Antibody–Drug Conjugate Efficacy in Neuroblastoma: Role of Payload, Resistance Mechanisms, Target Density, and Antibody Internalization

Samantha Buongervino1, Maria V. Lane1, Emily Garrigan1, Doncho V. Zhelev2, Dimitar S. Dimitrov2, and Kristopher R. Bosse13

ABSTRACT

Antibody–drug conjugates (ADC) are a targeted cancer therapy that utilize the specificity of antibodies to deliver potent drugs selectively to tumors. Here we define the complex interaction among factors that dictate ADC efficacy in neuroblastoma by testing both a comprehensive panel of ADC payloads in a diverse set of neuroblastoma cell lines and utilizing the glypican 2 (GPC2)-targeting D3-GPC2-PBD ADC to study the role of target antigen density and antibody internalization in ADC efficacy in neuroblastoma. We first find that DNA binding drugs are significantly more cytotoxic to neuroblastomas than payloads that bind tubulin or inhibit DNA topoisomerase 1. We additionally show that neuroblastomas with high expression of the ABCB1 drug transporter or that harbor a TP53 mutation are significantly more resistant to tubulin and DNA/DNA topoisomerase 1 binding payloads, respectively. Next, we utilized the GPC2-specific D3-GPC2-IgG1 antibody to show that neuroblastomas internalize this antibody/GPC2 complex at significantly different rates and that these antibody internalization kinetics correlate significantly with GPC2 cell surface density. However, sensitivity to pyrrolobenzodiazepine (PBD) dimers primarily dictated sensitivity to the corresponding D3-GPC2-PBD ADC, overall having a larger influence on ADC efficacy than GPC2 cell surface density or antibody internalization. Finally, we utilized GPC2 isogenic Kelly neuroblastoma cells with different levels of cell surface GPC2 expression to define the threshold of target density required for ADC efficacy. Taken together, DNA binding ADC payloads should be prioritized for development for neuroblastoma given their superior efficacy and considering that ADC payload sensitivity is a major determinant of ADC efficacy.

Introduction

Immunotherapeutic approaches utilizing chimeric antigen receptor (CAR) T cells for liquid malignancies have revolutionized anticancer therapy (1). However, similar CAR T-cell clinical successes in solid tumors have not been documented to date (2), making it imperative to explore alternative immune-based therapies for these cancer histotypes. Antibody–drug conjugates (ADC) have shown proof-of-concept for the treatment of solid tumors, such as the FDA-approved Sacituzumab govitecan (3), Trastuzumab emtansine (4), and Trastuzumab deruxtecan (5) for breast cancer, and Enfortumab vedotin (6) for urothelial carcinoma. In addition, ALK- (7), LGAL3SBP- (8), and GPC2-targeting (9, 10) ADCs were recently proven to be effective in neuroblastoma preclinical models. ADCs capitalize on the specificity of a tumor-targeting antibody to selectively deliver toxic payloads to cancer cells (11), and potentially offer distinct advantages over other types of immune-based therapies. For example, ADCs may be more tolerant of the heterogeneous antigen expression that can be found in solid tumors, as well as the immunosuppressive tumor microenvironment, by enacting diverse mechanisms of tumor killing such as bystander cell cytotoxicity (12) and immunogenic cell death (13), respectively. Although there has been a recent exponential increase in the preclinical development of ADCs for cancer, only a select few compounds have been fully optimized and proven to have an adequate therapeutic index to enable safe and efficacious dosing in humans (3–6, 14–17). The antitumor efficacy and clinical safety of an ADC is multifactorial, depending on the complex relationship of multiple antigen-dependent (tumor and normal cell surface antigen density, antibody specificity, and antibody/receptor internalization kinetics) and antigen-independent (payload sensitivity, linker chemistry, drug transporter expression, and mutations in drug response genes) factors. ADC payloads are a diverse and easily modifiable element of these therapeutics, and most can be broadly categorized into three main classes based on their mechanisms of action: microtubule or DNA binding drugs and DNA topoisomerase 1 inhibitors (11). Although ADCs capitalize on the specificity of tumor-specific antibodies, tumor selectivity can be inadequate and thus ADC dosing in humans can be limited by both toxicities that mirror those of the free payload, such as pleural/pericardial effusions and neutropenia/thrombocytopenia associated with PBD-containing ADCs (17–19) or peripheral neurotoxicities seen with tubulin-binding ADCs (20), in addition to toxicities that are unique to the ADC, such as the corneal toxicities observed with some tubulin-binding ADCs (21).

Embryonal cancers such as neuroblastoma are especially ideal to target with ADCs or other immunotherapeutic modalities as they represent a misappropriation of normal developmental processes and thus continue to selectively express lineage-specific cell surface molecules. In an unbiased screen to identify potential neuroblastoma
imunotherapeutic targets, we previously identified glypican 2 (GPC2), a glycosylphosphatidylinositol (GPI)-anchored cell surface proteoglycan, to be a robustly differentially expressed molecule in neuroblastoma and several other tumors that is transcriptionally regulated by the MYCN proto-oncogene, bHLH transcription factor (MYCN) protein and is essential for tumor growth (9, 10). We also developed a GPC2-targeting ADC comprised of a highly-specific, fully human GPC2 antibody (D3; SEQ ID No: 1 and 2; ref. 22) conjugated to pyrrolobenzodiazepine (PBD) dimers (D3-GPC2-PBD) through an enzymatic cleavable linker (9, 10). However, recent data have challenged the potential therapeutic index of PBD dimer payloads in humans (17, 18, 23), making it imperative to explore other drugs that may offer similar efficacy while providing a potentially wider therapeutic index in the clinic. Thus, here we test the efficacy of 11 unique ADC payloads in a large panel of clinically and genomically annotated neuroblastoma cellular models that recapitulate the diversity of the human clinical disease. Furthermore, we quantify the GPC2 cell surface density, D3-GPC2-IgG1 antibody internalization kinetics, and D3-GPC2-PBD ADC efficacy in these same neuroblastoma cell lines to comprehensively evaluate multiple factors that may dictate the efficacy of GPC2-targeting and other ADCs in neuroblastoma.

