BH3120, a PD-L1x4-1BB Bispecific Antibody, Exhibits Favorable Safety Profiles in Distinct *in vivo* and *ex vivo* Liver Toxicity Evaluations

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Jing Wang, Dongyang Li , Aibo Sun, Aihong Zhang, Jingmei Cai, Xiao Ma, Haixia Zhao, Jiangcheng Xu, Jiawang Liu, Kyoungwoo Lee

Beijing Hanmi Research Center, Beijing Hanmi Pharmaceutical Co., Ltd., People's Republic of China

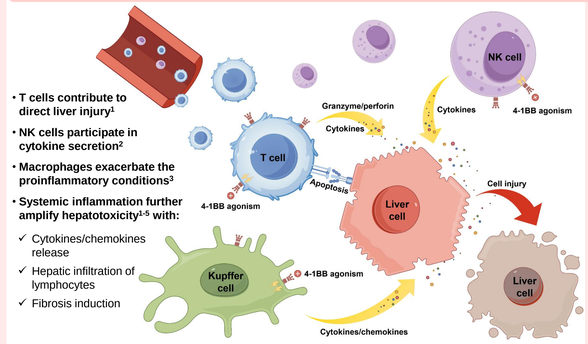
Abstract

BH3120 is a novel bivalent PD-L1/4-1BB bispecific antibody targeting both inhibitory (PD-1/PD-L1) and co-stimulatory (4-1BB clustering) signaling pathways. In multiple non-clinical safety evaluations, BH3120 has consistently shown minimal modulation of T cell functions in blood or normal tissues resulting in favorable safety profiles. The clinical evaluation of BH3120 as a monotherapy and in combination with a PD-1 inhibitor is under investigation (NCT06234397).

While early stage clinical evaluations of 4-1BB agonists as monotherapy or in combination with PD-1/PD-L1 inhibitors are generally associated with a certain level of liver abnormalities, the potential biomarkers or patient's backgrounds that can predetermine the risk of liver toxicity have not been discussed in detail. In the meantime, treatment discontinuation, disruption, or administration of corticosteroids due to liver toxicity would be the limitations of 4-1BB agonists in tumor management, and the mode of action and impact of 4-1BB agonists on liver inflammation need to be further investigated. To understand the potential risks of liver toxicity with BH3120, sensitive *in vivo* and *ex vivo* models for liver toxicity evaluations were established: a mouse model with mild liver inflammation induced by Concanavalin A, and human liver organoids co-cultured with immune cells to mimic the histological and physiological changes in patient's liver tissue.

In both models, BH3120 is not associated with significant elevation of liver inflammation markers, indicating minimal risk of liver toxicity that is in line with the safety results we previously reported. The property of BH3120 to minimize systemic immune modulation enables de-coupling of anti-tumor efficacy from systemic toxicities, and would provide flexibility in combination with different anti-tumor treatments.

4-1BB agonism associated liver immunotoxicity



- T cells contribute to direct liver injury[1]
- NK cells participate in cytokine secretion[2]
- Macrophages exacerbate the proinflammatory conditions[3]
- Systemic inflammation further amplify hepatotoxicity[1-5] with:
  - ✓ Cytokines/chemokines release
  - ✓ Hepatic infiltration of lymphocytes
  - ✓ Fibrosis induction

Figure 1. Main driven factors in immune mediated liver injury associated with 4-1BB agonism
4-1BB agonists as monotherapy or in combination with PD-1/PD-L1 inhibitors are reported to be associated with liver immunotoxicity. Mechanistic research indicates that hyperactivation of immune cells and the inflammatory cytokines are the main driven factors[4].

