based method ($p > 0.05$, data not shown), however for drug

Figure 2. Correlation between sensitivity and drug tolerance with affinity (cont.). Plots of affinity to (A) sensitivity of the SPR-based screening assay (ADC1), (B) sensitivity of the SPR-based confirmatory assay (ADC1), (C) sensitivity of the AlphaLISA-based screening assay (ADC1), (D) sensitivity of the AlphaLISA-based confirmatory assay (ADC1), (E) sensitivity of the AlphaLISA-based screening assay (ADC2), (F) sensitivity of the AlphaLISA-based confirmatory assay (ADC2), (G) drug tolerance (ratio) of the SPR-based screening assay (ADC1), (H) drug tolerance (ratio) of the SPR-based confirmatory assay (ADC1), (I) drug tolerance (hill slope) of the AlphaLISA-based screening assay (ADC1), (J) drug tolerance (hill slope) of the AlphaLISA-based confirmatory assay (ADC1), (K) drug tolerance (hill slope) of the AlphaLISA-based screening assay (ADC2), (L) drug tolerance (hill slope) of the AlphaLISA-based confirmatory assay (ADC2). ADC: Antibody drug conjugate; SPR: Surface plasmon resonance.

tolerance (hill slope) a significant correlation was found with the ratio ADC and PC in complex for the AlphaLISA-based assay (Figure 4B & D, Pearson correlation, $p = 0.009$ and $p = 0.003$, respectively), where lower values are associated with a greater hill slope (less drug tolerant). A trend was observed for screening and confirmatory assay sensitivity with the ratio ADC and PC in complex (Figure 4A & C, Pearson correlation, $p = 0.054$ and $p = 0.089$, respectively), where lower values are associated with better sensitivity (lower concentrations PC). As signal in the AlphaLISA is driven by formation of complexes incorporating donor and acceptor beads it is attractive to speculate that when limited PC compared with (labeled) ADC, as bridging partners, is required to form larger complexes it may result in a better sensitivity compared with a PC where this is not the case. The opposite may be true for drug tolerance where DOB may more easily compete with labeled ADC.

A prospective study with ADC2 in the AlphaLISA-based assay was performed to further investigate the trend for sensitivity. Three new PC were generated (PC15, PC16 and PC17) for which sensitivity was determined (see Table 1) as well as complex formation parameters by CG-MALS to complement PC1, PC2, PC4 and PC12 (see Supplementary Table 7). No correlation or trend was observed for the ratio of ADC and PC in complex with sensitivity. Furthermore no correlate or trend was found for drug tolerance (hill slope). This suggested that the complex formation parameters as determined by CG-MALS in this study have limited predictive value for sensitivity and drug tolerance in general.

Figure 3. Complex formation analysis by composition gradient-multiangle light scattering of PC1, 2, 4 and 9 with ADC1-DAR1. CG-MALS results of PC1, 2, 4 and 9 with ADC1-DAR1 are shown in panels (A)/(E), (B)/(F), (C)/(G) and (D)/(H), respectively. In blue are the measured light scattering signals, in red the fitted model based on reversible association an CVC, allowing a 1:1 stoichiometry (contribution to total fit in purple) and a 2:1 stoichiometry (contribution to total fit in grey). Component A (ADC1-DAR1) decreases from 100 to 0% in plateaus 1–15, indicated in dark green, whereas component B (PC) increases from 0 to 100% in plateaus 1–15, indicated in grey-black. The apparent MW corresponding to the measured light scattering signal is indicated on the right x-axis (data points in brown). Modeling shows PC9 (component B) has preference for a 1:1 stoichiometry (A1B1) with ADC1-DAR1 (component A) (panel D/H). PC1, 2 and 4 (component B) show the ability to easily form a 2:1 complex (A2B1) (panels (A)/(E), (B)/(F), (C)/(G), respectively). ADC: Antibody drug conjugate; CG: Composition gradient; CVC: Cross-virial coefficient; MALS: Multiangle light scattering; PC: Positive control.

As previously discussed, sample incubation times can play a role in observed sensitivity [14]. Sample incubation times were increased (2, 6 and 16 h) for a subset of PCs (PC1–2, PC4, PC12, PC15–17 and PC_{mix4}) and ADC2 to investigate if under these conditions a correlate with observed affinity or complex formation parameters could be found. A head-to-head comparison with a 1 h sample incubation time was made (N = 2 analytical runs for each time point). Variance (Levene's test, $p = 0.35$) and mean of blank individual samples and negative control pool remained similar. PC1, PC15 and PC16 showed a relevant increase in sensitivity (lower value) in time, the other PCs showed similar sensitivity at all incubation times (see Supplementary Figure 8). No trend was found with any of the complex formation parameters or the observed affinity. Although overall increasing incubation times may result in a better sensitivity for some PCs, we found during method development using ADC1 that increasing sample incubation time may occasionally have a detrimental effect on sensitivity and drug tolerance (see Supplementary Figure 9 for an example of the effect on drug tolerance for PC8).

As sensitivity, drug tolerance (hill slope), affinity and complex binding parameters may influence each other, additional statistical analysis (factor analysis) was performed to investigate any potential trends influenced by multiple factors together. No trends were noted, confirming the limited overall correlation based on the Pearson correlation analysis of two factors at a time.

