Coping with Pre-existing Antibodies in Innovative Cancer Immunotherapy

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Introduction

Novel therapeutic protein constructs are particularly prone to be recognized by pre-existing / cross-reactive antibodies, which could disturb proper evaluation of the anti-drug antibody (ADA) response. All categories of ADA responses, namely pre-existing or treatment-boosted and treatment-induced may impact efficacy and safety of a therapeutic protein.

Therefore, ability to correctly identify samples containing pre-existing antibodies is critical for appropriate bioanalytical assay assessment.

In the present study we developed a bioanalytical anti-drug-antibody (ADA) assay for a novel fusion protein, consisting of two molecules with endogenous counterparts fused. During assay development, we detected pre-existing antibodies in healthy individuals, which were identified to be IgM subclass antibodies. These pre-existing antibodies hampered the cut point determination for the evaluation of the drug treatment induced antibodies. A stepwise bioanalytical assay development approach comprising of MRD optimization in conjunction with simple statistical procedures led to the exclusion of these samples, allowing for an appropriate cut point determination.

In combination with validated minimum significant ratio (MSR) applied on the titer, this will allow proper identification of pre-existing antibodies, which may be treatment-unaffected or treatment-boosted and treatment-induced ADA.

Analytical Methods

In order to detect ADAs directed against the drug, we developed a homogeneous electrochemiluminescence bridging immunoassay.

In a first step, human serum samples are diluted and incubated in a conical (polypropylene) plate with a fixed concentration of biotinylated- and ruthenylated-drug to allow for the formation of drug/anti-drug complexes.

The formed complexes are then captured on a streptavidin-coated assay plate, and detected by electrochemiluminescence after washing (Figure 1).

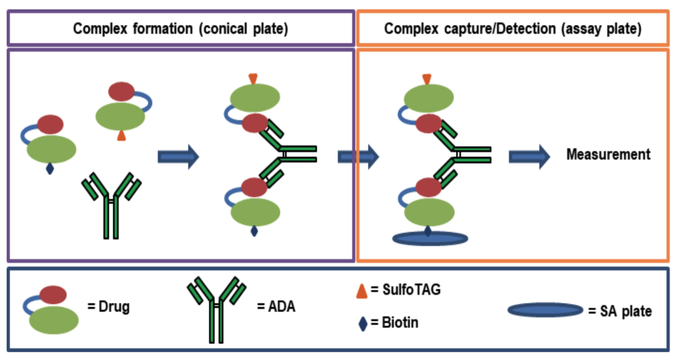

Figure 1: Scheme of the developed ADA Assay

Identification of Pre-existing Antibodies

During early stages of assay development, when individual serum samples at different dilutions are screened to optimize the MRD, we observed that some samples showed a signal significantly higher than the median signal of the 'population' (Table 1). This was also evidenced by the significant difference between the median and the average signal of the samples, as well as the overall high CVs.

These samples showed a consistently high signal at the different dilutions tested, suggesting the presence of a specific interference (i.e., pre-existing antibodies directed against the drug or drug-specific interfering factors) and not a matrix effect arising from the biological variability expected within a healthy population.

To identify this interference, two samples showing a high signal were first immunodepleted with protein G agarose beads (depletion of IgGs) or with protein G agarose beads + IgG anti-IgM (depletion of IgGs/IgMs), and then tested in the ADA assay set up. As a depletion control, a sample from a heathy subject with low signal in the assay was spiked with a polyclonal anti-drug antibody and treated in the same setting.

The results suggested that the reactivity was indeed due to pre-existing antibodies, likely IgMs: reactive samples showed a more dramatic reduction in their signal when they were treated with protein G agarose beads together with IgG anti-IgM rather than protein G alone (Table 2).

