Design and preclinical evaluation of CPL976-MMAE - novel, potent AXL-PD-L1 bispecific antibody conjugated with MMAE in targeted anticancer therapy.

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INTRODUCTION

Antibody-drug conjugates (ADCs) combine the specificity of antibodies with the potency of highly cytotoxic agents, reducing the off-target effects and improve effectiveness with more precise delivery of the payload to the tumor site. Strong correlation has been established between AXL and PD-L1 expression levels in many types of cancer. Therefore, targeting both proteins simultaneously ensure high cancer specificity in delivering the toxic payload. We have developed a bispecific antibody (BsAb), that strongly induces receptor internalization (Fig. 1). Our bispecific ADC CPL976-MMAE later described as CPBT0976-MMAE combines anti-AXL and anti-PD-L1 construct with toxic MMAE (Monomethyl auristatin E, a potent tubulin inhibitor toxin), for better targeting to solid tumor cells and greatly reducing off-target effects.

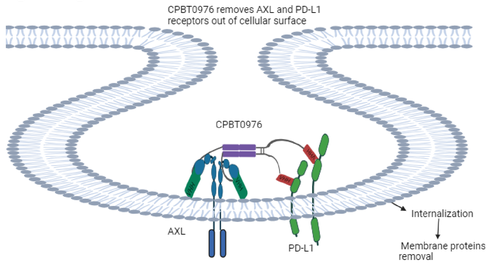


Figure 1. Proposed mechanism of action of CPBT0976

MATERIALS AND METHODS

Construction, Production and Conjugation of BsAb CPBT0976.

Potent PD-L1 and AXL binders were isolated from llama VHH libraries and re-formatted into multivalent bispecific constructs by the N- and C-terminal fusions with Fc fragment of human IgG1 (Fig. 2A). PD-L1-targeting VHH was fused directly to the hinge region while anti-AXL nanobody was connected to the C-terminus of Fc through a flexible glycine-serin (G4S) linker. Generation and production of BsAb was performed by standard DNA recombinant technologies, and BsAb was purified from transiently transfected cell culture supernatant by protein A chromatography.
BsAb CPBT0976 was site specific conjugated using GlycoConnect (GlyClick) technology to DBCO-VS-GlcA-PAB-MMAE payload (component of GlyCLICK® ADC MMAE kit, Genovis), which contains cleavable glycopeptide linker and a potent tubulin inhibitor toxin, Monomethyl Auristatin E (MMAE). Two-step enzymatic procedure (enzymatic trimming of the N-linked glycans by EndoS2 and terminal GlcNAc extension with N3-GalNAc using galactosyltransferase) transforms Fc-glycans present on IgG into two site-specific, azide-activated anchor points for the conjugation of alkyne-containing payload (Fig. 2C). The efficiency of the conjugation process was monitored with reducing SDS-PAGE after IdeS digestion of BsAbs. Glycan trimming resulted first in increased in-gel mobility of the protein (Fig. 2D, lane 2) and attachment of MMAE payload caused decreased in-gel mobility, confirming high efficiency of conjugation reaction, as no unconjugated CPBT0976 was detected (Fig. 2D, lane 3).

Evaluation of conjugation efficiency

Conjugation efficiency was estimated using RP-HPLC (Reverse Phase – High Performance Liquid Chromatography) with UV detection. The conjugate sample was denatured using β-mercaptoethanol before analysis. Due to change in polarity of bispecific antibody after conjugation of payload a shift in retention time of analyte was expected. The comparison of AUC (Area Under the Curve) of peaks which were detected for conjugated protein and free protein were used for estimation of conjugation efficiency.

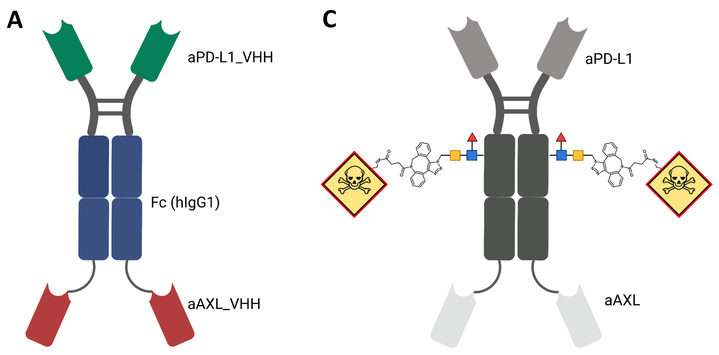
In vitro techniques used to verify biological activity and specificity of bispecific antibody CPBT0976-MMAE