### Materials and Methods

#### Cell lines

Human-derived neuroblastoma cell lines [NB-SD (RRID:CVCL_1124), NB69 (RRID:CVCL_1448), SK-N-AS (RRID:CVCL_5627), IMR-5 (RRID:CVCL_1306), SMS-SAN (RRID:CVCL_E218), NBL-S (RRID:CVCL_2136), NB-1643 (RRID:CVCL_0389), Kelly (RRID:CVCL_2092), and CHP-134 (RRID:CVCL_1124)] were obtained from the CHOP cell line bank or ATCC (Catalog No. CRL-3216). All cells were cultured in Corning RPMI 1640 medium containing 10% FBS, 2 mmol/L L-glutamine, 1% streptomycin/penicillin at 37 °C. HEK293T cells (RRID: CVCL_0063) were obtained from the ATCC (Catalog No. CRL-2302). All cells were cultured in Corning RPMI 1640 medium containing 10% FBS, 2 mmol/L L-glutamine, and 1% streptomycin/penicillin at 37 °C under 5% CO₂, and were used at an early passage from thaw. The genomic identity of each cell line was confirmed with genotyping using a Genelink 24 System (Promega) and cell lines were confirmed to be free of mycoplasma contamination via a MycoAlert Mycoplasma Detection Kit prior to use in experiments.

#### Isolation and preparation of D3-GPC2-IgG1

A naïve human Fab phage display library constructed from peripheral blood B cells of 50 healthy donors was used for selection of Fabs against purified recombinant GPC2 ectodomain (R&D Systems Inc., #2304) as described previously (9). Briefly, the isolated Fabs were expressed, purified, and tested for binding to the GPC2 ectodomain through ELISA and the best binder, designated as D3-GPC2-Fab, was converted to a full-length human IgG1. The full-length IgG1 DNA construct was transiently transfected into Freestyle 293-F cells (Thermo Fisher Scientific, #R79007, RRID:CVCL_D603) for antibody production and the D3-GPC2-IgG1 was purified on a protein A column.

#### Preparation of D3-GPC2-PBD

The purified D3-GPC2-IgG1 was directly used for glycan-based site-specific modification and conjugation as described previously (9). Briefly, DBCO-PEG4-PBD was used as the payload for the conjugation following the click chemistry-based approach. To maintain solubility of the DBCO-linker-PBD, the azide-attached antibody itself was diluted with propylene glycol to a final concentration of 33%. The solution of DBCO-linker-PBD in propylene glycol was added to the antibody solution with a drug-to-antibody mole ratio of approximately 4:1. The final concentration of propylene glycol in the conjugation reaction was 50%. The reaction was allowed to proceed for at least 4 hours at room temperature and purified by size exclusion chromatography and concentrated in PBS.

#### ADC payload and D3-GPC2-PBD ADC cytotoxicity assays

A panel of 11 neuroblastoma cell lines were plated in 96-well plates (typically between 1,000 and 5,000 cells/well) and treated with serial dilutions of each ADC payload, the D3-GPC2-PBD ADC, or vehicle the following day. After four additional days, cell viability was determined using a CellTiter-Glo assay (Promega) in a GloMax plate reader (Promega) according to the manufacturer’s instructions. Luminescence values were normalized to vehicle treated cells and data were analyzed in GraphPad Prism software to calculate IC₅₀. All cell lines were assessed for cytotoxicity in at least duplicate per drug concentration and each IC₅₀ assay was repeated at least twice. Means of biological replicates were used to compute the IC₅₀ values presented in the results section. The tubulin binding ADC payloads (MMAE (Catalog No. T1004), DM1 (Catalog No. LN-T-4582; Product Lot No. P1807L008192), DM4 (Catalog No. LN-T-7544; Product Lot No. P1706L008044), monomethyl dolastatin 10 (Catalog No. T1002; Product Lot No. P1710001150), HTI-286 (Catalog No. T1007), and DNA binding ADC payloads [PNU-159682 (Catalog No. D4001; Product Lot No. P1803L008114), PBD dimer (Catalog No. D4008), duocarmycin SA (Catalog No. D1001; Product Lot No. P120308054), and N-acetyl-calicheamicin γ1 (Catalog No. D4006)] were all purchased from Levena Biopharma and dissolved in DMSO. The topoisomerase I inhibitors [SN38 (HY-13704) and Dxd (HY-13631D)] were purchased from MedChem Express and similarly dissolved in DMSO. For all analyses when an IC₅₀ was not achieved, the highest tested dose was recorded and utilized in the summary IC₅₀ analyses.

#### D3-GPC2-IgG1 internalization

Cells were plated on an IncuCyte ZOOM live-cell monitoring system (Essen Bioscience, Inc.) and 5 µg/mL of D3-GPC2-IgG1 Red [generated using the IncuCyte FabFluor pH Red antibody labeling reagent (#4722) and D3-GPC2-IgG1 according to the manufacturer’s instructions] was added after 1 day. Treated wells of cells were quantified for red fluorescence (total object area) after background subtraction using Top-Hat and normalized to the % phase confluence of the identical cells.

#### Flow cytometry

For GPC2-directed flow cytometry, dissociated single cell suspensions were achieved with 0.02% EDTA in HBSS and cells were stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen) for 30 minutes in the dark on ice, washed with cold PBS, and incubated in the dark on ice for 30 minutes with the D3-GPC2-IgG1-PE antibody in 10% γ-Globulins (from human blood, Sigma-Aldrich, G4386), washed with cold PBS x 2, and fixed in 1% formaldehyde. Stained samples were run on a Beckman CytoFLEX S cytometer and analyzed using FlowJo software. To semiquantitate GPC2 cell surface expression, a BD Quantibrite Beads PE Phycocerythrin Fluorescence Quantitation Kit (#500495) was run in parallel with samples according to the manufacturer’s instructions. The D3-GPC2-IgG1 antibody was conjugated to PE using a PE-R-Phycocerythrin Conjugation Kit (Abcam, ab102918).
**Western blotting**

Whole-cell lysates were prepared with cell lysis buffer (Cell Signaling Technology, #9803), 1 mmol/L PMSF (Cell Signaling Technology, #8553), and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, P5726) and 3 (Sigma-Aldrich, P0044), briefly sonicated, rotated for 15 minutes at 4°C, centrifuged at 14,000 g at 4°C for 10 minutes, and then supernatant was removed and protein concentration was quantified by Bio-Rad bradford protein assays. Lysates were separated on 10% or 4% to 12% Bis-Tris gels (Life Technologies), transferred to a PVDF membrane, blocked in 5% nonfat milk in Tris-buffered saline.