	MRD			
Individual	1:5	1:10	1:20	1:40
CM/18-2666	135	80	64	53
CM/18-2695	48	45	47	44
CM/18-2693	61	54	51	44
CM/17-3035	42	44	47	43
CM/17-3029	48	45	46	43
CM/17-3031	53	47	45	42
CM/18-1538	144	88	68	54
CM/19-2101	71	60	52	47
CM/19-2124	63	54	49	45
CM/19-2091	58	51	48	46
CM/20-0959	916	520	285	167
CM/20-0964	75	60	51	49
CM/20-0975	45	44	44	44
CM/20-0976	225	109	73	54
CM/20-0977	121	84	66	54
CM/20-0928	47	46	45	43
CM/20-0931	60	50	48	44
CM/20-0938	49	45	45	44
Average	125	85	65	53
Median	60	52	49	44
CV %	161.96	130.47	85.56	53.96

Table 1: Heterogeneity of Individual Samples (red = samples with significantly high signal)

Individual	Control	Protein G	Protein G + αIgM 1	Protein G + αIgM 2
CM/20-0959	500	395	297	46
CM/20-0976	124	283	66.5	263.5
CM/19-0952	401	105	47	53.5

Table 2: Immunodepletion of pre-existing Antibodies (green = immunodepletion control).

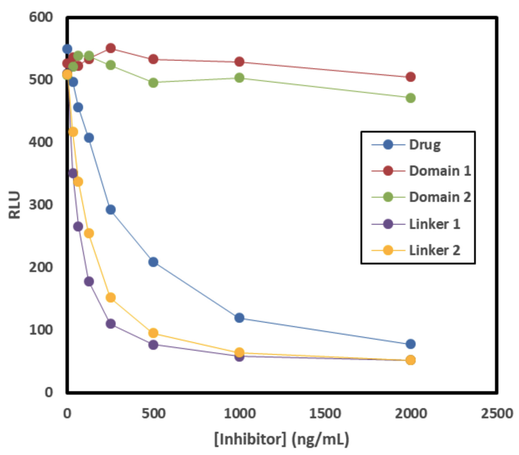

Figure 2: Characterization of Binding Epitope

In order to gain insight into the epitope(s) targeted by the pre-existing antibodies, we performed blocking experiments (confirmatory assay). Binding of pre-existing antibodies in sample CM/20-0959 (high levels) was blocked with either the drug, each of the individual domains, or two drug variants containing modified linkers (Figure 2).

Only the intact drug or the drug containing modified linkers were capable of inhibiting the binding of the pre-existing antibodies (Figure 2). Although the results were not unequivocal this data suggests that the epitope(s) recognized by the pre-existing antibodies arises from the novel interface created by the fusion protein.

Strategy to Mitigate Pre-existing Antibodies

Samples containing pre-existing antibodies have to be identified and excluded from cut point analysis (FDA Guidelines; Xue, L., et al.).

First, we defined the optimal MRD by applying iteratively the following procedure to the each MRD data presented in Table 1: The results obtained are shown in below:

1: Discard outliers identified in a box plot analysis

2: Evaluate remaining samples (variability, preliminary cut point correction factor, false-positive rate)

Figure 3: Strategy to define the optimal MRD

	MRD			
	1:5	1:10	1:20	1:40
Average	55	49	47	44
Median	53	47	47	44
CV %	18.58	12.02	5.57	4.30
Outliers	5/18	5/18	5/18	1/18
CF	1.39	1.19	1.11	1.09
FP %	7.69	7.69	0.00	38.46

Table 3: Evaluation of optimal MRD (CF = cut point correction factor; FP = false positive)

MRDs 1:5 and 1:10 showed overall the best performance with similar parameters (average and median values are close for each dilution, CV% are acceptable, correction factors and false-positive rates are within desirable values). However, since both showed similar false-positive rates, MRD 1:5 was selected due to the higher and more appropriate correction factor (see Devanarayan, V. et al. and citations within).

In order to confirm the selected MRD and evaluate the assay performance parameters, we analyzed individual samples in the screening and confirmatory formats following the strategy shown in Figure 4.

1: Screen naive samples (screening and confirmatory formats)

2: Discard outliers in a box plot analysis of screening format

3: Evaluate screening and confirmatory cut points

Figure 4: Strategy to confirm the optimal MRD and evaluate assay performance

Due to the identified pre-existing antibodies, we tested 60 healthy individual serum samples in order to obtain after outlier removal an adequate number of samples for cut point calculation, in both, screening and confirmatory assays (Table 4).

We identified pre-existing antibodies in approximately 27 % of the samples following this strategy. Elimination of these samples, and cut point calculation with the remaining samples lead to optimal performance parameters (Table 4, lower panel).

