Antibody-drug conjugate efficacy in neuroblastoma - role of payload, resistance mechanisms, target density, and antibody internalization

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Abstract

Antibody-drug conjugates (ADCs) 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 sensitivity 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 anti-cancer 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 (ADCs) 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. Additionally, ALK (7), LGALS3BP (8), and GPC2 (9,10) targeting 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. While 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 anti-tumor 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
While 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 immunotherapeutic targets, we previously identified glypican 2 (GPC2), a glycosylphosphatidylinositol (GPI)-anchored cell surface proteoglycan, to be a robustly differentially expressed molecule on 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 (22)] conjugated to pyrrolobenzodiazepine (PBD) dimers (D3-GPC2-PBD) through an enzymatic cleavable linker (9,10). However, recent data has 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_LF68), NB69 (RRID:CVCL_1448), SK-N-AS (RRID:CVCL_1700), NB-EbC1 (CVCL_E218), NBL-S (RRID:CVCL_2136), NB-1643 (RRID:CVCL_5627), IMR-5 (RRID:CVCL_1306), SMS-SAN (RRID:CVCL_7136), NGP (RRID:CVCL_2141), LA-N-5 (RRID:CVCL_0389), Kelly (RRID:CVCL_2092), and CHP-134 (RRID:CVCL_1124)] were obtained from the CHOP cell line bank or the Children’s Oncology Group Childhood Cancer Repository and HEK293T cells (RRID: CVCL_0063) were obtained from the American Type Culture Collection (ATCC, Cat # CRL-3216). All cells were cultured in Corning® RPMI 1640 medium containing 10% FBS, 2 mM 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 GenePrint® 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 previously described (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 previously described (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 1,000-5,000 cells/well) and treated with serial dilutions of each ADC payload, the D3-GPC2-PBD ADC, or vehicle the following day. After 4 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 IC$_{50}$s. All cell lines were assessed for cytotoxicity in at least duplicate per drug concentration and each IC$_{50}$ assay was repeated at least twice. Means of biological replicates were used to compute the IC$_{50}$ values presented in the results section. The tubulin binding ADC payloads [MMAE (Cat. No. T1004), DM1 (Cat. No. LN-T-4582; Product Lot No. P1807L008192), DM4 (Cat. No. LN-T-7544; Product Lot No. P1706L008044), monomethyl dolastatin 10 (Cat. No. T1002; Product Lot No. P1710001150), HTI-286 (Cat. No. T1007)], and DNA binding ADC payloads [PNU-159682 (Cat. No. D4001; Product Lot No. P1803L008114), PBD dimer (Cat. No. D4008), duocarmycin SA (Cat. No. D1001; Product Lot No. P120308054), and N-acetyl-calicheamicin γ1 (Cat. 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 IC$_{50}$ was not achieved, the highest tested dose was recorded and utilized in the summary IC$_{50}$ 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, 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 semi-quantitate GPC2 cell surface expression, a BD Quantibrite™ Beads PE Phycoerythrin Fluorescence Quantitation Kit (#340495) 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-Phycoerythrin Conjugation Kit (Abcam, ab102918).

**Western blotting**

Whole-cell lysates were prepared with cell lysis buffer (Cell Signaling Technology, #9803), 1 mM 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 x 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 4-12% Bis-Tris gels (Life Technologies), transferred to a PVDF membrane, blocked in 5% non-fat milk in Tris-buffered saline 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_2070042), Cleaved PARP (Asp214) (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).

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 isogenic cells 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 previously described (25,26).