### Table 1. ADC payload classes, mechanisms of action, and dosing.

<table>
<thead>
<tr>
<th>Payload</th>
<th>Payload class</th>
<th>Mechanism of action</th>
<th>ABCB1 substrate</th>
<th>Membrane permeable</th>
<th>FDA approved ADCs</th>
<th>ADC dosing (per cycle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomethyl</td>
<td>Tubulin (auristatin)</td>
<td>Inhibit tubulin polymerization, bind vinca binding site (45)</td>
<td>Yes (29, 30)</td>
<td>Yes (41)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>Tubulin (auristatin)</td>
<td>Inhibit tubulin polymerization, bind vinca binding site (45)</td>
<td>Yes (29, 30)</td>
<td>Yes (41)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DM1</td>
<td>Tubulin (maytansinoid)</td>
<td>Microtubule destabilizer, bind maytansine binding site (29)</td>
<td>Yes (32)</td>
<td>Yes (42)</td>
<td>Trastuzumab emtansine (4)</td>
<td>3.6 mg/kg</td>
</tr>
<tr>
<td>DM4</td>
<td>Tubulin (maytansinoid)</td>
<td>Microtubule destabilizer, bind maytansine binding site (29)</td>
<td>Yes (33)</td>
<td>Yes (42)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MMAE</td>
<td>Tubulin (auristatin)</td>
<td>Inhibit tubulin polymerization, bind vinca binding site (29)</td>
<td>Yes (29, 31)</td>
<td>Yes (43, 44)</td>
<td>Brentuximab vedotin (14)</td>
<td>1.8 mg/kg</td>
</tr>
<tr>
<td>H1-286</td>
<td>Tubulin (maytansinoid)</td>
<td>Inhibit tubulin polymerization, bind vinca binding site (45)</td>
<td>Yes (45)</td>
<td>Yes (45)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PNU-159682</td>
<td>DNA</td>
<td>Alkylates dsDNA, metabolite of anthracycline nemorubicin (46)</td>
<td>No (46)</td>
<td>Yes (44)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>N-acetyl-</td>
<td>Calicheamicin γ1</td>
<td>Interacts with DNA minor groove and cleaves DNA (47)</td>
<td>Yes (48)</td>
<td>Yes (49)</td>
<td>Gemtuzumab ozogamicin (16)</td>
<td>9 mg/m²</td>
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<tr>
<td>N-acetyl-</td>
<td>Calicheamicin γ1</td>
<td>Interacts with DNA minor groove and cleaves DNA (47)</td>
<td>Yes (48)</td>
<td>Yes (49)</td>
<td>Inotuzumab Ozogamicin (16)</td>
<td>1.8 mg/m²</td>
</tr>
<tr>
<td>Duocarmycin SA</td>
<td>DNA</td>
<td>Alkylates DNA (50)</td>
<td>Unk.</td>
<td>Yes (51)</td>
<td>Loncastuximab tesirine (17)</td>
<td>0.15 mg/kg</td>
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<tr>
<td>PBD dimer</td>
<td>DNA</td>
<td>Crosslinks DNA in minor groove (52)</td>
<td>No (52)</td>
<td>Yes (52)</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Dxd</td>
<td>DNA</td>
<td>Inhibits DNA topoisomerase 1 (53)</td>
<td>No (54)</td>
<td>Yes (55)</td>
<td>Trastuzumab deruxtecan (5)</td>
<td>5.4 mg/kg</td>
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<tr>
<td>SN-38</td>
<td>DNA</td>
<td>Inhibits DNA topoisomerase 1 (53)</td>
<td>Yes (56)</td>
<td>Yes (57)</td>
<td>Sacituzumab govitcan (3)</td>
<td>10 mg/kg</td>
</tr>
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</table>

### Table 2. Neuroblastoma cell line panel genomics and clinical covariates.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clinical phenotype</th>
<th>Patient gender</th>
<th>Primary tumor site/cell origin</th>
<th>Stage</th>
<th>TP53b</th>
<th>ALKc</th>
<th>MYCNb</th>
<th>Other mutation/Ampb</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB-SD</td>
<td>Relapse</td>
<td>M</td>
<td>Unk./BM</td>
<td>4</td>
<td>C176F</td>
<td>FI174L</td>
<td>Amp</td>
<td>ARID1A S1985fs*13</td>
</tr>
<tr>
<td>NB-EcC1</td>
<td>Relapse</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>4</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
<td>KRAS G12D</td>
</tr>
<tr>
<td>SMS-SAN</td>
<td>Diagnosis</td>
<td>F</td>
<td>Adrenal/BM</td>
<td>4</td>
<td>WT</td>
<td>FI174L</td>
<td>Amp</td>
<td>—</td>
</tr>
<tr>
<td>NB-1643</td>
<td>Diagnosis</td>
<td>M</td>
<td>R9/Rp</td>
<td>4</td>
<td>WT</td>
<td>R1275Q</td>
<td>Amp</td>
<td>—</td>
</tr>
<tr>
<td>LA-N-5</td>
<td>Diagnosis</td>
<td>M</td>
<td>Unk./BM</td>
<td>4</td>
<td>WT</td>
<td>R1275Q</td>
<td>Amp</td>
<td>—</td>
</tr>
<tr>
<td>CHP-134</td>
<td>Post-mortem</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>4</td>
<td>WT</td>
<td>WT</td>
<td>Amp</td>
<td>—</td>
</tr>
<tr>
<td>NB69</td>
<td>Diagnosis</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>3</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
<td>NTRK3 Q759K</td>
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<tr>
<td>NBL-S</td>
<td>Diagnosis</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>3</td>
<td>WT</td>
<td>WT</td>
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<td>Post-treatment</td>
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<td>C141W</td>
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<td>Amp</td>
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<tr>
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<td>Diagnosis</td>
<td>M</td>
<td>Abd./abd.</td>
<td>Unk.</td>
<td>WT</td>
<td>WT</td>
<td>Amp</td>
<td>ATM R189K</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>Relapse</td>
<td>F</td>
<td>Adrenal/BM</td>
<td>4</td>
<td>Nulld</td>
<td>WT</td>
<td>NA</td>
<td>NTRK3 Q759K</td>
</tr>
</tbody>
</table>