BH3120 shows no enhancement of T cell mediated hepatocyte damage

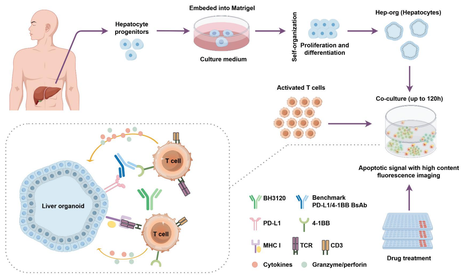
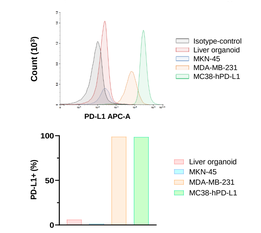
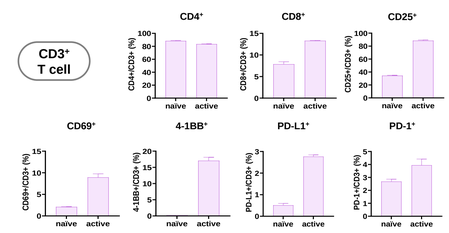


Figure 3. Immunotoxicity assay in human liver organoids This model investigates T cell mediated hepatocyte injury using a human liver organoid model co-cultured with activated T cells. The system aims to provide mechanistic insights into immune-related liver toxicity.
(A) Human liver organoids, derived from primary hepatocyte progenitors, were analyzed by flow cytometry for PD-L1 expression and compared to tumor cell lines (MKN-45, MDA-MB-231 and MC38-hPD-L1). The liver organoids exhibit positive PD-L1 signal, but significantly lower than that of PD-L1-positive tumor cell lines. **(B)** Human T cells were isolated from PBMCs, activated using an anti-CD3 antibody, and analyzed by FACS. Activated T cells upregulate activation markers including CD25 and CD69, along with increased expression of PD-1, PD-L1, and 4-1BB. **(C)** The liver organoids, which is PD-L1 positive, were co-cultured with activated T cells, and the interaction between the PD-L1 expressing organoids and T cells were observed in this experimental setup by a confocal microscope. **(D)** Liver organoid viability is monitored during co-culture with or without activated T cells, by an ImageXpress Confocal HT.ai High-Content Imaging System. Liver organoids exhibit progressively increasing apoptotic signals over time by the activated T cells, demonstrating enhanced apoptosis in the organoids and suggesting a role of activated T cell in immune-mediated liver injury.

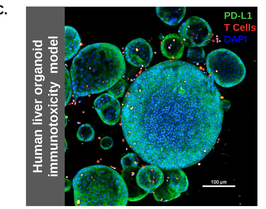
A. PD-L1 expression in human liver organoid



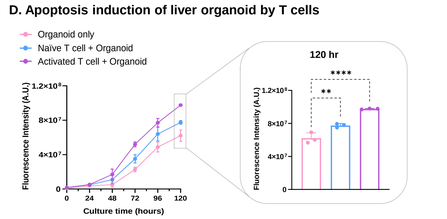
B. Modulation of T cell markers



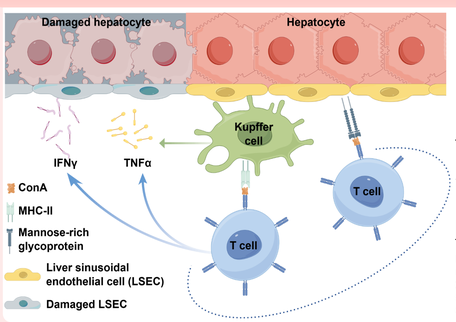
C.



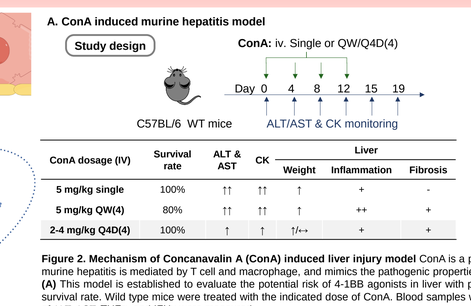
D. Apoptosis induction of liver organoid by T cells



BH3120 demonstrates favorable liver safety in a Concanavalin A induced murine hepatitis model



A. ConA induced murine hepatitis model



ConA dosage (IV)	Survival rate	ALT & AST	CK	Liver		
				Weight	Inflammation	Fibrosis
5 mg/kg single	100%	↑↑	↑↑	↑	+	-
5 mg/kg QW(4)	80%	↑↑	↑↑	↑	++	+
2-4 mg/kg Q4D(4)	100%	↑	↑	↑/↔	+	+