**Quantitation 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, duocarmycin SA (Duo SA), and N-acetyl-calicheamicin γ1 (NAC)], and DNA topoisomerase I [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 half-maximal inhibitory concentration (IC₅₀) 96 hours after treatment with each free ADC payload in 11 unique neuroblastoma cell lines with diverse genomic drivers and clinical co-variates (Table 2 and Figure 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 pM, range = 8.0 - 39.8 pM for DNA binding drugs (n=4) versus mean IC₅₀ = 943 pM, range = 119 - 1,769 pM for tubulin binding drugs (n=5) and mean IC₅₀ = 1,725 pM, range = 1,230 - 2,220 pM for DNA topoisomerase I inhibitors (n=2); Figure 1B]. PNU-159682 was the most potent payload overall (neuroblastoma cell line median IC₅₀ = 8.0 pM, range = 3.2 - 43.7 pM), followed by the other DNA interacting drugs (Figure 1A, B). The most potent tubulin binding payload was dolastatin 10 (neuroblastoma cell line median IC₅₀ = 119 pM, range = 43.8 - 435 pM) and Dxd was minimally more potent than SN-38 in the DNA topoisomerase 1 inhibitor drug family (neuroblastoma cell line median IC₅₀ = 1,230 pM, range = 586 - >10,000 pM; Figure 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 pM drug treatment in the SMS-SAN (MYCN amplified) and
NB69 (MYCN non-amplified) neuroblastoma cell lines and observed upregulation of most of these proteins after payload treatment in these cellular models (Figure 1C). Finally, we compared the median IC_{50} for each of the 3 payload families tested (DNA or tubulin binders and DNA topoisomerase I 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 (Figure 1D).

**ABCB1 overexpression and mutant TP53 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 Figure 2A) (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. 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; Figure 2B). Next, to determine if high levels of ABCB1 or BCL2 might be imparting resistance to individual or classes of ADC payloads, we compared IC_{50}s for each drug across cohorts of high versus low ABCB1 and BCL2 expressing cell lines (Figure 2C and Supplementary Figure 1A). Neuroblastoma cell lines expressing high levels of ABCB1 were significantly more resistant to the tubulin binders dolastatin 10, DM1, DM4, and MMAE (Figure 2C, left), but not the other ADC payloads tested. We similarly compared ADC
payload IC₅₀s across BCL2 high versus low expression cohorts (Supplementary Figure 1A, 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 findings, we co-treated the high-ABCB1 expressing cell lines NB-EbC1 and NB69 with the ABCB1 inhibitor tariquidar (28) and the DM1 and MMAE ADC payloads. Inhibition of ABCB1 in these cell lines significantly decreased the DM1 and MMAE IC₅₀s (Figure 2D and E), which importantly was not the result of tariquidar alone inducing changes in NB69 or NB-EbC1 cell growth (Supplementary Figure 1B and C). These data are consistent with these tubulin binding payloads being previously described as ABCB1 substrates in other cells (Table 1) (29-33).

We next cohorted 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 1 inhibiting payloads Dxd and SN-38 (Figure 2C, middle). However, cohorting this cell panel by the presence of an activating mutation in the ALK receptor tyrosine kinase (ALK) gene (7) (Table 2), 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 Figure 1A, middle and right). Finally, comparing ADC payload IC₅₀s in MYCN amplified versus MYCN non-amplified neuroblastoma cell lines, we found that the DNA damaging N-acetyl-calicheamicin γ1 payload was significantly more cytotoxic to MYCN amplified cell lines, but no difference was found in cytotoxicity to the other payloads (Figure 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 (Figure 3A, 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; Figure 3C, D) and quantified the D3-GPC2-PBD ADC induced cytotoxicity (IC$_{50}$) for each of the same 11 neuroblastoma cell lines (Figure 3E, 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 IC$_{50}$ ($r = -0.09$ to $-0.20$; $p = 0.55 - 0.79$; Figure 3G, H and Supplementary Figure 1D). In fact, cell lines that were sensitive to PBD dimers ($n = 7$ of 11 cell lines; PBD dimer IC$_{50} <$40 pM), with over a 6-fold-range in D3-GPC2-IgG1-Red internalization [area under the curve (AUC) of 779,130 - 4,943,575], all responded robustly to the GPC2 ADC (IC$_{50}$ range 1.6 – 6.1 pM), except for the low D3-GPC2-IgG1 antibody internalizing NBL-S cell line (D3-GPC2-IgG1-Red AUC = 793,069; ADC IC$_{50} >$5,000 pM; Figure 3I). However, the CHP-134 cell line similarly internalized a low amount of antibody (D3-GPC2-IgG1-Red AUC = 779,130), yet was remarkably more susceptible to PBD dimers (IC$_{50} = 5.7$ versus 27.0 pM for NBL-S), and thus was ultimately also very sensitive to the D3-GPC2-PBD ADC (IC$_{50} = 1.6$ pM; Figure 3I).
Conversely, NB-1643 was only moderately sensitive to PBD dimers (IC$_{50}$ = 49.3 pM), 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 (IC$_{50}$ = 3.0 pM; Figure 3I).

