Integrated Evaluation of Payload-, Fc-, and Target-mediated Mechanisms of ADCs Using Streamlined Complementary Platforms

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Abstract

This study demonstrates an integrated approach for the comprehensive characterization of antibody–drug conjugates (ADCs) using complementary bioanalytical platforms. The objective was to evaluate target binding, internalization, and both payload- and Fc-mediated cytotoxic mechanisms to generate a holistic understanding of ADC mode of action.

ADC binding affinity and kinetic parameters were characterized by surface plasmon resonance microscopy (SPRM) to assess cell-surface receptor engagement. Internalization was measured in engineered tumor cell lines expressing relevant antigens (e.g., BCMA, CD33) using enzyme-fragment complementation (EFC)-based PathHunter® internalization assays. Functional cytotoxicity was quantified using the KILR® Cytotoxicity platform, providing direct readouts of target-cell death. Fc-effector functions, including antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP), were evaluated using effector cell models to distinguish immunemediated from payload-driven mechanisms.

Here we show characterization data for 2 different ADCs that demonstrated high-affinity binding by SPRM, and efficient internalization by PathHunter assays. In KILR assays, the ADCs produced a concentration-dependent cytotoxic response distinct from its unconjugated parental antibody. Comparative analysis revealed that payload-mediated killing dominated overall cytotoxicity, while measurable ADCC and ADCP activities reflected retained Fc function. Integration of biophysical and cell-based results provided mechanistic resolution of ADC function at multiple levels. Combining SPRM, PathHunter internalization assays, and KILR cytotoxicity enables comprehensive ADCs characterization encompassing binding, uptake, and multi-mechanistic cytotoxicity. This integrated workflow supports discovery, optimization, and lot-release testing by linking molecular properties to functional outcomes, enhancing mechanistic understanding critical for ADC development.

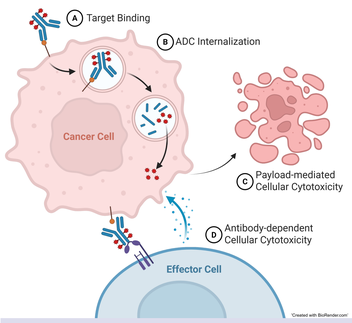

Figure 1. Antibody Drug Conjugate (ADC) Mechanisms of Action (MOA). ADC efficacy is shaped by antigen specificity, internalization efficiency, payload potency, and Fc-mediated immune engagement. ADCs mediate targeted cytotoxicity through a multi-step process starting with antibody recognition of a specific cell-surface antigen (A). The ADC–antigen complex is internalized (B) via endocytosis and trafficked to endosomes and lysosomes, where linker cleavage releases the cytotoxic payload, which induces cell death through DNA damage, microtubule disruption, or other mechanisms. Beyond direct killing, ADCs can drive immune effector functions through their Fc domain (C & D), including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), or other applications.

Key Takeaways

- A streamlined, end-to-end workflow enables a multi-mechanistic ADC characterization.
- SPR microscopy (SPRM) accelerates target-specific ADC binding and kinetics assessment.
- PathHunter assays are homogeneous, no-wash cell-based platform to rapidly quantify receptor internalization, enabling direct comparison of ADCs and parent antibodies.
- The KILR platform includes over 40 tumor models and provides a sensitive, homogeneous, and target-specific assay format for detecting direct killing of target cells through a variety of different mechanisms such as payload-mediated cytotoxicity, ADCC, ADCP, CDC, and T-cell redirection mediated by antibodies, ADC, TILs or CAR-Ts, and more.
- Eurofins DiscoverX can generate novel target cell models or customize current assays as well as perform assay optimization and IND-enabling characterization studies to support your specific program needs.

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Workflow and Assay Designs to Interrogate MOAs of ADCs

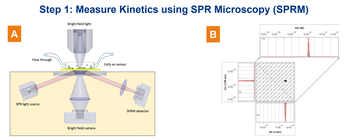


Figure 2. SPR microscopy (SPRM) principles. A. SPRM uses a light source to induce surface plasmon resonance on a sensor chip with cells, detecting changes in the dielectric constant from molecular binding. ADCs are delivered via automated microfluidics to enable simultaneous SPR and Bright-field imaging. B. The sensing area is divided into 600 regions of interest (ROI)s, with each ROI's response during kinetic injections recorded. Binding curves are fitted to extract ka and kd, and isoaffinity scatter plots visualize heterogeneity. Gaussian fits of the histograms along each axis and the diagonal provide mean and standard deviation for ka, kd, and K_D values.

