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# FECH, a novel metabolic target influencing CAR T-cell phenotype and function

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## Abstract

Recent phase I/II clinical trials have demonstrated that chimeric antigen receptor (CAR) T cells targeting the disialogangliosade GD2 represent a promising therapeutic option for pediatric patients with relapsed or refractory high-risk neuroblastoma (NB). However, incomplete and heterogeneous clinical responses highlight the need to improve CAR T-cell efficacy and persistence. We previously demonstrated the therapeutic benefit of combining the dual insulin-like growth factor 1 receptor/insulin receptor (IGF1R/IR) inhibitor linsitinib (LIN) with third-generation GD2.CAR T cells in diffuse intrinsic pontine glioma, where LIN induced tumor cell death and modulated the CAR T-cell phenotype. Here, we extended these findings to NB and explored the mechanisms of LIN-mediated CAR T-cell modulation. LIN, in combination with CAR T cells, significantly enhanced antitumor activity in LIN-sensitive NB cell lines. Mechanistically, we investigated ferrochelatase (FECH), a mitochondrial enzyme involved in heme biosynthesis, and a known off-target of LIN. LIN treatment or selective FECH inhibition with N-methyl protoporphyrin IX reduced intracellular heme, attenuated activation and exhaustion marker expression and promoted central memory characteristics associated with improved in vivo CAR T-cell persistence and functionality. Both treatments similarly decreased ATP production by reducing glycolysis and mitochondrial respiration in chronical antigen-activated CAR T cells. Collectively, these data reveal a dual mechanism of action for LIN, combining direct tumor cell cytotoxicity with metabolic reprogramming of CAR T cells linked to heme biosynthesis. These findings identify heme metabolism as regulator of CAR T-cell phenotype and function and support further investigation of FECH to enhance therapeutic efficacy in NB and beyond.

**Keywords** GD2.CAR T cells, Heme biosynthesis, Ferrochelatase, Neuroblastoma

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**To the editor,**

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and remains a leading cause of pediatric cancer-related mortality. The disease predominantly affects infants and young children and exhibits marked clinical heterogeneity, ranging from spontaneous regression to aggressive, therapy-refractory metastatic disease, underscoring the urgent need for innovative and durable therapeutic strategies [1, 2]. Chimeric antigen receptor (CAR) T cells targeting the disialoganglioside GD2 have shown encouraging antitumor activity in early-phase clinical trials for NB; however, durable responses remain uncommon [3–5]. Limited efficacy has been attributed to insufficient T-cell persistence, immunosuppressive tumor microenvironments, antigen heterogeneity and CAR T-cell exhaustion, highlighting the need for rational strategies to enhance CAR T-cell functionality and durability [6–9].

Based on our prior identification of the dual insulin-like growth factor 1 receptor/insulin receptor (IGF1R/IR) inhibitor linsitinib (LIN) as a small-molecule enhancer of CAR T-cell function in diffuse midline pontine glioma [10], we investigated whether LIN could similarly improve GD2.CAR T-cell efficacy in NB and explored the underlying mechanisms. LIN significantly enhanced CAR T-cell mediated cytotoxicity in LIN-sensitive NB cells, independently of GD2 expression levels (Fig. 1A; Fig. S1A-C). In contrast, the LIN-resistant NB cell ACN did not exhibit increased susceptibility to CAR T-cell killing (Fig. 1B), indicating that tumor-intrinsic sensitivity contributes to the observed synergy.

Importantly, LIN also exerted tumor-independent effects on CAR T cells. Phenotypic analyses revealed selective attenuation of activation and exhaustion marker expression and enrichment of a central memory phenotype in $CAR^+$ T cells activated either by GD2 antigen (Fig. 1C) or by NB cells (Fig. S1D). As CAR T cells lacked IGF1R and IR expression irrespective of activation status (Fig. S1E), these findings suggested an off-target mechanism underlying LIN-mediated T-cell modulation.

Mechanistically, we investigated ferrochelatase (FECH), a mitochondrial enzyme that catalyzes the final step of heme biosynthesis and a reported off-target of LIN [11]. We confirmed FECH engagement by LIN in NB cells and CAR T cells using cellular thermal shift assays at concentrations that were non-cytotoxic to CAR T cells (Fig. 1D; Fig. S1F). LIN treatment reduced intracellular heme levels and induced accumulation of protoporphyrin IX (PpIX) (Fig. 1E-F). Pharmacologic inhibition of FECH with N-methyl protoporphyrin IX (NMPP) recapitulated the heme-depleting effects of LIN, validating FECH as a functionally relevant LIN target.

Although LIN reduced NB cell proliferation as expected (Fig. 1G), neither LIN nor NMPP impaired CAR T-cell viability, expansion or cytotoxic capacity (Fig. 1G-J). GD2.CAR T-cell mediated tumor killing was enhanced by LIN whether CAR T cells were pre-treated or exposed concomitantly during co-culture, whereas NMPP did not enhance antitumor activity. Interferon-γ production trended toward attenuation, while granzyme B and TGF-β secretion were significantly decreased (Fig. 1K), consistent with a lower inflammatory response in CAR T cells recently associated with better response and less toxicity [12].

