LETTER OPEN

Bifunctional small molecules targeting PD-L1/CXCL12 as dual immunotherapy for cancer treatment

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Dear Editor,

Inhibiting PD-1/PD-L1 interaction is a highly promising therapeutic modality.[1] However, due to the low overall response rate in patients, researchers have attempted to combine PD-L1 inhibitors with other antitumor agents for cancer therapy. Studies have shown that combination immunotherapy of PD-L1 antibodies with CXCL12 inhibitors exhibited synergistic and better antitumor efficacy than monotherapy, indicating the potential clinical utility of targeting both PD-L1 and CXCL12 as dual immunotherapy to treat cancer.[2,3] However, there are several disadvantages for combination therapy, including unpredictable PK/PD and overlapping toxicities. A potential alternative to combination therapy would be to use a single molecule with dual or multi-targeting capability, as the PK/PD of a single molecule is easily predictable. For example, dual-targeting bispecific antibodies (bsAbs) have gained significant attention in the field of anticancer drug discovery in recent years. Many PD-1/PD-L1-based bsAbs (e.g., anti-PD-L1/TGF-β, anti-PD-1/CTLA-4, and anti-PD-1/LAG-3) have entered clinical trials as dual immunotherapy for treating cancer. However, bsAbs-based dual immunotherapies also suffer from the common drawbacks (e.g., immunogenicity, poor pharmacokinetics) as antibodies, thus it would be of high significance to develop small molecule PD-L1 inhibitor-based dual immunotherapy, as small molecules may overcome the above drawbacks of antibodies.

We have previously reported PD-L1-targeting bifunctional molecules as potential anticancer agents.[4] To continue our interest in this area, we designed a set of compounds targeting both PD-L1 and CXCL12 simultaneously as potential dual immunotherapy based on the hypothesis that PD-L1 and CXCL12 are two critical biomacromolecules controlling the immunosuppressive tumor microenvironment. Firstly, we analyzed the pharmacophores of PD-L1 inhibitors and CXCL12 inhibitors (Fig. 1a). The tail group of PD-L1 inhibitors and the hydroxyl moiety of CXCL12 inhibitors were exposed to solvent, making them suitable sites for conjugating the two inhibitors via a linker. Thus, twenty-one bifunctional molecules were designed, synthesized (Supplementary Scheme S1), and bioevaluated (Supplementary Table S1).

Among them, **CP21** showed the strongest PD-L1-inhibitory effects with IC_{50} of 78.6 nM (HTRF assay). Furthermore, **CP21** displayed similar binding affinity (SPR assay) to both h(human)PD-L1 ($K_D = 66.9$ nM, Fig. 1b) and m(mouse)PD-L1 ($K_D = 70.1$ nM) (supplementary Fig. S1a). In addition, CD (Circular dichroism) assay revealed that when hPD-L1 or mPD-L1 was mixed with **CP21**, the conformation of their secondary structures changed similarly, as compared to the vehicle which contains only hPD-L1 (Fig. 1c) or mPD-L1 (Supplementary Fig. S1b). Moreover, the microscale thermophoresis (MST) assay confirmed that **CP21** could bind to mPD-L1 with a K_D of 654.1 nM (Supplementary Fig. S1c, d). Next, the binding affinity of **CP21** to hCXCL12 and mCXCL12 was also determined by SPR and CD. **CP21** bound to hCXCL12 ($K_D = 160$ nM, SPR) and mCXCL12 ($K_D = 76.5$ nM, SPR) in a dose-dependent manner (supplementary Fig. S1e, f). Furthermore, CD spectroscopy revealed an altered conformation in the secondary structures of hCXCL12 and mCXCL12 upon the addition of **CP21** (Supplementary Fig. S1g, h). The above results suggest that **CP21** could cross-bind to hPD-L1/hCXCL12 or mPD-L1/mCXCL12 proteins with high affinity.

To further investigate the possible binding modes of these dual-acting compounds, we conducted molecular docking analysis for **CP21** with PD-L1 and CXCL12 proteins. As expected, **CP21** fitted nicely in the inner cavity of the PD-L1 dimer (Fig. 1d). Calorimetric data (Fig. 1e and supplementary Fig. S2a) from the ITC assay suggested that the excellent binding affinity of **CP21** to PD-L1 was primarily due to hydrophobic interactions, consistent with molecular modeling studies. Additional molecular docking study also suggested that **CP21** could cross-bind with h/m PD-L1 through different modes (supplementary Fig. S2b–e). Furthermore, **CP21** bound well to the hydrophobic cleft formed by CXCL12 (supplementary Fig. S2f), which was further confirmed by ITC analysis (supplementary Fig. S2g, h). The well-defined molecular modeling studies of **CP21** in PD-L1 and CXCL12 may explain the high binding affinity of **CP21** to its target proteins.