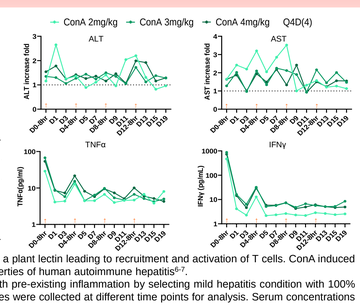
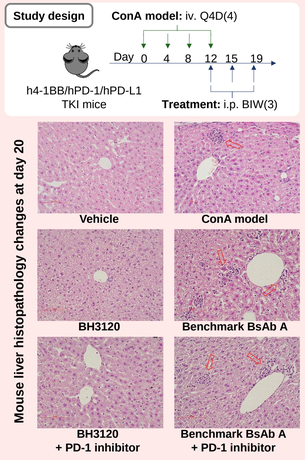
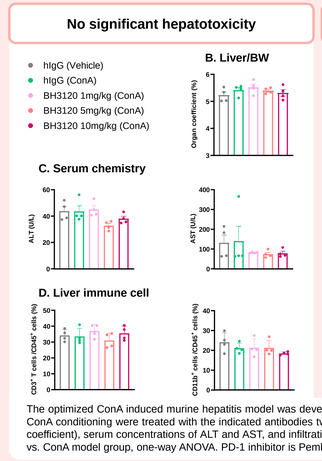


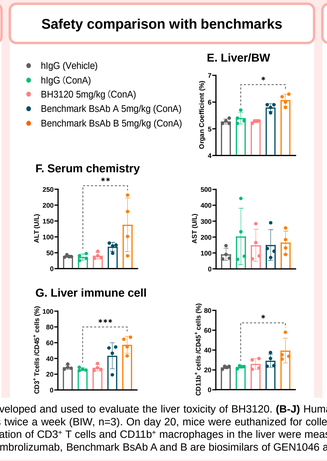
Figure 2. Mechanism of Concanavalin A (ConA) induced liver injury model ConA is a plant lectin leading to recruitment and activation of T cells. ConA induced murine hepatitis is mediated by T cell and macrophage, and mimics the pathogenic properties of human autoimmune hepatitis[6-7].
(A) This model is established to evaluate the potential risk of 4-1BB agonists in liver with pre-existing inflammation by selecting mild hepatitis condition with 100% survival rate. Wild type mice were treated with the indicated dose of ConA. Blood samples were collected at different time points for analysis. Serum concentrations of ALT, AST, TNFα, and IFNγ were measured.



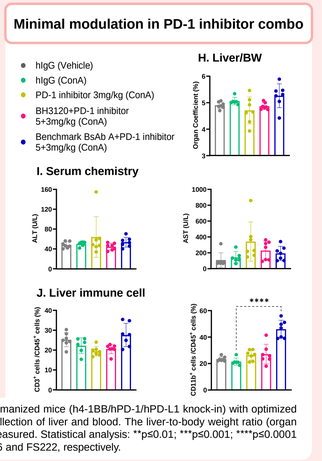
No significant hepatotoxicity



Safety comparison with benchmarks



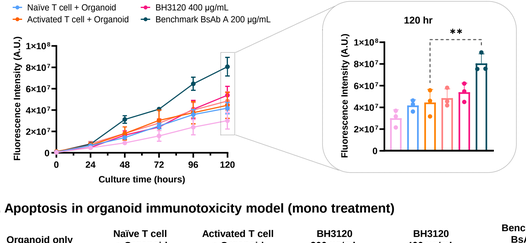
Minimal modulation in PD-1 inhibitor combo



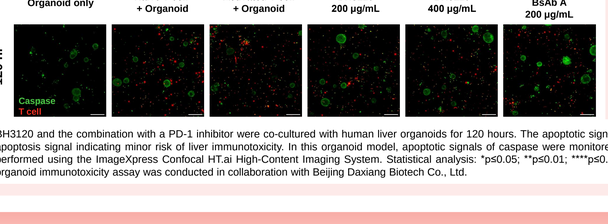
The optimized ConA induced murine hepatitis model was developed and used to evaluate the liver toxicity of BH3120. **(B-J)** Humanized mice (h4-1BB/hPD-1/hPD-L1 knock-in) with optimized ConA conditioning were treated with the indicated antibodies twice a week (BIW, n=3). On day 20, mice were euthanized for collection of liver and blood. The liver-to-body weight ratio (organ coefficient), serum concentrations of ALT and AST, and infiltration of CD3⁺ T cells and CD11b⁺ macrophages in the liver were measured. Statistical analysis: **p≤0.01; ***p≤0.001; ****p≤0.0001 vs. ConA model group, one-way ANOVA. PD-1 inhibitor is Pembrolizumab, Benchmark BsAb A and B are biosimilars of GEN1046 and FS222, respectively.

