CPL976, an innovative bispecific antibody targeting AXL and PD-L1 axis as a potential new anticancer therapeutic.

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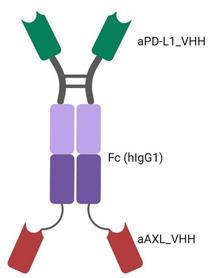
INTRODUCTION

Bispecific antibodies rapidly emerge as a powerful therapeutic tool in cancer treatment. In oncology therapies use of the bispecific antibodies targeting more than one antigen on cancer cells enhances the specificity of the therapy, increases its effectiveness and decreases the resistance rate. This approach may be use to target the defense mechanism of the cancer cells, such as high-level expression of the immune checkpoint ligands, like PD-L1 or growth factor receptors like EGFR, VEGFR and AXL. The latest reports revealed the correlation between expression level of the PD-L1 and AXL/EGFR1 in cancers. Notably, the disruption of the AXL signaling pathway leads to a reduction of the PD-L1 level on the cell surface. Adoption of this mechanism into oncologic therapies may help to achieve the enhanced immune response against cancer cells. Moreover, the use of the bispecific antibodies simultaneously targeting PD-L1 and AXL provoke internalization of the target to maximize the biological effect of the receptor/ligand complexes (AXL/GAS6, PD-1/PD-L1) formation inhibition.

MATERIALS AND METHODS

Bispecific antibody CPBT0976 development

Bispecific tetravalent biparatopic anti-AXL/anti-PD-L1, single chain antibody, contained humanized lama-origined heavy chain variable domains (VHH) as N- and C-terminal fusion with the human IgG1 Fc fragment, in which: the VHH fragment that specifically binds PD-L1 is contained as an N-terminal fusion with the human IgG1 Fc fragment and the VHH fragment that specifically binds AXL contained as C-terminal fusion with Fc human IgG1.



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

Fig.1. Schematic structure of CPBT0976 and table with names of referent compounds and monospecific single arms.

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

Antibodies' biological activity and specificity were studied with the Western blot technique on breast cancer cell line (MDA-MB-231), automated Cytation5 Imager with BioSpa incubator (cytotoxicity with Sytox Green™ dye, scratch assay with BioTek AutoScratch). Bispecific binding potential to AXL and PD-L1, and its effect on receptor internalization were evaluated with the use of surface plasmon resonance (SPR), Western Blot technique, flow cytometry (FACS), and pH-dependent dye assays (Zenon™ pHrodo™ iFL IgG Labeling Reagents). The efficacy of the antibody in the tumor was checked in a syngeneic mice model (C57BL/6) bearing murine colon cancer cells (MC-38).