To validate **CP21** as a dual inhibitor of CXCL12 and PD-L1 in vitro, we first evaluated the effects of **CP21** on CXCL12-mediated Ca^{2+} cellular responses in THP-1 cells using a calcium flux assay.[5] As shown in Fig. 1f, **CP21** dose-dependently inhibited CXCL12-induced calcium responses as measured by spectrofluorometry. Flow cytometry analysis further confirmed that **CP21** abolished the response of CXCL12-mediated Ca^{2+} flux (supplementary Fig. S3a). Moreover, **CP21** dose-dependently inhibited CXCL12-mediated cell migration (supplementary Fig. S3b).

To investigate the immunomodulatory effects of **CP21**, we assessed the mortality rate of HepG2 cells utilizing a Jurkat T&HepG2 cell co-culture model. As illustrated in Fig. 1g, **CP21** dose-dependently promoted the death of HepG2 cells with effects similar to that of BMS-1233 (supplementary Fig. S4a, b). The immunomodulatory effects of **CP21** were further confirmed by the MDB-MB-231& PBMC, Hep3B&PMBC, and Jurkat T&B16-F10 co-culture models (supplementary Fig. S4c–g). Collectively, these results suggest that **CP21** could target both CXCL12 and PD-L1 in vitro.

The pharmacokinetic properties of **CP21** were evaluated in male SD rats with **CP21** administered intravenously (i.v.) and orally (p.o.). As detailed in Table S2, oral gavage of **CP21** (18 mg/kg) exhibited low but acceptable plasma exposure ($AUC_{(0-t)} = 127.5 \pm 4.8$ ng/mL h). While intravenous administration of **CP21** (1 mg/kg) displayed more favorable pharmacokinetic properties, such as higher exposure ($AUC = 2032.5 \pm 11.44.8$ ng/mL h), and lower clearance rate (0.5 ± 0.1 L/h/kg).

Having identified the excellent PD-L1/CXCL12 inhibitory potency in vitro for the bifunctional molecule **CP21**, we next evaluated the anticancer activity of **CP21** in vivo using a mouse

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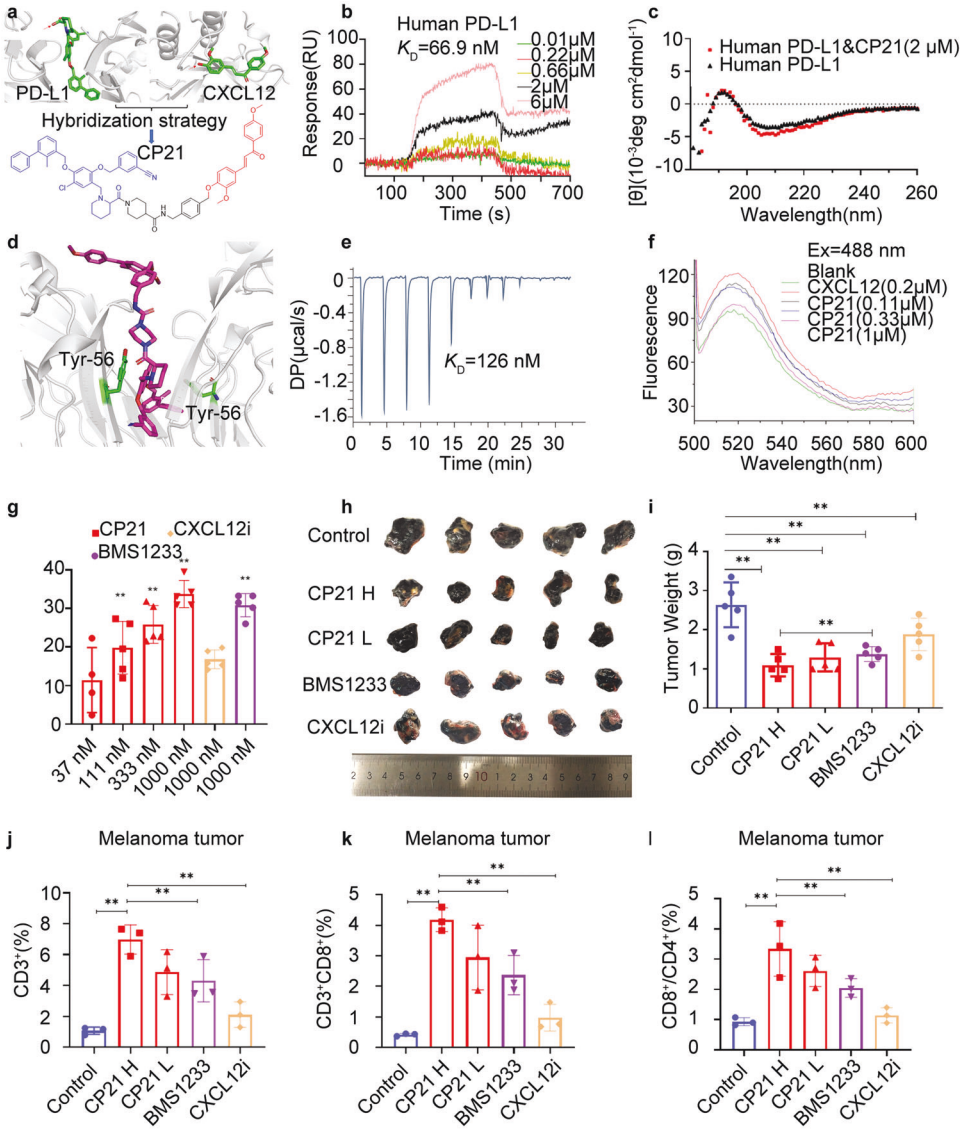