BH3120 does not enhance T cell mediated liver organoid apoptosis

E. Liver organoid apoptotic signal (mono treatment)

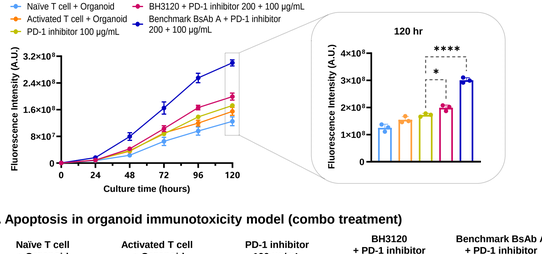


G. Apoptosis in organoid immunotoxicity model (mono treatment)

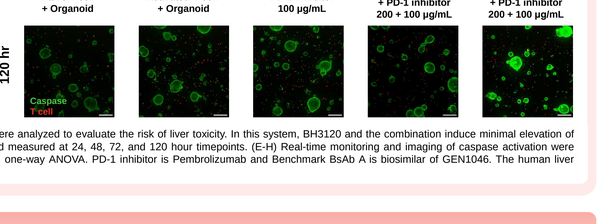


Combination of BH3120 and a PD-1 inhibitor induces minimal apoptosis

F. Liver organoid apoptotic signal (combo treatment)



H. Apoptosis in organoid immunotoxicity model (combo treatment)



BH3120 and the combination with a PD-1 inhibitor were co-cultured with human liver organoids for 120 hours. The apoptotic signals were analyzed to evaluate the risk of liver toxicity. In this system, BH3120 and the combination induce minimal elevation of apoptosis signal indicating minor risk of liver immunotoxicity. In this organoid model, apoptotic signals of caspase were monitored and measured at 24, 48, 72, and 120 hour timepoints. (E-H) Real-time monitoring and imaging of caspase activation were performed using the ImageXpress Confocal HT.ai High-Content Imaging System. Statistical analysis: *p≤0.05; **p≤0.01; ****p≤0.0001, one-way ANOVA. PD-1 inhibitor is Pembrolizumab and Benchmark BsAb A is biosimilar of GEN1046. The human liver organoid immunotoxicity assay was conducted in collaboration with Beijing Daxiang Biotech Co., Ltd.

Conclusion

- **Hepatotoxicity remains a major safety concern in the clinical application of 4-1BB targeted anti-tumor immunotherapies. BH3120, a PD-L1/4-1BB bispecific antibody with potentially minimal toxicity risk, is designed to selectively activate T cells within the tumor microenvironment while de-coupling their activation in normal tissues, thereby reducing or mitigating systemic immune-related adverse effects, in particular the risk of hepatotoxicity.**
- **Historical data has demonstrated the safe profile of BH3120 in animal models including both humanized mice and non-human primates. In newly established *in vivo* and *ex vivo* hepatotoxicity evaluation models, Concanavalin A induced murine hepatitis model and human liver organoid immunotoxicity model, BH3120 alone or in combination with a PD-1 inhibitor did not induce significant elevation of liver enzymes or infiltrated immune cells, indicating minimal T cell activity and reduced risk of hepatotoxicity. The results of these intensive liver toxicity evaluations suggest potentially broad application of BH3120 in clinical settings.**
- **BH3120 has demonstrated favorable preclinical safety, which holds significant implications for further development. In particular, these safety data suggest potential application of BH3120 in the context of additional combination regimens. The clinical safety of BH3120 is currently under investigation as a monotherapy and in combination with a PD-1 inhibitor.**

Reference:
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The schematic diagrams were drawn by FigDraw

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