MSLN and MUC1 dual-targeting CAR-T with conditional activation and tumor specificity to improve clinical safety and efficacy

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SUMMARY

MSLN and MUC1 are highly expressed in numerous solid tumors, making them potential targets for solid tumor treatment. However, these targets also show varying levels of expression in key human tissues, raising the risk of 'on-target, off-tumor' effects in CAR-T therapy. Furthermore, the tumor immune microenvironment (TME) exerts a strong immunosuppressive effect on CAR-T cells, rendering them prone to exhaustion and impairing their ability to maintain a long-lasting anti-tumor response.

In this study, a dual-targeting CAR-T (designated BZE2203) for MUC1 and MSLN was developed and validated in both pre-clinical and clinical studies. The anti-MSLN VHH was engineered with a pH-dependent characteristic, enabling CAR-T cells to exhibit cytotoxicity only at acidic pH levels within the TME. The anti-MUC1 VHH was selected based on antigen density dependence to ensure it does not exert cytotoxic effects on cells with low MUC1 expression in normal tissues. Additionally, the CAR-T cells were armed with a secreting anti-PD1 VHH to counteract the immunosuppressive effects of the TME on CAR-T cells and tumor-infiltrating lymphocytes. The Cao-V3 xenograft animal model was used to assess the safety and efficacy of BZE2203 CAR-T in vivo. In an investigator-initiated trial (IIT), patients, including two with platinum-resistant ovarian cancer, were enrolled and infused with a low dose of BZE2203 CAR-T cells to preliminarily evaluate the safety and efficacy of the product.

In vitro experiments demonstrated that BZE2203 CAR-T cells exerted cytotoxicity against MSLN-positive target cells only in acidic pH, not neutral pH, and targeted MUC1-positive tumor cells in a density-dependent manner. Importantly, no cytotoxic effect was observed on normal human primary lung cells with low MUC1 expression. Upon activation by target cells, CAR-T cells secreted high levels of anti-PD1 VHHs. BZE2203 CAR-T showed significant anti-tumor effects in the Cao-V3 animal model. In the IIT clinical trial, patients did not experience severe safety issues, such as cytokine storms or neurotoxicity, commonly associated with CAR-T treatments. Notably, two ovarian cancer patients achieved partial remission.

Our results suggest that pH-dependent and antigen-density-dependent CAR-T strategies can mitigate on-target, off-tumor toxicities, showing significant potential to improve both safety and efficacy in the clinical treatment of solid tumors.

METHOD

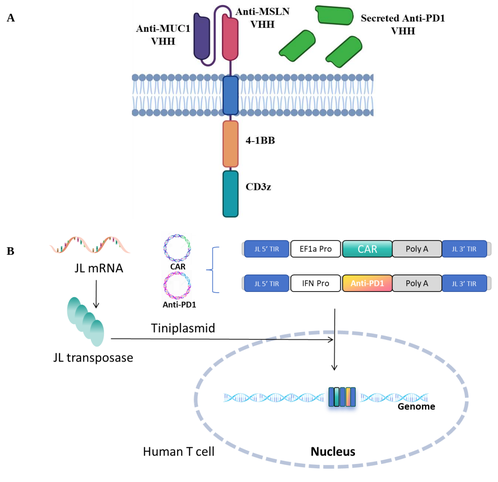

Figure 1. Product design. A) Schematic diagram of the CAR-T structure. **B)** Delivery system using transposase mRNA and Tiniplasmids.

RESULTS

Table 1. Affinity characterization of MUC1 or MSLN VHHs and bispecific VHHs

Ligand	Analyte	PH6.0 k_{on} (1/Ms)	PH6.0 k_{off} (1/s)	PH6.0 K_d (M)	PH7.4 k_{on} (1/Ms)	PH7.4 k_{off} (1/s)	PH7.4 K_d (M)
Anti-MUC1 VHH	MUC1	1.55E+05	1.83E-03	1.18E-08	7.65E+04	1.96E-03	2.56E-08
Bispecific VHH	MUC1	3.75E+04	9.82E-04	2.62E-08	3.75E+04	1.36E-03	3.63E-08
Anti-MSLN VHH	MSLN	1.21E+04	2.54E-03	2.10E-07	No binding		
Bispecific VHH	MSLN	1.29E+04	2.62E-03	2.02E-07			

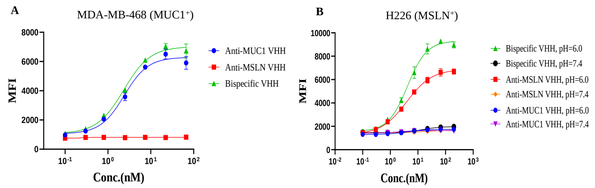

Figure 2. VHHs and bispecific VHH binding to target-positive cells. A) The binding activity of bispecific VHH on MUC1-positive cells was comparable to that of the anti-MUC1 parental VHH; **B)** Bispecific VHH exhibited pH-dependent binding to MSLN-positive cells.