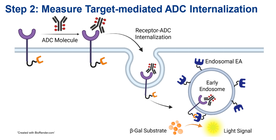


Figure 3. Overview of the PathHunter Internalization Assay. ADC efficacy requires receptor binding, endocytosis, and intracellular payload delivery. Target cells stably express the receptor fused to enhanced ProLabel® (ePL), while the complementary fragment, or Enzyme Acceptor (EA), resides in the cytoplasm. Upon ADC binding, receptor-ADC complexes internalize via endocytosis into early endosomes, bringing fragments into proximity to form active β-gal. The resulting chemiluminescent signal is proportional to the extent of internalization.

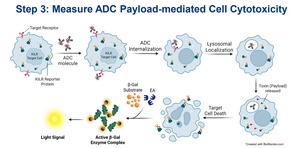


Figure 4. Overview of KILR ADC Payload Cytotoxicity Assay. KILR target cells expressing the antigen, stably produce a housekeeping KILR Reporter Protein tagged with enhanced ePL. Following ADC treatment, payload-induced cell death releases ePL, which combines with EA to form active β-gal, generating a chemiluminescent signal proportional to cytotoxicity.

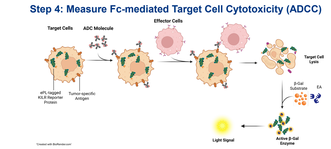


Figure 5. Overview of the KILR ADCC Assay. KILR Target Cells expressing the antigen are stably engineered with a housekeeping KILR Reporter Protein tagged with enhanced ePL. Upon incubation with effector cells and antibody, killing releases ePL, which complements with the enzyme acceptor (EA) in the detection reagent to form active β-gal. Substrate hydrolysis generates a chemiluminescent signal measurable on standard luminometers.

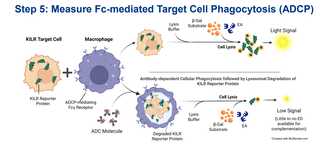
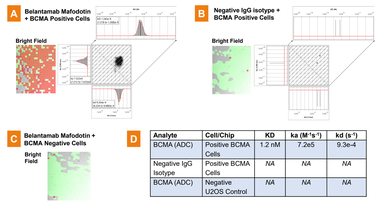


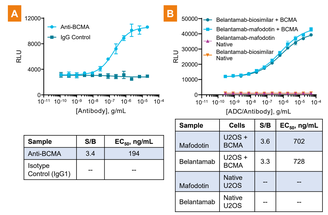
Figure 6. Overview of the KILR ADCP Assay. KILR Target Cells expressing the antigen are engineered to stably express a housekeeping protein tagged with the ePL fragment. PBMC-derived monocytes are differentiated with M-CSF for 7 days to generate M0. Opsonized KILR target cells are co-cultured with macrophages for 24 hours. After lysis, β-gal complementation is measured: high signal indicates minimal killing, low signal indicates greater killing.

Case Study 1: Functional Characterization of Belantamab Mafodotin (Anti-BCMA)



D	Analyte	Cell/Chip	KD	ka ($M^{-1}s^{-1}$)	kd (s^{-1})
	BCMA (ADC)	Positive BCMA Cells	1.2 nM	7.2e5	9.3e-4
	Negative IgG Isotype	Positive BCMA Cells	*NA*	*NA*	*NA*
	BCMA (ADC)	Negative U2OS Control	*NA*	*NA*	*NA*

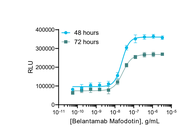
Figure 7. Assessment of ADC binding to BCMA Internalization Cell Line using SPR microscopy (SPRM). Affinity isotherm plots were generated from hundreds of responsive ROIs. Kinetic interaction distributions are summarized in the histograms. SPR and Bright Field images along with kinetic data for A. belantamab mafodotin (an ADC) with BCMA positive cells, B. IgG isotype control with BCMA positive cells, and C. belantamab mafodotin with parental BCMA negative cells. D. Results summary table.