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RESULTS

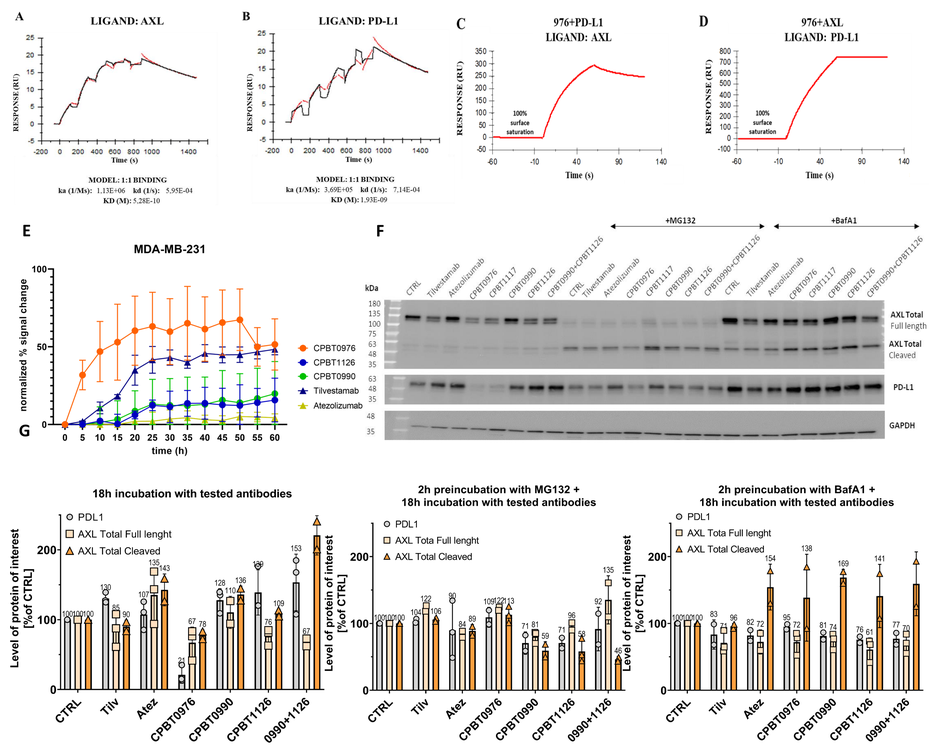

Fig.2. CPBT0976 bispecific antibody simultaneously binds to both molecular targets and induces their internalization and degradation via proteasomal and endosomal pathway. A,B: kinetic parameters of CPBT0976 binding to AXL **(A)** and PD-L1 **(B)**, acquired by SPR technique; fit was acquired with Biacore Evaluation software. **C, D:** binding profiles of CPBT0976 forming a ternary complex with AXL and PD-L1. Full ligand (**C**: AXL , **D**: PD-L1) saturation was established with an injection of CPBT0976, and second antigen was then injected to confirm a secondary binding event. Fit was acquired with Biacore Evaluation software. **E:** Internalization of bispecific antibodies and single arm controls (n=3). Antibodies were labeled with pH-dependent fluorescent dye, which signal increases with decrease of the pH. The data are presented as normalized to signal obtained from dye control group. **F:** Protein level of molecular targets of CPBT0976: AXL, PD-L1 and loading control (GAPDH) in non-treated cells vs cells pre-treated with inhibitors of protein degradation (proteasome inhibitor, MG132 and inhibitor of lysosomal acidification, BafilomycinA1); representative Western Blot image. **G:** densitometry of the protein bands was performed in ImageJ software and results were shown as % of control (N=2; error bars: SD).

RESULTS

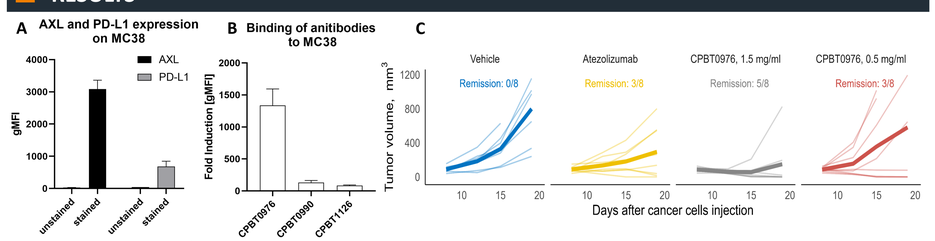

Fig.4. CPBT0976 Bispecific antibody binds to murine PD-L1 and AXL and in dose dependent manner induces tumor growth inhibition in mice with intact immune system. A: Expression of AXL and PD-L1 on cell surface of murine colon cancer cells MC-38 (FACS); **B:** binding contribution of monoclonal antibodies anti-PD-L1 and anti-AXL in bispecific antibodies to AXL and PD-L1 on MC-38 cells surface (FACS); **C:** Volume of tumors in C57BL/6 mice bearing murine colon cancer cells (MC-38) after bi-weekly intraperitoneal injection of 6 doses of vehicle, reference antibody (Atezolizumab) and CPBT0976.