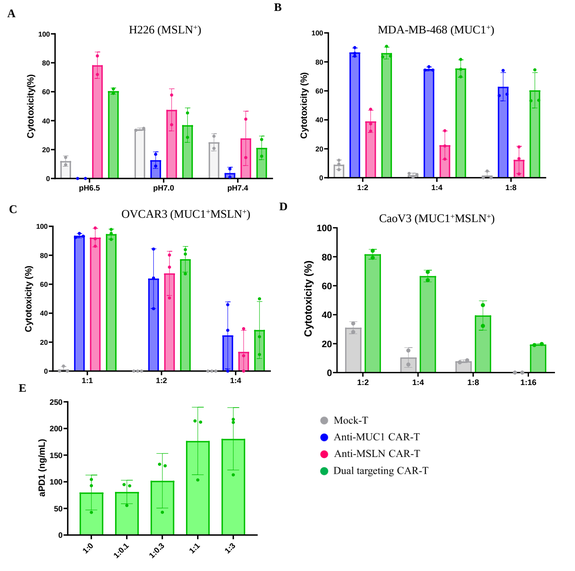

Figure 3. Specific killing of BZE2203 CAR-T cells against MUC1 and MSLN-positive tumor cells. A) BZE2203 CAR-T exhibited pH-dependent killing against MSLN-high expressing cell lines; **B)** BZE2203 CAR-T demonstrated potent killing against MUC1-positive cell lines at different E:T ratios; **C-D)** CAR-T cytotoxicity against MUC1 and MSLN double-positive ovarian cancer cell lines; **E)** Secretion of anti-PD1 VHH concentration by CAR-T cells when co-cultured with OVCAR3 cells at different E:T ratios.

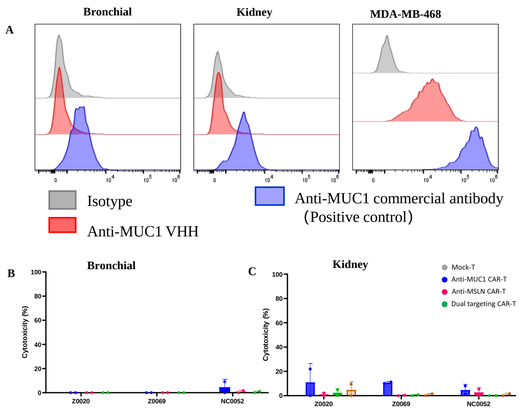

Figure 4. *In vitro* evaluation of BZE2203 CAR-T "on target, off-tumor" safety risk. A) Analysis of MUC1 expression in human primary cells using anti-MUC1 VHH and a commercial antibody, with MDA-MB-468 as a control. **B-C)** BZE2203 CAR-T showed no cytotoxicity against MUC1-low expressing human bronchial primary cells and kidney primary cells.

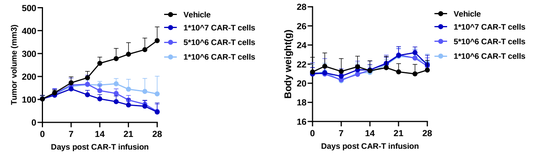

Figure 5. BZE2203 CAR-T cells demonstrate robust efficacy in ovarian cancer Cao-V3 xenograft models. A) Tumor volume measurements by caliper at various study time points; **B)** Body weight measurements across study time points.

Table 2. Phase 1, single-arm, open-label, dose-escalation, and expanded cohort study of BZE2203-A-01 in late-stage solid tumor patients who have failed standard treatments (NCT06327997).