Abbreviations: dsDNA, double-stranded DNA; Unk., unknown.

Western blotting

Whole-cell lysates were prepared with cell lysis buffer (Cell Signaling Technology, #9803), 1 mmol/L PMSF (Cell Signaling Technology, #8553), and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, P5726) and 3 (Sigma-Aldrich, P0044), briefly sonicated, rotated for 15 minutes at 4°C, centrifuged at 14,000 × g at 4°C for 10 minutes, and then supernatant was removed and protein concentration was quantified by Bio-Rad Bradford protein assays. Lysates were separated on 10% or 4% to 12% Bis-Tris gels (Life Technologies), transferred to a PVDF membrane, blocked in 5% nonfat milk in Tris-buffered saline.

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<td>Relapse</td>
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<td>4</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
<td>KRAS G12D</td>
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<td>SMS-SAN</td>
<td>Diagnosis</td>
<td>F</td>
<td>Adrenal/BM</td>
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<td>Amp</td>
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<td>WT</td>
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<td>Amp</td>
<td>—</td>
</tr>
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<td>Diagnosis</td>
<td>M</td>
<td>Unk./BM</td>
<td>4</td>
<td>WT</td>
<td>R1275Q</td>
<td>Amp</td>
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<td>Post-mortem</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>4</td>
<td>WT</td>
<td>WT</td>
<td>Amp</td>
<td>—</td>
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<td>NB69</td>
<td>Diagnosis</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>3</td>
<td>WT</td>
<td>WT</td>
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<td>NBL-S</td>
<td>Diagnosis</td>
<td>M</td>
<td>Adrenal/adrenal</td>
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<td>Nulld</td>
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<td>NTRK3 Q759K</td>
</tr>
</tbody>
</table>

Abbreviations: Abd., abdomen; Amp, MYCN or other indicated gene amplified cell line; BM, bone marrow; F, female; LN, lymph node; M, male; NA, MYCN non-amplified cell line; RP, retroperitoneal; Unk., unknown; WT, wild-type.

As previously described (58–60).

As previously described (25, 26).

Subclone of IMR32, IMR32 clinical information shown.

Deletion of one TP53 allele and expression of C-terminally truncated TP53 variants from remaining TP53 allele due to a deletion in the intron 9/exon 10 junction, although SK-N-AS cells may maintain some partial TP53 functionality (9).
and Tween-20 (TBS-T), and blotted using standard protocols. Membranes were typically incubated at 4°C overnight in primary antibody, washed x 3 in TBS-T, then incubated in 1:2,000 diluted HRP-labeled secondary antibody at room temperature for 1 hour, washed an additional x 3 with TBS-T, and then developed with a chemiluminescent reagent (SuperSignal West Femto; Thermo Fischer Scientific). The following primary antibodies were used: β-actin (1:5,000; Cell Signaling Technology, #4967, RRID:AB_330288), cleaved caspase-3 (1:1,000; Cell Signaling Technology, #9664, RRID:AB_2070642), cleaved PARP (A2024; 1:1,000; Cell Signaling Technology, #9541, RRID:AB_331426), phospho-histone H2A.X (Ser139; γH2AX; 1:1,000; Cell Signaling Technology, #2577, RRID:AB_2118010), MDR1/ABCB1 (E1Y7S; 1:1,000; Cell Signaling Technology, #13978, RRID:AB_2798357), and BCL2 (D55G8; 1:1,000; Cell Signaling Technology, #4223, RRID:AB_1903909).

Figure 1.
DNA interacting ADCs payloads are significantly more cytotoxic to neuroblastomas. A, Heatmap showing the log IC50 values for each ADC payload tested in 11 unique neuroblastoma cell lines. Payloads ranked from left to right by the cell line panel median log IC50 value. B, Plot showing median ADC payload IC50 value grouped by drug family across the panel of neuroblastoma cell lines. Horizontal line in each group denotes mean of each payload family. C, Western blot analysis of the neuroblastoma SMS-SAN and NB69 cell lines 72 hours after treatment with 50 and 200 pmol/L of duocarmycin SA, N-acetyl-calicheamicin γ1, PBD dimer, or PNU-159682 DNA binding ADC payloads. D, Plot showing payload class median IC50 for each neuroblastoma cell line. IC50 is in A, B, and D represent summary data from at least two independent experiments. NAC, N-acetyl-calicheamicin γ1; Duo SA, duocarmycin SA; Dol 10, monomethyl dolastatin 10; MMAE, monomethyl auristatin E; cPARP, cleaved PARP; cCaspase-3, cleaved caspase-3 (*, P < 0.05).