Bispecific antibodies chosen for conjugation were first characterized in vitro for the interaction with the extracellular domain of human AXL and PD-L1 using surface plasmon resonance and flow cytometry. After the conjugation with MMAE, ADCs cytotoxicity and selectivity were evaluated in the human breast cancer model (MDA-MB-31) with high expression of both targets, AXL and PD-L1. The human embryonic kidney cell line (HEK-293) with minimal expression of targets was used as a negative control, the expression of both targets was confirmed by Western Blot technique (Fig. 4A). The cytotoxic effect of the CPBT0976-MMAE conjugate was tested with use of the fluorescent nucleic acid stain which enters only cells with compromised cell membranes. Cells were stained with Hoechst for total cell nuclei count and cultured with addition of CPBT0976-MMAE conjugate, its single arm controls (CPBT0990-MMAE and CPBT01126-MMAE) or referent control conjugate (Tilvestamab-MMAE). Results are shown as % of dead cells in each timepoint (Fig. 3)
The ability of CPBT0976-MMAE to bind to PD-L1 and AXL on MDA-MB-231 cell line was exanimated by flow cytometry. The MDA-MB-231 cell line was treated with CPBT0976 and CPBT0976-MMAE and the amount of bonded antibodies were detected with a PE-labelled Goat anti-Human IgG Fc antibody. The Flow cytometry analysis was performed on a Attune NxT flow cytometer (Invitrogen) using the Attune software. The results were compared to the control stained by the secondary antibody (PE-labelled Goat anti-Human IgG Fc) (Fig 4B).

In vivo evaluation of CPBT0976-MMAE

The efficacy of conjugates were evaluated in xenograft mouse model. The CB17-SCID (CB17/Icr-Prkdcscid) mice were implanted subcutaneously with human MDA-MB-231 cancer cells and received the test conjugates intravenously to assess tumor growth inhibition. Conjugates were given according to scheme: 6 doses, administered twice a week (Fig. 4C).

RESULTS



ID	Molecular target
CPBT0976 (CPL976)	AXL/aPDL1
CPBT0990	PDL1
CPBT1126	AXL

Fig.2. Conjugation of anti-PD-L1 × anti-AXL BsAb CPBT0976 with a cytotoxic payload. A) Schematic representation of bispecific antibody. Anti-PD-L1 VHH domains (green) were linked to the hinge region (grey) and anti-AXL nanobodies (red) by flexible G4S linker to the C-terminal end of the human IgG1 Fc fragment (blue); **B)** Table with names of bispecific and monospecific single arms; **C)** Two molecules of MMAE (shown as red/yellow diamonds) are conjugated to anti-PD-L1 × anti-AXL BsAb CPBT0976 via click chemistry at Asn297 (EU numbering) after glycan remodeling with GlycoConnect site specific bioconjugation technology; **D)** The efficiency of the conjugation and purity of obtained ADC were analyzed with reducing SDS-PAGE. Untreated BsAb CPBT0976 (1st line) was first trimmed with EndoS2, leaving partially deglycosylated sample of lower molecular mass (2nd line), which was then conjugated with MMAE payload, resulting in molecular mass shift of approx. 2 kDa (3rd line), as compared with deglycosylated sample (976 dG).

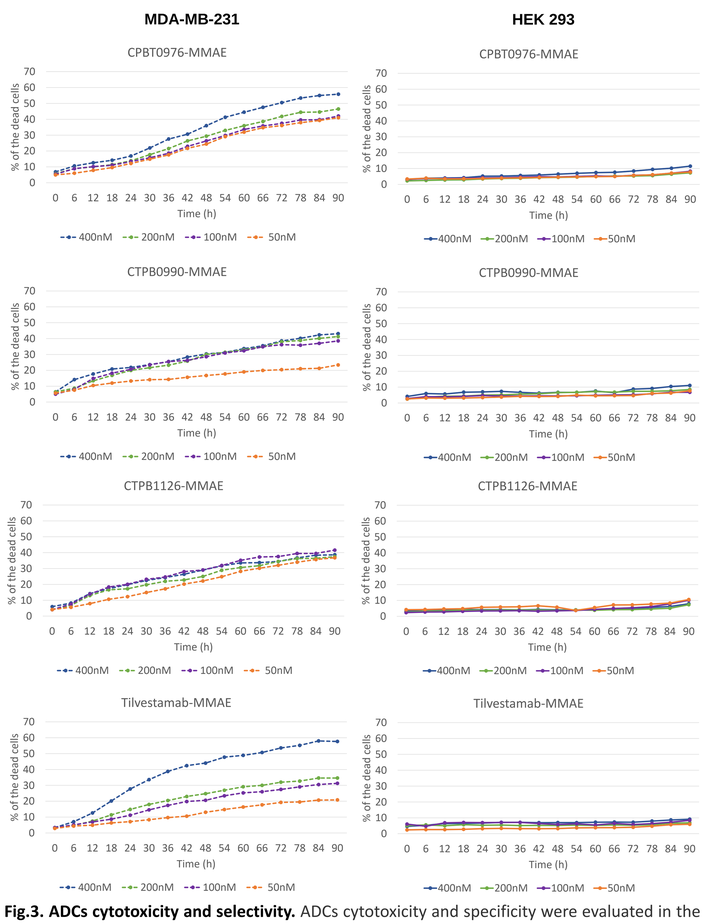


Fig.3. ADCs cytotoxicity and selectivity. ADCs cytotoxicity and specificity were evaluated in the human breast cancer model – MDA-MB-231 cell line (left) with high expression of both targets, AXL and PD-L1, using SytoX and Hoechst. The human embryonic kidney cell line - HEK-293 (right) with minimal expression of targets, was used as a negative control.