Key inclusion criteria	Key exclusion criteria
•Patients with advanced solid tumors diagnosed by histopathological examination, with at least one measurable lesion according to RECIST 1.1 criteria • Eligible patients must have tumor tissue samples with confirmed MUC1 or MSLN expression and PD-L1 positivity by IHC. • Expected survival time ≥ 3 months • ECOG score 0-1	• Patients who have previously received mesothelin-targeted therapy, cell therapy, gene therapy products (including CAR-T cell therapy), or any domestic or foreign T cell therapy • Patients with any uncontrolled active infections, coagulation disorders, or other major illnesses • Patients with autoimmune diseases under treatment, immune-related diseases (such as organ transplantation), or those on long-term immunosuppressive drugs (e.g., glucocorticoids) • Patients with evident brain metastases, or those with a history of central nervous system diseases

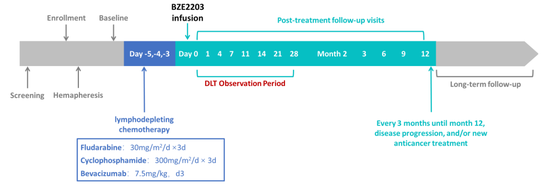

Figure 6. Schematic diagram of research process

Table 3. Phase 1 dose-escalation clinical results

		Subject 1	Subject 2
Characteristics	**Age**	60	67
	Sex	F	F
	Baseline ECOG	1	1
	Tumor Type	PRROC	PRROC
	Past medical history	Type 2 diabetes/ Sleep disorders	Grade 1 hypertension/ Grade 2 Platelet count decreased
	Diagnosis time(Months)	67	22
	Number of previous treatment lines	3L	3L
	Previous PD-L1 treatment history	1L (Atezolizumab)	3L (Enwalimab)
	CA125 (0-35U/ml)	33.5	15151
Safety	**TEAE（Grade）**		
	White blood cell count decreased	2	1
	Neutrophil count decreased	3	1
	Lymphocyte count decreased	-	1
	Platelet count decreased	-	3
	Anaemia	-	2
	Gamma-glutamyl transferase increased	-	2
	Blood alkaline phosphatase increased	-	1
	Blood lactate dehydrogenase increased	-	1
	Bile acids increased	1	-
	Blood triglycerides increased	2	-
	Blood cholesterol increased	1	-
	Asthenia	2	1
	Hypertension	1	-
	Chest tightness	-	1
	No DLT, SAE, CRS, ICANS.		
Efficacy	**ORR**	PR(Day 28)	PR(Day 28)
	Best Tumor Shrinkage	-100%（Month 4）	-46.7%（Month 3）
	PFS	217 Days	112 Days
	OS	NR	NR
PK	The CAR-T Cmax reached 89,783 copies/μg DNA, and anti-PD-1 nanobodies Cmax reached 108,450 pg/mL, detectable for 7 months. The time to peak CAR-T expansion and anti-PD-1 nanobodies was between Day 18 and Day 21 post-infusion.		

ECOG: Eastern Cooperative Oncology Group; **PR: Partial** Response; **PRROC**: Platinum resistant recurrent ovarian cancer; **TEAE**: Treatment Emergent Adverse Event; **NR**: Not Reached

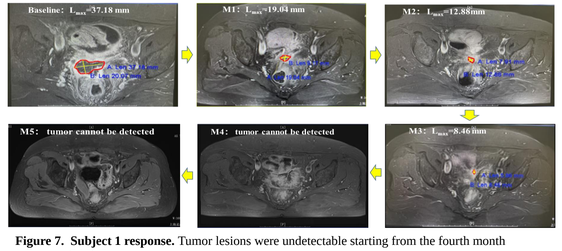

Figure 7. Subject 1 response. Tumor lesions were undetectable starting from the fourth month following CAR-T infusion.

CONCLUSIONS

1. Targeting MUC1 and MSLN, the bispecific VHH demonstrated strong binding to target-positive tumor cell lines, minimal binding to MUC1-low expressing primary cells, and lost binding to MSLN at pH 7.4.
2. BZE2203 CAR-T exhibited potent cytotoxicity against tumor cells while sparing human primary cells. The conditional activation design of this CAR-T effectively addresses the "on-target, off-tumor" toxicity issue.
3. *In vivo* study showed that BZE2203 CAR-T had substantial anti-tumor effects in the Cao-V3 animal model.
4. In a clinical setting, two ovarian cancer patients achieved partial remission after CAR-T infusion, with no dose-limiting toxicities, CRS, or graft-versus-host disease observed.