Generation of GPC2 isogenic clones
GPC2 cDNA in the gateway donor vector pDONR221 was purchased from Harvard PlasmID Repository (plasmid ID: HsCD00045342) and cloned into the pLenti CMV Puro DEST (w118–1) vector, which was a gift from Eric Campeau (24) and was purchased via Addgene (plasmid #17452), with the Gateway LR clonase enzyme (Invitrogen) via the manufacturer’s protocol to make the GPC2 pLenti CMV Puro vector. The GPC2 over-expression plasmid was transfected along with pMD2.G (encoding envelope plasmid VSV-G) and psPAX2 (packaging plasmid) into HEK293T cells utilizing FuGENE 6. The pMD2.G (RRID:Addgene_12259) and psPAX2 (RRID:Addgene_12260) plasmids were a kind gift from the laboratory of Dr. Robert Schnepp. The virus-containing supernatant was collected 48 and 72 hours later and filtered with 0.45 μm nitrocellulose membranes. The Kelly cell line was transduced with this GPC2
lentivirus-containing supernatant. To increase transduction efficiency, virus was added to cells in the presence of 8 μg/mL polybrene (Sigma). Virus was removed after 24 hours, and transduced cells were replenished with medium supplemented with puromycin (Sigma) at an optimized concentration for bulk selection. Once fully selected with puromycin, GPC2 isoforms were then plated at a 1 cell/well dilution in 96-well plates. Each single-cell-derived clone was expanded and differential cell-surface GPC2 expression was confirmed by flow cytometry.

Neuroblastoma cell line profiling

Neuroblastoma cell lines were profiled by RNA and targeted DNA sequencing as described previously (25, 26).

Quantitative and statistical analysis

Differences between groups were presented as the mean ± error as noted in the figure legends. Experimental sample numbers (n) are indicated in the figures, figure legends, and results section where applicable. All t tests were two sided and P values < 0.05 were considered statistically significant. All statistical analysis was done with GraphPad Prism.

Results

DNA binding ADC payloads are significantly more cytotoxic to neuroblastoma cells

We selected a comprehensive panel of ADC payloads (n = 11; Table 1) including drugs that interact with tubulin (monomethyl dolastatin 10 (Dol-10), monomethyl auristatin E (MMAE), DM1, DM4, and HTI-286), DNA (PNU-159682, PBD dimer, doxorubicin SA (Duo SA), and N-acetyl-calicheamicin γ1 (NAC)), and DNA topoisomerase 1 (SN38 and Dxd (Exatecan derivative for ADC)) to define which payloads and/or payload drug classes were most potent in inducing neuroblastoma cell cytotoxicity. Importantly, each ADC payload selected for testing here is membrane permeable (Table 1). We assessed the IC50 96 hours after treatment with each free ADC payload in 11 unique neuroblastoma cell lines with diverse genomic drivers and clinical covariates (Table 2 and Fig. 1A). Neuroblastoma cell lines were significantly more sensitive to the family of DNA binding payloads than either the group of tubulin or DNA topoisomerase 1 interacting ADC payloads [mean IC50 = 25.6 pmol/L, range = 8.0–39.8 pmol/L for DNA binding drugs (n = 4)] versus mean IC50 = 943 pmol/L, range = 119–1,769 pmol/L for tubulin binding drugs (n = 5) and mean IC50 = 1,725 pmol/L, range = 1,230–2,220 pmol/L for DNA topoisomerase 1 inhibitors (n = 2); Fig. 1B]. PNU-159682 was the most potent payload overall (neuroblastoma cell line median IC50 = 8.0 pmol/L, range = 3.2–43.7 pmol/L), followed by the other DNA interacting drugs (Fig. 1A and B). The most potent tubulin binding payload was dolastatin 10 (neuroblastoma cell line median IC50 = 119 pmol/L, range = 43.8–435 pmol/L) and Dxd was minimally more potent than SN-38 in the DNA topoisomerase 1 inhibitor drug family (neuroblastoma cell line median IC50 = 1,230 pmol/L, range = 586–10,000 pmol/L; Fig. 1B). To confirm the on-target DNA damaging effects of the most potent DNA-interacting payloads (duocarmycin SA, N-acetyl-calicheamicin γ1, PBD dimer, and PNU-159682), we next quantified protein markers of DNA damage (γH2AX) and apoptosis (cleaved caspase-3 and cleaved PARP) 72 hours after 50 and 200 pmol/L drug treatment in the SMS-SAN (MYCN amplified) and NB69 (MYCN non-amplified) neuroblastoma cell lines and observed upregulation of many of these proteins after payload treatment in these cellular models (Fig. 1C). Finally, we compared the median IC50 for each of the three payload families tested (DNA or tubulin binders and DNA topoisomerase 1 inhibitors) for each of the 11 neuroblastoma cell lines and found that for each cell line, DNA binding drugs were the most potent ADC payload family, further confirming their superior potency in inducing neuroblastoma cytotoxicity (Fig. 1D).

ABCBI overexpression and mutant TPS53 impart selective ADC payload resistance in neuroblastoma

Given that there was significant variability in ADC payload-induced cytotoxicity across this neuroblastoma cell line panel, we next looked to assess potential mediators of neuroblastoma ADC payload resistance. First, we collated possible drug resistance mutations along with quantifying the expression of common drug transporters, ADC-specific resistance genes (27), and other potential mediators of neuroblastoma drug resistance in this cell line panel from our prior DNA and RNA sequencing analyses, respectively (Table 2 and Fig. 2A; refs. 25, 26). Considering the differential expression of the ATP binding cassette subfamily B member 1 (ABCB1) and the BCL2 apoptosis regulator (BCL2) mRNA in this cell line panel, we first chose to focus on these genes and further validate expression differences by Western blot analysis. ABCB1 and BCL2 protein expression generally correlated with RNA expression (r = 0.90; P < 0.001 for ABCB1 and r = 0.53; P = 0.10 for BCL2; Fig. 2B). Next, to determine if high levels of ABCB1 or BCL2 might be imparting resistance to individual or classes of ADC payloads, we compared IC50s for each drug across cohorts of high versus low ABCB1 and BCL2 expressing cell lines (Fig. 2C; Supplementary Fig. S1A). Neuroblastoma cell lines expressing high levels of ABCB1 were significantly more resistant to the tubulin binders dolastatin 10, DM1, DM4, and MMAE (Fig. 2C, left), but not the other ADC payloads tested. We similarly compared ADC payload IC50s across BCL2 high versus low expression cohorts (Supplementary Fig. S1A, left), but found no significant differences in susceptibility to any individual ADC payload or payload family in high versus low BCL2 expressing cells. To further validate the ABCB1

Figure 2.