RESULTS

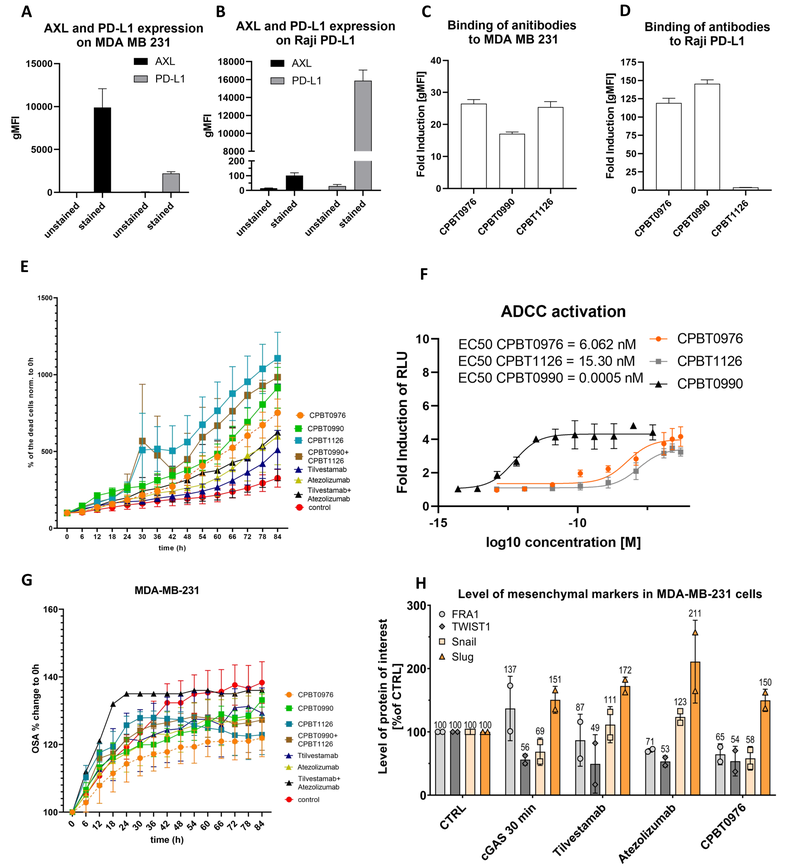

Fig.3. Biological activity of CPBT0976 results from simultaneous binding to both molecular targets and induction of AXL and PD-L1 internalization and degradation. **A-B:** Expression of AXL and PD-L1 on cell surface of MDA-MB-231 and Raji PD-L1 cells, analyzed by Flow Cytometry; **C-D:** binding contribution of monoclonal antibodies anti-PD-L1 and anti-AXL in bispecific antibodies binding to AXL and PD-L1 on MDA-MB-231 and Raji PD-L1 cells surface (FACS). **E:** Cytotoxic effect on MDA-MB-231 cells. The data are presented as % of dead cells after normalization to non-treated control. **F:** EC50 and intensity of ADCC signaling stimulation by CPBT0976 and monospecific antibodies after binding to PD-L1 and AXL on MDA-MB-231 and CD16 on Jurkat NFAT CD16. **G:** Effect on migratory potential of MDA-MB-231 cells. Data shown as change in object sum area (OSA) normalized to non-treated control. **H:** Level of mesenchymal markers in MDA-MB-231 measured by densitometry of protein bands from Western Blots (N=2; error bars: SD).

CONCLUSIONS

We present CPBT0976 (CPL976), an innovative bispecific antibody, with excellent binding parameters, strong biological effect on molecular targets presentation on the cell surface, and promising *in vitro* and *in vivo* results (even after two weeks recovery time, tumors did not regrow in C57BL/6 mice). These combined properties may establish a new generation of anticancer antibodies that effectively block cancer development and break the PD-1/PD-L1 axis in patients with primary and secondary resistance to PD-1/PD-L1 targeted therapies. Such unique properties might also be later use as an effective carrier of toxic payloads in antibody-drug conjugate format.