Fig. 1 **a** Design of bifunctional molecules targeting PD-L1/CXCL12 based on the pharmacophores of CXCL12 inhibitors and PD-L1 inhibitors. **b** The binding affinity of **CP21** to hPD-L1 as measured by SPR. **c** CD spectra of hPD-L1 in complex with or without **CP21**. **d** Docking of **CP21** to hPD-L1 (PDB code: 6R3K). **e** ITC study for **CP21** in complex with hPD-L1. **f** Intracellular Ca^{2+} release was measured by spectrofluorometry in THP-1 cells. **g** Effects of **CP21** and BMS-1233 (positive control) on HepG2 cell mortality in Jurkat T cell&HepG2 co-culture system ($n = 5$, $^{**}P < 0.05$). **h** Therapeutic effects of **CP21** (**h**, 125 mg/kg; **l**, 75 mg/kg), BMS-1233, and CXCL12i (inhibitor) against B16-F10 melanoma tumors in mice ($n = 5$). **i** Average tumor weights ($^{**}P < 0.05$, $n = 5$). Percentages of $CD3^+$ cells (**j**) and $CD3^+CD8^+$ cells (**k**), and the ratio of $CD8^+/CD4^+$ cells (**l**) in tumor tissues ($n = 3$, $^{**}P < 0.05$)

B16-F10 model. As presented in Fig. 1h, i, **CP21** dose-dependently decreased tumor weights and volumes. Oral administration of **CP21** at 125 mg/kg exhibited a tumor growth inhibition (TGI) of 58.5%, better than either of the monotherapies. In addition, the body weights of mice did not change markedly during the treatment (supplementary Fig. S5). In addition, **CP21** exhibited similarly high in vivo antitumor efficacy in a CT-26 colon syngeneic tumor model (supplementary Fig. S6).

To examine the immunomodulatory effects of **CP21**, a flow cytometry assay was carried out to analyze the TILs (tumor-infiltrating lymphocytes). As shown in supplementary Fig. S7 (representative examples), the percentage of TILs ($CD3^+$) in the **CP21** treatment group was dose-dependently higher than that in either of the monotherapy groups. In addition, data from multiple repeated experiments/animals also showed that the percentages of $CD3^+$, $CD3^+CD8^+$, and the ratio of $CD8^+/CD4^+$ T cells were dose-dependently/significantly increased in the **CP21** treatment groups, higher than either of the monotherapy groups (Fig. 1j–l). The above results indicate that **CP21** could activate the immune microenvironments in tumors.

In addition, serum biochemistry showed that **CP21** did not induce hepatotoxicity and nephrotoxicity in mice (Supplementary Fig. S8a). Importantly, **CP21** treatment did not cause cardiotoxicity based on the creatine kinase isoenzyme assay (Supplementary Fig. S8b). Furthermore, H&E staining revealed that there was no apparent morphological aberration in the **CP21** treatment group (Supplementary Fig. S8c), which was in agreement with the results of serum biochemistry.

Collectively, **CP21** is a bifunctional small molecule targeting PD-L1 and CXCL12 with high affinities. It also demonstrated high in vivo antitumor efficacy by activating the immune system. Hence, **CP21** represents a first-in-class dual PD-L1/CXCL12 inhibitor deserving further investigation as a potential dual immunotherapy agent for cancer treatment.

DATA AVAILABILITY

All the data used for the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

B.C., W.W., and T.L. conceived the project, performed experiments, analyzed data, and wrote the manuscript. H.C., W.P., and Y.X. helped with the experimental design. S.L. and J.C. supervised and supported the study. J.C. edited the language, conceived the study, and provided guidance for the whole study. All authors have read and approved the article.

ADDITIONAL INFORMATION

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Competing interests: The authors declare no competing interests.

Ethics: Protocols for animal experiments (B16-F10 and CT-26 tumor model) were approved by the NIACEC (National Institutional Animal Care & Ethical Committee) at SMU (Southern Medical University) and Hubei Polytechnic University (Assurance Number: HBPU-IACUC-MED-2022-1122). All animal studies were conducted strictly in accordance with the guidelines set by the Chinese Regulations of Laboratory Animals and Laboratory Animal Requirements of Environment and Housing Facilities.

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