ABCBI overexpression and TPS53 mutation impart selective resistance to specific ADC payloads in neuroblastoma cells. A, Heatmap summarizing expression of common drug transporter and resistance genes across the neuroblastoma cell line panel. B, ABCB1 and BCL2 western blot of neuroblastoma cell line panel. C (left), Plots showing IC50 data in ABCB1 low versus high expressing neuroblastoma cell line cohorts for DNA (top), tubulin (middle), and DNA topoisomerase 1 (bottom) interacting payloads. NB-ESt1, NB69, NBL-S, and IMR-5 represent ABCB1 high expressing cell lines as indicated in B (middle). Plots showing IC50 data in TPS53 mutant versus wild-type (WT) neuroblastoma cell line cohorts for DNA (top), tubulin (middle), and DNA topoisomerase 1 (bottom) interacting payloads. 5K-N-AS, NB-SD, and NGP represent TPS53 mutated cell lines as indicated in Table 2. C (right), Plots showing IC50 data in MYCN amplified (amp) versus MYCN non-amplified (NA) neuroblastoma cell line cohorts for DNA (top), tubulin (middle), and DNA topoisomerase 1 (bottom) interacting payloads. NB-SD, SMS-SAN, NB-1643, LA-N-5, CHP-134, NGP, and IMR-5 represent MYCN amplified cell lines as indicated in Table 2. D, NB69 DMI (top) and MMAE (bottom) IC50 with and without co-treatment with the ABCB1 inhibitor tariquidar. E, NB-ESt1 DM1 (top) and MMAE (bottom) IC50 with and without co-treatment with the ABCB1 inhibitor tariquidar. IC50 data in C represent the mean of at least 2 biological replicates. Horizontal lines in C represent cell line cohort median value. Data in D and E represent the mean ± SEM of at least three biological replicates. FPKM, Fragments Per Kilobase of transcript per Million mapped reads; MYCN amp, MYCN amplified cell lines; MYCN NA, MYCN non-amplified cell lines; WT, wild-type (*, P < 0.05; **, P < 0.01; ***, P < 0.001). See also Supplementary Fig. S1.
findings, we cotreated the high-ABCBl expressing cell lines NB-EbC1 and NB69 with the ABCBl inhibitor tariquidar (28) and the DM1 and MMAE ADC payloads. Inhibition of ABCBl in these cell lines significantly decreased the DM1 and MMAE IC50 (Fig. 2D and E), which importantly was not the result of tariquidar alone inducing changes in NB69 or NB-EbC1 cell growth (Supplementary Figs. S1B and S1C). These data are consistent with these tubulin binding payloads being previously described as ABCBl substrates in other cells (Table 1; refs. 29–33).

Next we co-hosted this neuroblastoma cell line panel according to the presence or absence of a tumor protein p53 (TP53) mutation (Table 2) to determine if a hypomorphic TP53 protein might also impart selective ADC payload resistance in neuroblastoma cells. Cell lines harboring a mutated TP53 protein had significantly higher IC50 values for the duocarmycin SA and PNU-159682 DNA binding payloads, along with both the DNA topoisomerase I inhibiting payloads Dsd and SN-38 (Fig. 2C, middle). However, co-hosting this cell panel by the presence of an activating mutation in the ALK receptor tyrosine kinase (ALK) gene (Table 2; ref. 7), or by cell lines derived at the time of diagnosis or post-treatment, showed no significant difference in payload sensitivity across these different cohorts (Supplementary Fig. S1A, middle and right). Finally, comparing ADC payload IC50 in MYCN non-amplified neuroblastoma cell lines, we found that the DNA damaging N-acetyl-calicheamicin y1 payload was significantly more cytotoxic to MYCN-amplified cell lines, but no difference was found in cytotoxicity to the other payloads (Fig. 2C, right).

Antibody internalization kinetics and target molecule density are more minor determinants of ADC efficacy in neuroblastoma

In addition to payload potency, the efficacy of an ADC also depends on antigen-dependent factors, such as cell surface density of the targeted molecule and the ability of the antibody/receptor interaction to induce cellular internalization of the ADC. Thus, we next utilized the recently developed GPC2-directed D3-GPC2-PBD ADC (9, 10), comprised of a fully human D3-GPC2-IgG1 conjugated to PBD via an enzymatically cleavable valine-alanine (VA) linker, as a model compound to study the interplay of some of these additional elements and their contribution to ADC efficacy in neuroblastoma. First, we quantified GPC2 cell surface density in this same panel of 11 neuroblastoma cell lines using flow cytometry studies with the D3-GPC2-IgG1 antibody (Fig. 3A and B). We also assessed the D3-GPC2-IgG1/GPC2 internalization kinetics using a labeled D3-GPC2-IgG1 that fluoresces red upon acidification in the lysosome (D3-GPC2-IgG1-Red; Fig. 3C and D) and quantified the D3-GPC2-PBD ADC induced cytotoxicity (IC50) for each of the same 11 neuroblastoma cell lines (Fig. 3E and F). GPC2 cell surface density significantly correlated with D3-GPC2-IgG1-Red antibody internalization (r = 0.75; P = 0.01), but neither of these GPC2 measurements correlated with D3-GPC2-PBD IC50 (r = −0.09 to −0.20; P = 0.55–0.79; Figure 3G and H; Supplementary Fig. S1D). In fact, cell lines that were sensitive to PBD dimers (n = 7 of 11 cell lines; PBD dimer IC50 < 40 pmol/L) had, with a 0.6-fold-range in D3-GPC2-IgG1-Red internalization (AUC of 779,130–4,943,575), all responded robustly to the GPC2 ADC IC50 range 1.6–6.1 pmol/L, except for the low D3-GPC2-IgG1 antibody internalizing NBL-S cell line (D3-GPC2-IgG1-Red AUC = 793,069; ADC IC50 > 5,000 pmol/L; Fig. 3I). However, the CHP-134 cell line similarly internally a low amount of antibody (D3-GPC2-IgG1-Red AUC = 779,130), yet was remarkably more susceptible to PBD dimers (IC50 = 5.7 pmol/L vs. 27.0 pmol/L for NBL-S), and thus was ultimately also very sensitive to the D3-GPC2-PBD ADC (IC50 = 1.6 pmol/L; Fig. 3I). Conversely, NB-1643 was only moderately sensitive to PBD dimers (IC50 = 49.3 pmol/L), yet internalized the maximum amount of antibody in this cell panel (D3-GPC2-IgG1-Red AUC = 5,419,312), and thus was also found to be very sensitive to the D3-GPC2-PBD ADC (IC50 = 3.0 pmol/L; Fig. 3I).