Sample	S/B	EC_{50}, ng/mL
Anti-BCMA	3.4	194
Isotype Control (IgG1)	--	--

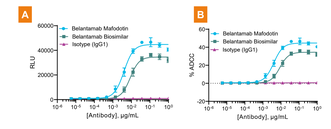
Sample	Cells	S/B	EC_{50}, ng/mL
Mafodotin	U2OS + BCMA	3.6	702
Belantamab	U2OS + BCMA	3.3	728
Mafodotin	Native U2OS	--	--
Belantamab	Native U2OS	--	--

Figure 8. Evaluation of ability of ADC to stimulate internalization of BCMA. A. Control anti-BCMA antibody mediates BCMA internalization after overnight incubation with PathHunter BCMA-expressing cells, while isotype control does not. B. Potency of internalization of BCMA by belantamab mafodotin (the ADC) is essentially equivalent to that mediated by belantamab (native antibody) alone. The ADC and antibody do not mediate any increase in signal in the parental U2OS cells that do not express BCMA.



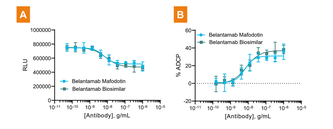
Time Point Sample	S/B	EC_{50}, ng/mL
48 Hours	4.4	23.4
72 Hours	4.1	25.4

Figure 9. Evaluation of the ability of the ADC to mediate cytotoxicity in MM1.R cancer model. Belantamab mafodotin was incubated with MM.1R cells that endogenously express BCMA for 48 or 72 hours at 37°C followed by the detection of cell killing by addition of KILR detection reagent. Note that a robust assay window can be detected in as little as 48 hours with similar assay window (S/B) and EC_{50} values.



C	Sample	S/B	E_{Max}	EC_{50}, ng/mL
	Belantamab Mafodotin	46	45%	4
	Belantamab Antibody	49	35%	8.7

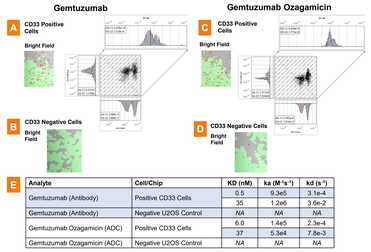
Figure 10. Evaluation of effector mediated functions (e.g., ADCC and ADCP) of Fc domain of ADC and parent (unconjugated antibody) in the KILR platform. Belantamab mafodotin and unconjugated parent antibody (belantamab) are competent to mediate ADCC in KILR MM.1R cells using engineered effector (KILR CD16) cells, while isotype control is inactive. A. Raw ADCC RLU, B. % ADCC data, and C. results summary table.



C	Sample	S/B	E_{Max}	EC_{50}, ng/mL
	Belantamab Mafodotin	1.4	31%	6.4
	Belantamab Antibody	1.6	37%	7.8

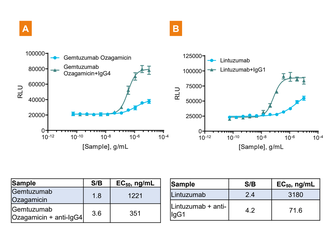
Figure 11. Evaluation of effector-mediated ADCP function of Fc domain of an ADC and its native antibody in KILR platform. Belantamab mafodotin (ADC) and unconjugated parent antibody (belantamab) mediate ADCP in KILR MM.1R cells using M0 macrophages as effector cells used at an E:T ratio of 5:1. A. Raw ADCP RLU, B. % ADCP data, and C. results summary table.

Case Study 2: Functional Characterization of the ADC Gemtuzumab Ozagamicin (Anti-CD33)



E	Analyte	Cell/Chip	KD (nM)	ka ($M^{-1}s^{-1}$)	kd (s^{-1})
	Gemtuzumab (Antibody)	Positive CD33 Cells	0.5	9.3e5	3.1e-4
			35	1.2e6	3.6e-2
	Gemtuzumab (Antibody)	Negative U2OS Control	*NA*	*NA*	*NA*
	Gemtuzumab Ozagamicin (ADC)	Positive CD33 Cells	6.0	1.4e5	2.3e-4
			37	5.3e4	7.8e-3
	Gemtuzumab Ozagamicin (ADC)	Negative U2OS Control	*NA*	*NA*	*NA*