Conclusion

PCs used in development, validation and quality control of ADA assays are surrogates for ADA at best. Ten to greater fold differences in sensitivity between PCs were found in ECLIA-based, SPR-based and AlphaLISA-based assays. Investigations into drug tolerance also saw dissimilar behavior between PCs. Understanding the drivers

Figure 3. Complex formation analysis by composition gradient-multiangle light scattering of PC1, 2, 4 and 9 with ADC1-DAR1 (cont.). CG-MALS results of PC1, 2, 4 and 9 with ADC1-DAR1 are shown in panels (A)/(E), (B)/(F), (C)/(G) and (D)/(H), respectively. In blue are the measured light scattering signals, in red the fitted model based on reversible association an CVC, allowing a 1:1 stoichiometry (contribution to total fit in purple) and a 2:1 stoichiometry (contribution to total fit in grey). Component A (ADC1-DAR1) decreases from 100 to 0% in plateaus 1–15, indicated in dark green, whereas component B (PC) increases from 0 to 100% in plateaus 1–15, indicated in grey-black. The apparent MW corresponding to the measured light scattering signal is indicated on the right x-axis (data points in brown). Modeling shows PC9 (component B) has preference for a 1:1 stoichiometry (A1B1) with ADC1-DAR1 (component A) (panel D/H). PC1, 2 and 4 (component B) show the ability to easily form a 2:1 complex (A2B1) (panels (A)/(E), (B)/(F), (C)/(G), respectively). ADC: Antibody drug conjugate; CG: Composition gradient; CVC: Cross-virial coefficient; MALS: Multiangle light scattering; PC: Positive control.

behind assay sensitivity is of key interest in method development of assays that are used to detect ADA. In this study we have not been able to confirm a link between affinity and sensitivity or drug tolerance. In theory, one may expect a correlate or trend between affinity and sensitivity when simple 1:1 interactions are involved and/or large differences in affinity exist (e.g., μM vs pM affinity), in practice however, complex formation may play an important role in ADA assays. We have demonstrated that in some cases the ability to form complexes with at least two ADC molecules and the ratio between ADC to PC in complexes may be linked to drug tolerance and sensitivity in some cases. However, overall the complex formation parameters as determined by CG-MALS had limited to no predictive value for sensitivity and drug tolerance. The parameters as investigated in this study may either not capture complex formation sufficiently, or complex formation may only play a limited role in determining sensitivity and drug tolerance as investigated here. In addition it must be noted that assays may intrinsically differ, among others, in solid phase characteristics, acid dissociation conditions, incubation times and buffer constitution, all potentially able to directly or indirectly influence the interaction between PCs and the investigated compound. Reporting PC characteristics, without a correlation or trend of those characteristics to sensitivity and drug tolerance in the assay may be of limited value.

The associated confidence level for the CP and CPF had a clear impact on sensitivity reported for screening and confirmatory assays, thereby resulting in confirmatory assays in general being less sensitive than screening assays. For the SPR-based method, the variance between individual drug-naïve samples was reduced to a larger degree than we observed for other assays when taking the ratio of (confirmatory ADC) spiked and nonspiked samples, resulting in a more sensitive confirmatory assay. Although screening and confirmatory assays would preferably have the same sensitivity, this may in practice be difficult to achieve.

Figure 4. Scatter plots of sensitivity and drug tolerance (hill slope) to the ratio ADC and positive control in complex with ADC1 for the AlphaLISA-based assays. Scatter plots of (A) sensitivity and (B) drug tolerance (hill slope) to the ratio ADC and PC in complex with ADC1 for the AlphaLISA-based screening assay. Scatter plots of (C) sensitivity and (D) drug tolerance (hill slope) to the ratio ADC and PC in complex with ADC1 for the AlphaLISA-based confirmatory assay. ADC: Antibody drug conjugate; PC: Positive control.

In conclusion, assay sensitivity and drug tolerance may be driven by multiple factors as illustrated in this study. This highlights the relative nature of drug tolerance as well as sensitivity and as such caution should be taken in interpreting a reported value, especially in the interpretation of study sample results.

Future perspective

Bioanalytical scientists are required to characterize and at later development stages validate the assays that are used to detect ADA. However, the reported assay characteristics depend on the PC used. It is key to understand the characteristics of PC used in these assays that drive sensitivity and drug tolerance as a first step in understanding relevance of the assays for ADA assessment. We would like to encourage other bioanalytical scientists to publish their data on PC characteristics in relation to sensitivity and drug tolerance in order to build an understanding of what drives assay sensitivity and drug tolerance. The next stage, however difficult, would be to identify and characterize ADA and immune complexes found in subjects for various compounds and indications and further refine assays to detect ADA of interest, thereby ultimately allowing a better understanding of clinical events in relation to ADA characteristics.

Summary points

Sensitivity & drug tolerance

- Assay sensitivity in the presence of drug (drug tolerance and sensitivity) are relative and highly dependent on the assay and positive control (PC) used.
- As may be expected, the selected confidence limit may show a significant impact on reported sensitivity.
- Comparisons between assays using a single PC may be misleading when assessing sensitivity with and without the presence of drug-on-board.

Correlations of binding characteristics to sensitivity & drug tolerance

- Stoichiometry and complex formation as investigated here may correlate to assay sensitivity and sensitivity in the presence of drug in some cases, however, overall may have limited to no predictive value.
- Surprisingly, no relation between affinity of PCs and sensitivity or drug tolerance was found in the study described here.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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