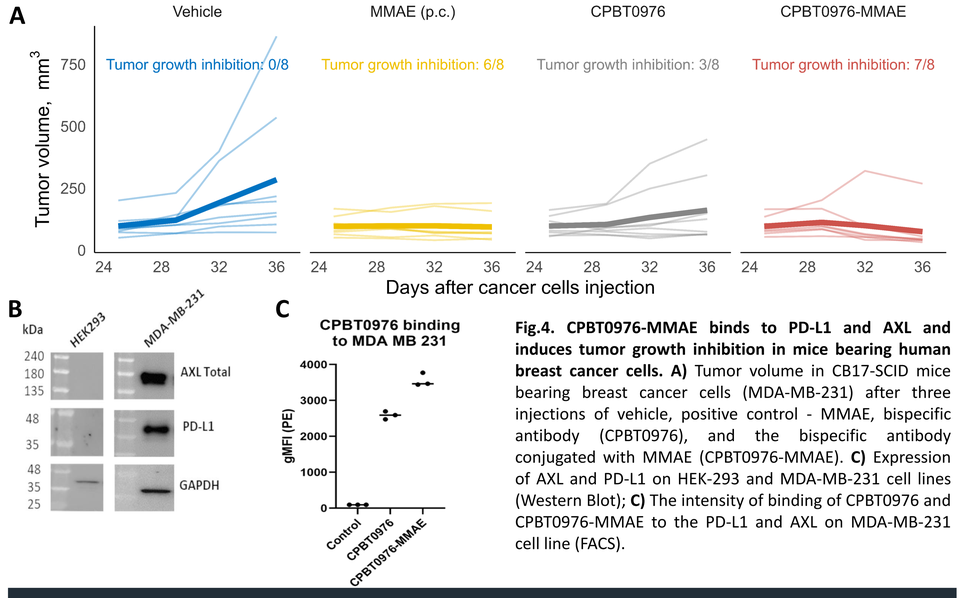


Fig.4. CPBT0976-MMAE binds to PD-L1 and AXL and induces tumor growth inhibition in mice bearing human breast cancer cells. A) Tumor volume in CB17-SCID mice bearing breast cancer cells (MDA-MB-231) after three injections of vehicle, positive control - MMAE, bispecific antibody (CPBT0976), and the bispecific antibody conjugated with MMAE (CPBT0976-MMAE). **C)** Expression of AXL and PD-L1 on HEK-293 and MDA-MB-231 cell lines (Western Blot); **C)** The intensity of binding of CPBT0976 and CPBT0976-MMAE to the PD-L1 and AXL on MDA-MB-231 cell line (FACS).

REFERENCES

[1] Sun *et al*. Regulation and Function of the PD-L1 Checkpoint. Immunity (2018) 48, 434-452.
[2] Loe *et al*. Monomethyl auristatin E-conjugated anti-EGFR antibody inhibits the growth of human EGFR-positive non-small cel lung cancer. Cancer Chemotherapy and Pharmacology (2019), 84, 61-72.
[3] Engelsen *et al*. Dissecting the Role of AXL in Cancer Immune Escape and Resistance to Immune Checkpoint Inhibition (2022) Front. Immunol. 13:869676.
[4] Shim *et al*. Bispecific Antibodies and Antibody-Drug Conjugates for Cancer Therapy: Technological Considerations. Biomolecules (2020) 26;10(3):360.

CONCLUSIONS

We present CPBT0976 (CPL976), an innovative bispecific antibody, with excellent binding parameters, strong biological effect on receptor presentation on the cell surface, and promising *in vitro* and *in vivo* results which after effective conjugation process (98% efficiency) reach 20% higher cytotoxic effect than achieved by incubation with single arm ADCs. The effect of the highest dose (400nM) of CPBT0976-MMAE is comparable to the same concentration of conjugate of referent (Tilvestamab). However, in the case of Tilvestamab-MMAE the cytotoxic effect was dose dependent, while for the CPBT0976-MMAE the dose response was much smaller what can suggest the higher effectiveness of ADCs with our bispecific antibody. CPBT0976-MMAE, as well as its single arm controls (CPBT0990-MMAE, CPBT1126-MMAE) has a target-specific cytotoxic effect on MDA-MB-231 cells, what was confirmed with lack of effect on viability of HEK-293 cells which are negative for the targets. Moreover, conjugating the MMAE to the CPBT0976 antibody does not influence its binding to the target proteins. CPBT0976-MMAE inhibits the tumor growth in 7 from 8 mice.

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