Total and Free VEGF Assays with High Drug Tolerance for the Anti-VEGF Inhibitors Avastin® (Bevacizumab), Lucentis® (Ranibizumab), and Eylea® (Aflibercept)

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Translating Science to Medicine

Introduction

Anti-vascular endothelial growth factor (VEGF) treatment with bevacizumab, ranibizumab, and aflibercept are therapies approved globally for several cancers and age-related macular degeneration. Patents for Avastin®, Lucentis®, and Eylea® are due to expire between 2019 and 2020 in the US and between 2021 and 2022 in Europe, and there are estimated to be around 15 biosimilars of bevacizumab alone under development. Measurement of total endogenous VEGF and free VEGF not bound to the drug after their dosing are important biomarkers used to demonstrate pharmacokinetics and efficacy of these drugs.

Total VEGF assays require high drug tolerance and sample processing steps to dissociate VEGF from the drug. These processing steps typically negatively impact accuracy and precision. Free VEGF has typically been measured with commercial ELISA kits. However, recent studies show these kits do not accurately measure total or free VEGF.

Use of commercial VEGF assays for development of new anti-VEGF biologics and their biosimilars would therefore be highly problematic and development of de novo methods is necessary. Here we demonstrate assays for accurate and precise measurement of total and free VEGF in human serum with electrochemiluminescence that are not subject to interference from bevacizumab, ranibizumab, and aflibercept.

Design and Validation of Free and Total Ligand Assays

Challenges

For biologic and biosimilar drugs, multiple forms of the drug and target ligand exist with a dynamic binding equilibrium occurring both in the body and during bioanalysis. Assays must be designed with challenging sample preparation steps, appropriate capture and detection ligands, and assay conditions to not disturb in vivo equilibrium.

Guidelines for validation of these assays is limited as current US FDA and EMA guidelines focus only on total drug and ligand assays.

Solutions

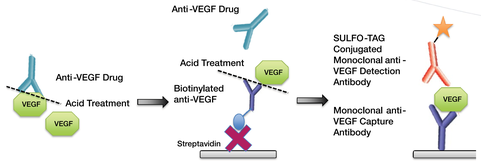
Accurate determination of total and free ligand can be used in conjunction with clinical response as a more rapid biomarker for PK modeling, dosing, and efficacy.

Screening of capture ligands and optimization of sample dilution, incubation times, and buffers allows minimization of perturbations in drug-ligand equilibrium.

Not all aspects of validation guidelines for biomarker assays can be applied to free ligand assays but precision and accuracy, selectivity, and parallelism tests can be adapted. "Free analyte QCs" which contain both target analyte and drug can be used to develop QCs based on the binding affinity of the drug.

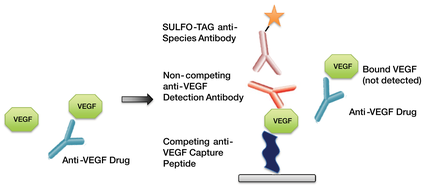
Methods

Total VEGF Format



The total VEGF assay was based on a solid phase extraction with acid dissociation (SPEAD) method that was modified for this biomarker assay. Briefly, samples containing VEGF and drug were pretreated with a novel dissociation and neutralization buffer (Somru Bioscience) to remove interfering drug before incubation with a biotinylated anti-VEGF antibody. Samples were then incubated on a streptavidin coated plate, dissociated again and transferred to a Meso Scale Discovery VEGF assay for detection.

Free VEGF Format



We simultaneously developed a sensitive method for the measurement of free VEGF in serum containing bevacizumab, ranibizumab, and aflibercept using electrochemiluminescence. Peptides and antibodies binding to VEGF were screened for their ability to compete for the drug binding site. A peptide from Somru BioScience competing for the drug binding site was used to capture free VEGF in samples without disturbing VEGF bound to drug and a non-competing antibody was used as a detection antibody with a sulfo-tag secondary antibody.

Results

Total VEGF Results:

Table 1. Precision and Accuracy of QC Samples with no Drug Present

	QC A (400 pg/mL)	QC B (160 pg/mL)	QC C (32.1 pg/mL)
Batch 1	385	146	28.8
	350	143	25.0
	393	141	32.0
Batch 2	381	188	27.5
	425	160	39.7
	458	179	34.2
Mean	399	160	31.0
% Recovery	99.7	99.8	97.2
% CV	9.50	12.5	17.0
n	6	6	6

Table 2. Precision and Accuracy of QC Samples with 1250 ng/mL Bevacizumab (Avastin®)