Conclusions and Discussion

Pre-existing antibodies may have a deleterious impact during cut point determination of ADA immunoassays, therefore, it is crucial to detect and exclude them from any development activity in order to appropriately detect treatment/drug-induced or -boosted anti-drug antibodies, which may negatively impact safety and efficacy of the biotherapeutic.

A simple approach that we followed ensures a correct identification, without obscuring samples containing the undesired interference, and resulting in the selection of an adequate data set for cut point calculation.

The notion that the identified IgM antibodies are likely directed to a novel epitope arising from the fusion of two endogenous proteins suggests a cross-reactive mechanism for their generation. Therefore, it will be of interest to understand if these pre-existing antibodies are boosted following treatment and if safety and efficacy data obtained from the clinical studies ahead will be impacted.

	Screening		Confirmatory	
Individual	RLU	Box plot	RLU	% inh
CM/19-0942	53		48	8.57
CM/19-0985	76		46	39.47
CM/19-0964	51		48	5.88
CM/19-0974	49		46	6.12
CM/19-0959	53		48	10.38
CM/19-0989	50		46	7.07
CM/19-0976	56		48	15.18
CM/19-0965	52		48	6.80
CM/19-0987	51		47	6.93
CM/19-0949	52		46	12.50
CM/19-0948	49		49	1.02
CM/19-0966	52		48	6.80
CM/19-0968	58		48	16.52
CM/19-0960	60		54	9.24
CM/19-0970	60		49	19.17
CM/19-0940	122	Outlier	48	60.49
CM/19-0957	133	Outlier	59	56.02
CM/19-0952	55		47	13.76
CM/19-0946	119	Outlier	52	56.30
CM/19-0965	51		46	9.90
CM/19-0947	51		48	5.94
CM/19-0951	51		47	7.84
CM/19-0963	79		51	35.44
CM/19-0954	48		47	2.11
CM/19-0981	63		50	20.80
CM/19-0986	157	Outlier	51	67.73
CM/19-0971	48		47	2.11
CM/19-0980	57		45	20.35
CM/19-0984	87		53	39.66
CM/19-0983	52		49	5.83
CM/20-0962	52		49	5.83
CM/20-0961	88		50	43.18
CM/20-0960	50		49	2.02
CM/20-0958	62		62	0.81
CM/20-0957	60		49	19.17
CM/20-0956	49		46	5.15
CM/20-0953	60		50	17.50
CM/20-0952	51		50	1.96
CM/20-0978	51		47	7.84
CM/20-0975	55		48	12.73
CM/20-0974	55		45	18.18
CM/20-0963	56		48	15.18
CM/20-0964	88		48	45.14
CM/20-0965	69		55	20.29
CM/20-0966	67		49	27.07
CM/20-0967	58		51	12.93
CM/20-0968	53		47	11.32
CM/20-0969	48		47	3.13
CM/20-0970	49		47	5.10
CM/20-0971	112	Outlier	50	55.36
CM/20-0938	55		48	12.73
CM/20-0972	97	Outlier	70	27.98
CM/20-0973	203	Outlier	83	59.36
CM/20-0935	52		48	8.65
CM/20-0934	204	Outlier	51	74.94
CM/20-0945	50		47	6.06
CM/20-0928	51		46	9.90
CM/20-0944	64		48	24.41
CM/20-0943	51		50	0.99
CM/20-0977	119	Outlier	51	56.96

Total samples	60
Outliers	9
CF	1.136
iCP (%)	22.74
False positive	6/60 (10 %)
True positive	16/60 (27 %)

Table 4: Evaluation of optimal MRD (CF = cut point correction factor; FP = false positive)

References

1. Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products. Guidance for Industry. Draft Guidance. US FDA 2016
2. Xue, L., Clements-Egan, A., Amaravadi, L., Birchler, M., Gorovits, B., Liang, M., Myler, H., Purushothama, S., Starcevic Manning, M., Sung, C. Recommendations for the Assessment and Management of Pre-existing Drug-Reactive Antibodies During Biotherapeutic Development. AAPS J. 19(5): 1576-1586 (2017).
3. Devanarayan, V., Smith, W.C., Brunelle, R.L., Seger, M.E., Krug, K., Bowsher, R.R. Recommendations for Systematic Statistical Computation of Immunogenicity Cut Points. AAPS J. 19(5):1487-1498 (2017).