Finally, to further assess the role of differential cell surface GPC2 expression on D3-GPC2-PBD ADC efficacy, we engineered a panel of 7 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 (Figure 4A, B). These data confirmed a correlation between GPC2 cell surface density and ADC cytotoxicity (Figure 4B-D), and in fact showed a strict GPC2 cell surface density threshold to achieve ADC efficacy (IC$_{50}$ < 10 pM; black box in Figure 4D), above which no significant additional ADC efficacy benefits were observed. Finally, to ensure the observed differences in D3-GPC2-PBD ADC IC$_{50}$ 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 (Figure 4E, 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. Additionally, 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-ABCB1 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, schlafen 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 IC₅₀s 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. While 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.

While 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 non-cleavable 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, non-cleavable ADC linkers may offer a larger therapeutic index than their cleavable counterparts given their increased stability in the human circulation, along with also offering the potential advantage of converting a payload from an ABCB1 substrate into a non-substrate by increasing hydrophilicity 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 enfortumab 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...
deruxtecan. 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.
Acknowledgments

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References


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# 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 Dolastatin 10</td>
<td>Tubulin (auristatin)</td>
<td>Inhibit tubulin polymerization, bind vinca binding site (40)</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 (12)</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></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Polatuzumab vedotin (15)</td>
<td>2.4 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Enfortumab vedotin (6)</td>
<td>3.75 mg/kg</td>
</tr>
<tr>
<td>HTI-286</td>
<td>Tubulin (hemiasterlin)</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-calicheamicin γ1</td>
<td>DNA</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>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></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>-</td>
<td>-</td>
</tr>
<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>Loncastuximab tesirine (17)</td>
<td>0.15 mg/kg</td>
</tr>
<tr>
<td>Dxd</td>
<td>DNA topoiseromerase 1</td>
<td>Inhibits DNA topoiseromerase 1 (53)</td>
<td>No (54)</td>
<td>Yes (55)</td>
<td>Trastuzumab deruxtecan (5)</td>
<td>5.4 mg/kg</td>
</tr>
<tr>
<td>SN-38</td>
<td>DNA topoiseromerase 1</td>
<td>Inhibits DNA topoiseromerase 1 (53)</td>
<td>Yes (56)</td>
<td>Yes (57)</td>
<td>Sacituzumab govitecan (3)</td>
<td>10 mg/kg</td>
</tr>
</tbody>
</table>

*Potentially less interaction with ABCB1 than other anti-microtubule agents.
dsDNA, double-stranded DNA; Unk., unknown.
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 (^a)</th>
<th>TP53 (^b)</th>
<th>ALK (^b)</th>
<th>MYCN (^b)</th>
<th>Other mutation/ Amp (^b)</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>F1174L</td>
<td>Amp</td>
<td>ARID1A S1985fs*13</td>
</tr>
<tr>
<td>NB-Ebc1</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>F1174L</td>
<td>Amp</td>
<td>-</td>
</tr>
<tr>
<td>NB-1643</td>
<td>Diagnosis</td>
<td>M</td>
<td>RP/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>
</tr>
<tr>
<td>NBL-S</td>
<td>Diagnosis</td>
<td>M</td>
<td>Adrenal/adrenal</td>
<td>3</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>NGP</td>
<td>Post-treatment</td>
<td>M</td>
<td>Unk./lung</td>
<td>4</td>
<td>A159D</td>
<td>C141W</td>
<td>WT</td>
<td>CDK4 Amp MDM2 Amp</td>
</tr>
<tr>
<td>IMR-5</td>
<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>Null (^d)</td>
<td>WT</td>
<td>NA</td>
<td>NRAS Q61K</td>
</tr>
</tbody>
</table>