	QC A (400 pg/mL)	QC B (160 pg/mL)	QC C (32.1 pg/mL)
Batch 1	385	146	28.8
	350	143	25.0
	393	141	32.0
Batch 2	381	188	27.5
	425	160	39.7
	458	179	34.2
Mean	399	160	31.0
% Recovery	99.7	99.8	97.2
% CV	9.50	12.5	17.0
n	6	6	6

Table 3. Precision and Accuracy of QC Samples with 1000 ng/mL Ranibizumab (Lucentis®)

	QC A (400 pg/mL)	QC B (160 pg/mL)	QC C (32.1 pg/mL)
Batch 1	338	144	41.8
	439	116	15.9
	322	132	23.1
Batch 2	366	144	39.7
	379	148	28.6
	388	146	35.3
Mean	372	138	31.0
% Recovery	93.0	86.4	95.7
% CV	11.0	9.00	32.7
n	6	6	6

Table 4. Precision and Accuracy of QC Samples with 100 ng/mL Aflibercept (Eylea®)

	QC A (400 pg/mL)	QC B (160 pg/mL)	QC C (32.1 pg/mL)
Batch 1	417	163	49.9
	418	134	32.9
	477	149	38.0
Batch 2	445	119	34.5
	483	160	34.8
	437	180	31.9
Mean	446.4	150.8	37
% Recovery	111.6	94.3	115.3
% CV	6.4	14.5	18.0
n	6	6	6

Figure 1a: Interference Test of Total VEGF by Bevacizumab (Avastin®)

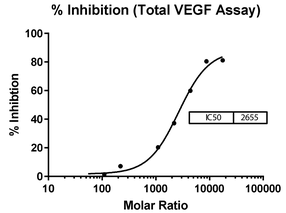
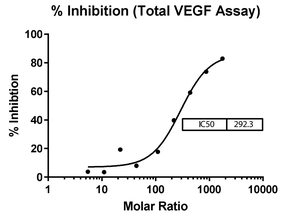
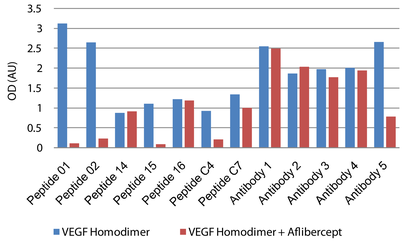


Figure 1b: Interference Test of Total VEGF by Ranibizumab (Lucentis®)



Free VEGF Results:

Figure 2: Screening of Capture Ligands Competing for the Aflibercept Binding Site

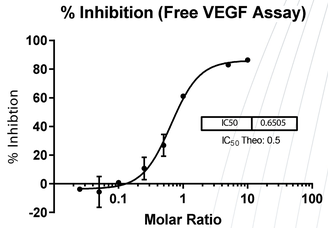


Peptides and antibodies binding to VEGF were screened for their ability to compete for the drug binding site. Peptides 01, 02, 15, and C4 exhibited the best competition for VEGF binding as potential capture ligands in the free VEGF assay.

Table 5: Precision and Accuracy of Free VEGF Assay in Samples with Aflibercept (Eylea®)

Sample	Theoretical Free VEGF (pg/mL)	Mean Concentration Found (pg/mL)	Accuracy (%)	Precision (%)
1	1030	866	-15.7	0.115
2	1002	892	-11.1	6.37
3	537	357	-33.4	1.04
4	141	152	7.64	1.84
5	98.4	78.5	-20.3	6.67

Figure 3: Interference Test of Free VEGF by Aflibercept (Eylea®)



Conclusion & Future Work

The results from both VEGF assays indicate they are "validatable" and have high drug tolerance for aflibercept, bevacizumab, and ranibizumab well above the Cmax for intravitreal use.

Future experiments will validate the assays according to FDA Bioanalytical Guidance for biomarker assays and industry best practices for total and free ligand assays including verifying assay and precision, matrix effect, parallelism, and specificity.

Novel Aspect

Our total VEGF assay is the first report of an accurate assay for total VEGF in the presence of all three current anti-VEGF drugs (aflibercept, bevacizumab, and ranibizumab). Optimization of assay conditions and buffers allowed measurement of true accuracy instead of relative accuracy and successfully removed interfering drug. This is especially significant for aflibercept due to its extremely high affinity for VEGF.

Free target ligand assays are also challenging because of the need to find capture and detection antibodies based on the drug binding site. In addition, these assays are typically only used with relative accuracy because it is difficult to prevent "contamination" of total VEGF during the assay. We optimized assay conditions, buffers, and a competing capture peptide to develop the first reported accurate assay for free VEGF in the presence of aflibercept without contamination of total VEGF.

Acknowledgements

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