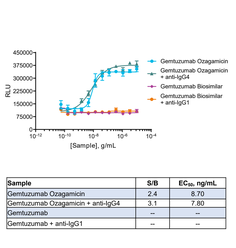
Figure 12. Assessment of Gemtuzumab and Gemtuzumab Ozagamicin binding to PathHunter CD33 Internalization Cell Line using SPRM. SPR and Bright Field images along with kinetic data for A. gemtuzumab with CD33 positive cells, B. gemtuzumab with parental CD33 negative cells, C. gemtuzumab ozagamicin with CD33 positive cells, and D. gemtuzumab ozagamicin with parental CD33 negative cells. E. Results summary table.



Sample	S/B	EC_{50}, ng/mL
Gemtuzumab Ozagamicin	1.8	1221
Gemtuzumab Ozagamicin + anti-IgG4	3.6	351

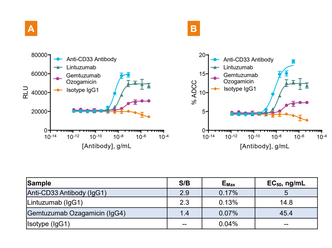
Sample	S/B	EC_{50}, ng/mL
Lintuzumab	2.4	3180
Lintuzumab + anti-IgG1	4.2	71.6

Figure 13. Evaluation of ability of ADC to stimulate internalization of CD33 in the presence and absence of cross-linking antibody. For both antibodies, cross-linking significantly improved potency of CD33 antigen internalization in PathHunter U2OS CD33-expressing cells. A. Gemtuzumab ozagamicin mediates CD33 internalization after 2 hours incubation. B. Lintuzumab (IgG1) mediates CD33 internalization after 5 hours incubation.



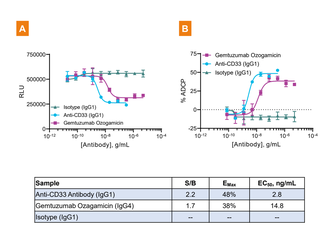
Sample	S/B	EC_{50}, ng/mL
Gemtuzumab Ozagamicin	2.4	8.70
Gemtuzumab Ozagamicin + anti-IgG4	3.1	7.80
Gemtuzumab	--	--
Gemtuzumab + anti-IgG1	--	--

Figure 14. Evaluation of ability of the ADC to mediate cytotoxicity in HL-60 target cells in the presence and absence of cross-linking antibody. Gemtuzumab ozagamicin mediates cytotoxicity (quantified with KILR detection kit) after 72 hours incubation with KILR HL-60 target cells while a gemtuzumab antibody without payload is inactive.



Sample	S/B	E_{Max}	EC_{50}, ng/mL
Anti-CD33 Antibody (IgG1)	2.9	0.17%	5
Lintuzumab (IgG1)	2.3	0.13%	14.8
Gemtuzumab Ozagamicin (IgG4)	1.4	0.07%	45.4
Isotype (IgG1)	--	0.04%	--

Figure 15. Evaluation of effector mediated functions (ADCC) of Fc domain of ADC and parent (unconjugated antibody) in KILR platform. Gemtuzumab ozagamicin (IgG4) and two IgG1-formatted anti-CD33 antibodies are competent to mediate ADCC in KILR HL-60 cells using primary human PBMCs, while isotype control is inactive; A. Raw ADCC RLU, B. % ADCC data, and C. results summary table.



Sample	S/B	E_{Max}	EC_{50}, ng/mL
Anti-CD33 Antibody (IgG1)	2.2	48%	2.8
Gemtuzumab Ozagamicin (IgG4)	1.7	38%	14.8
Isotype (IgG1)	--	--	--

Figure 16. Evaluation of effector mediated functions (ADCP) of Fc domain of ADC and unconjugated anti-CD33 antibody in KILR platform. Gemtuzumab ozagamicin (IgG4) and an unconjugated anti-CD33 (IgG1) antibody mediate ADCP in KILR HL-60 cells using M0 macrophages as effector cells, while an IgG1 isotype control is inactive; A. Raw ADCP RLU, B. % ADCP data, and C. results summary table.