Finally, to further assess the role of differential cell surface GPC2 expression on D3-GPC2-PBD ADC efficacy, we engineered a panel of seven GPC2 isogenic clones from the MYCN amplified Kelly neuroblastoma cell line, which has a heterozygous deletion of the GPC2 locus, to eliminate the effect of genomic variability on these analyses (Fig. 4A and B). These data confirmed a correlation between GPC2 cell surface density and ADC cytotoxicity (Fig. 4B–D), and in fact showed a strict GPC2 cell surface density threshold to achieve ADC efficacy (IC50 < 10 pmol/L; black box in Fig. 4D), above which no significant additional ADC efficacy benefits were observed. Finally, to ensure the observed differences in D3-GPC2-PBD ADC IC50 in these cell lines was not due to a difference in sensitivity to the PBD payload itself, we treated a subset of these cell lines with PBD alone and found no correlation between sensitivity to PBD and GPC2 cell surface density (Fig. 4E and F). Taken together, these data reveal the critical interplay between antigen-dependent and antigen-independent factors and their effect on ADC efficacy, and importantly identifies payload potency as a critical determinant of ADC efficacy in neuroblastoma.

Discussion

Immunotherapeutic approaches have revolutionized the treatment of several cancers. However, much of what drives the efficacy of these different therapies, their mechanisms of resistance, and how best to optimize each therapy to maximize safety and efficacy in humans remains unknown. ADCs are an attractive type of immunotherapy especially for solid tumors, as they enable selective targeting of tumor-specific, cell surface molecules that are not found widely on normal tissues. Furthermore, ADCs are often endowed with diverse mechanisms of tumor killing, which is particularly ideal for the often heterogeneous antigen expressing, immunosuppressive microenvironment state of solid tumors. However, due to lack of prior systematic investigation of different ADC payload classes in neuroblastoma, drug selection for neuroblastoma targeting ADCs to date has been achieved by arbitrary selection of payloads based on efficacy in other cancer histotypes (7–9, 34). Thus, our first aim here was to assess the potency of membrane permeable ADC payloads in a diverse panel of neuroblastoma cellular models to define which payloads would be most optimal to utilize in ADCs targeting this pediatric cancer. We also aimed to use our recently developed GPC2-targeting D3-GPC2-PBD ADC to define how antigen dependent factors, such as target cell surface density and antibody internalization kinetics, also contribute to ADC efficacy in neuroblastoma. Finally, the parallel RNA and DNA profiling of this neuroblastoma cell line panel allowed us to determine potential mediators of ADC payload resistance in neuroblastoma.

Importantly, we found that ADC payloads that interact with DNA are the most potent class of drugs for neuroblastoma. Although TP53 mutation may be a biomarker for DNA interacting payload resistance, TP53 mutations are an infrequent event in patients with both newly diagnosed and relapsed neuroblastoma (35). Furthermore, we found that MYCN amplified neuroblastomas are equally, or potentially even more susceptible, to the potent DNA binding payloads as their MYCN non-amplified cell line counterparts. Taken together, these data support the superior efficacy of DNA binding ADC payloads even in the most clinically aggressive subset of high-risk neuroblastoma patients that have relapsed disease and/or MYCN amplified tumors. In
Cell surface antigen density and antibody internalization are other factors involved in ADC efficacy. 

A, Representative GPC2 flow cytometry histograms for 11 neuroblastoma cell lines. 

B, Summary of neuroblastoma cell line GPC2 cell surface densities. 

C, Plot of D3-GPC2-IgG1-Red internalization kinetics for 11 neuroblastoma cell lines. 

D, Summary of neuroblastoma cell line D3-GPC2-IgG1-Red internalization AUC from C. 

E, Plot of relative neuroblastoma cell growth after treatment with increasing concentrations of the GPC2 ADC D3-GPC2-PBD. 

F, Summary of neuroblastoma cell line D3-GPC2-PBD ADC IC50s. 

G, Plot of D3-GPC2-IgG1-Red internalization AUC versus D3-GPC2-IgG1-Red internalization kinetics for the 11 neuroblastoma cell lines. 

H, Plot of D3-GPC2-IgG1-Red internalization AUC versus D3-GPC2-PBD ADC IC50s for the 11 neuroblastoma cell lines. 

I, Plot of D3-GPC2-PBD ADC IC50 versus PBD dimer IC50 for the 11 neuroblastoma cell lines. 

Relative D3-GPC2-IgG1-Red internalization for each cell line indicated. Very high D3-GPC2-IgG1-Red internalizing cell lines represent a D3-GPC2-IgG1-Red AUC of >3 million, high represents an AUC of 2 to 3 million, moderate represents an AUC of 1 to 2 million, and low represents an AUC of <1 million. Data in B and F represent mean ± SEM of at least two independent experiments and data in A, C, D, and E represent data from a representative experiment repeated at least two independent times. See also Supplementary Fig. S1.
Figure 4.