\(^a\) As previously described (58-61).
\(^b\) As previously described (25,26).
\(^c\) Subclone of IMR32, IMR32 clinical information shown.
\(^d\) 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).

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

Figure 1. DNA interacting ADCs payloads are significantly more cytotoxic to neuroblastomas.

(A) Heatmap showing the log IC_{50} 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 IC_{50} value.

(B) Plot showing median ADC payload IC_{50} 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 of the neuroblastoma SMS-SAN and NB69 cell lines 72 hours after treatment with 50 and 200 pM of duocarmycin SA, N-acetyl-calicheamicin γ1, PBD dimer, or PNU-159682 DNA binding ADC payloads.

(D) Plot showing payload class median IC_{50} for each neuroblastoma cell line.

IC_{50}s in A, B, and D represent summary data from at least 2 independent experiments.

NAC, N-acetyl-calicheamicin γ1; PBD, pyrrolobenzodiazepine dimer, Duo SA, duocarmycin SA; Dol 10, monomethyl dolastatin 10; MMAE, monomethyl auristatin E; cPARP, cleaved PARP; cCaspase-3, cleaved caspase-3.

*, p < 0.05.

Figure 2. ABCB1 overexpression and TP53 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-EbC1, NB69, NBL-S, and IMR-5 represent ABCB1 high expressing cell lines as indicated in B.

(C, middle) Plots showing IC₅₀ data in TP53 mutant versus wild-type (WT) neuroblastoma cell line cohorts for DNA (top), tubulin (middle), and DNA topoisomerase 1 (bottom) interacting payloads. SK-N-AS, NB-SD, and NGP represent TP53 mutated cell lines as indicated in Table 2.

(C, right) Plots showing IC₅₀ data in MYCN amplified (amp) versus MYCN non-amplified (NA) harboring 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 DM1 (top) and MMAE (bottom) IC₅₀s with and without co-treatment with the ABCB1 inhibitor tariquidar.

(E) Nb-EbC1 DM1 (top) and MMAE (bottom) IC₅₀s 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 3 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 Figure 1.
Figure 3. 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 area under the curve (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 IC_{50}s.

(G) Plot of D3-GPC2-IgG1-Red internalization AUC versus GPC2 cell surface density for the 11 neuroblastoma cell lines (r = 0.75, p = 0.01).

(H) Plot of D3-GPC2-IgG1-Red internalization AUC versus D3-GPC2-PBD ADC IC_{50} for the 11 neuroblastoma cell lines (r = -0.20, p = 0.55).

(I) Plot of D3-GPC2-PBD ADC IC_{50} versus PBD dimer IC_{50} for the 11 neuroblastoma cell lines (r = 0.79, p = 0.006). Relative D3-GPC2-IgG1-Red internalization for each cell line indicated. Very high D3-GPC2-IgG1 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 2 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 Figure 1.

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$_{50}$s for GPC2 isogenic Kelly cell lines across a range of different GPC2 cell surface densities (GPC2 molecules/cell noted in parentheses on the x-axis).

Data in B, D, and F represent mean ± SEM of at least 2 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.
Molecular Cancer Therapeutics

Antibody-drug conjugate efficacy in neuroblastoma - role of payload, resistance mechanisms, target density, and antibody internalization

Samantha N. Buongervino, Maria V. Lane, Emily Garrigan, et al.

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