Defining the GPC2 cell surface density required for D3-GPC2-PBD efficacy. A, Representative GPC2 flow cytometry histograms for GPC2 isogenic Kelly cell line panel. B, Summary of GPC2 cell surface density for GPC2 isogenic Kelly cell line panel. C, Plot of relative GPC2 isogenic Kelly cell line growth after treatment with increasing concentrations of the D3-GPC2-PBD ADC. D, Plot of the D3-GPC2-PBD ADC IC_{50} versus GPC2 cell surface density for the GPC2 isogenic Kelly cell line panel. E, Plot of relative GPC2 isogenic Kelly cell line growth after treatment with increasing concentrations of free PBD across a range of different GPC2 cell surface densities (GPC2 molecules/cell noted in parentheses in legend). F, Plot of PBD and D3-GPC2-PBD ADC IC_{50} for GPC2 isogenic Kelly cell lines across a range of different GPC2 cell surface densities (GPC2 molecules/cell noted on the x-axis). Data in B, D, and F represent mean ± SEM of at least two independent experiments. For D, SEM is indicated for both the ADC IC_{50} and GPC2 molecule per cell values. Data in A, C, and E are representative data from experiments done at least two independent times. US, unstained.
addition, the cytotoxicity of the DNA binding payloads tested here were not significantly affected by high levels of the common drug-transporter ABCB1. Conversely, many of the tubulin-binding payloads more commonly used in the oncology clinic were significantly less potent in high-ABC1 expressing neuroblastoma cells. Clearly many other neuroblastoma cell intrinsic factors may be influencing the sensitivity to this diverse panel of ADC payloads and it is possible that many of these factors are unique to this cancer histotype. For example, schlaften family member 11 (SLFN11) expression has recently been shown to be integral in DNA-damage induced cell death in several adult cancers, including sensitivity to the DNA binding and DNA topoisomerase inhibiting drug classes studied here (36). In considering SLFN11 expression across this cell line panel and these data’s relevance to this study, we found that the NB-EbC1 cell line is the only model that has any appreciable SLFN11 expression. However, in general we did not observe the NB-EbC1 cell line to be more sensitive to the DNA binding and DNA topoisomerase 1 inhibiting drug class compounds studied here. Thus, the exquisite sensitivity to DNA binding drugs here is not driven by SLFN11 and, more broadly, drug resistance mechanisms need to be studied in a cancer histotype-specific manner.

By defining the GPC2 cell surface density, kinetics of GPC2 antibody internalization, and D3-GPC2-PBD ADC IC50 in this cell line panel in parallel, we were also able to begin to elucidate the complex interplay of these factors in combination with payload potency in dictating overall ADC efficacy in neuroblastoma cells. Although we found a strong correlation between the density of GPC2 on neuroblastoma cells and D3-GPC2-IgG1 antibody internalization, the susceptibility of each cell line to the PBD payload was the most critical driver of D3-GPC2-PBD ADC potency in these studies. Remarkably, in PBD dimer susceptible cell lines, very minimal antibody internalization is needed to achieve potent ADC efficacy (e.g., CHP-134). These data suggest it is imperative to carefully consider the selection of payload when designing new ADC therapeutics for preclinical testing.

Although this study focused on the potency of different ADC payloads, future work should also determine the role of linker chemistry in the overall efficacy of ADCs in neuroblastoma. Different ADC linkers, such as noncleavable versus chemical or enzymatic labile linkers, may also have a critical role in dictating what ADC therapeutics are ultimately the most cytotoxic to neuroblastoma cells (37). For example, noncleavable ADC linkers may offer a larger therapeutic index than their cleavable counterparts given their increased stability in the human circulation, along with also differing the potential advantage of converting a payload from an ABCB1 substrate into a nonsubstrate by increasing hydrophobicity of the intracellularly released drug (38, 39). Thus, the effect of different linker chemistries on ADC efficacy and resistance also warrants careful consideration and rigorous testing in neuroblastoma and other cancers.

A potential limitation of this study is the ability to ultimately translate these in vitro findings to clinical care. The therapeutic window of ADCs, like other small molecules, is generally defined by the differential effects of the ADC on the tumor versus vital normal tissues, not by their relative in vitro potencies. Furthermore, the human drug exposure for ADCs containing PBD and other DNA binding payloads are significantly lower than ADCs conjugated to DNA topoisomerase 1 or tubulin binding drugs, and these differences in drug exposure should be considered when interpreting the results of these studies. For example, as shown in Table 1, initial dosing of the FDA-approved PBD-containing ADC loncastuximab tesirine is 12- to 25-fold lower than the MMAE payload-containing ADCs polatuzumab vedotin, brentuximab vedotin, and enfotumab vedotin, 24-fold lower than the DM1-containing ADC trastuzumab emtansine, and 36-fold lower than the DNA topoisomerase 1 inhibitor Dxd-conjugated ADC trastuzumab duxetanec. These dosing differences should be considered when interpreting the 24-, 66-, and 46-fold increase in neuroblastoma potency (cell line median IC50) of PBD versus MMAE, DM1, and Dxd payloads, respectively. However, given that the therapeutic index of an ADC is also defined by the differential expression of the target molecule, a tumor-specific protein such as GPC2 may allow for higher doses of PBD or other DNA binding payload ADCs to be tolerated in the clinic, thus potentially minimizing these payload dosing differences in some circumstances.

Taken together, here we used a comprehensive panel of ADC payloads and the GPC2-targeting D3-GPC2-PBD ADC to define the major factors of ADC potency in neuroblastoma. For this often lethal embryonal cancer, DNA binding payloads should be prioritized for future ADC development given their superior efficacy coupled with the fact that payload sensitivity is a major determinant of ADC efficacy.

**Authors’ Disclosures**

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**Authors’ Contributions**

S.N. Buenger: conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. M.V. Lane: conceptualization, data curation, software, formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. E. Garrett: data curation, formal analysis, validation, investigation, visualization, methodology. D.V. Zhelev: validation, investigation, methodology. D.S. Dimitrov: Resources, data curation, formal analysis, supervision, funding acquisition, validation, methodology, project administration. K.R. Bosse: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing—review and editing.

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