Comprehensive phenotypic profiling demonstrated that LIN and NMPP similarly modulated CAR T-cell differentiation programs. Both treatments reduced expression and/or the frequency of activation and exhaustion markers, including CD69, CD38, CD95, PD-1, LAG-3 and TIM-3, in both $CD4^+$ and $CD8^+$ CAR T-cell subsets

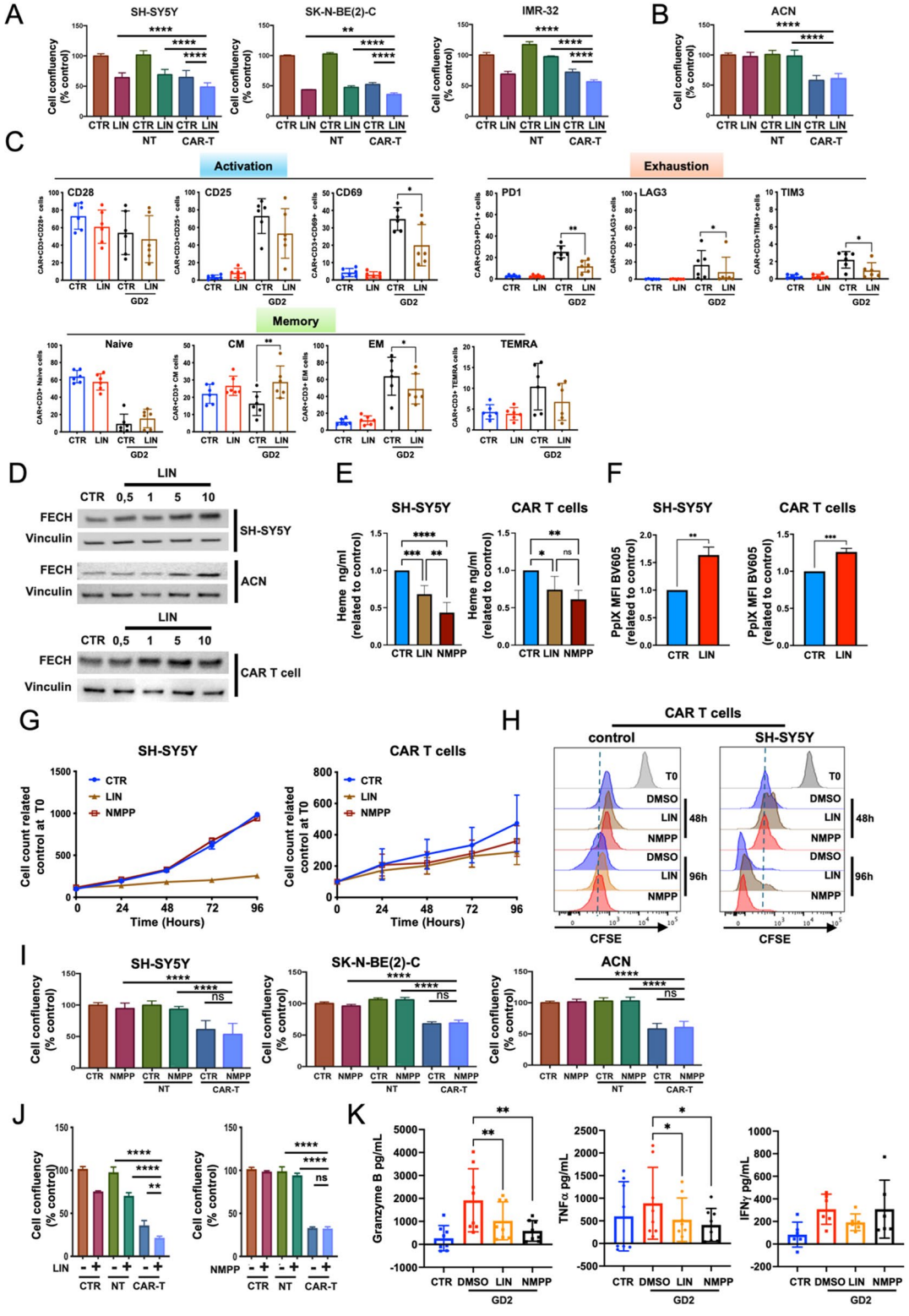


**Fig. 1** (See legend on next page.)

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**Fig. 1** Linsitinib targets ferrochelatase to modulate heme metabolism without impairing CAR T-cell antitumor activity. (**A-B**) Bar graphs showing CAR T-cell cytotoxicity in the presence of LIN or DMSO (CTR) in (**A**) LIN-sensitive and (**B**) LIN-resistant neuroblastoma (NB) cell lines. NT: non-transduced T cells Data are shown as mean ± SD of n=3, representative on 3 technical repeats. Statistical significance was determined using Ordinary one-way ANOVA and Turkey's multiple comparison test. $^{**}P < 0.01$, $^{****}P < 0.0001$; (**C**) Bar graphs representing the phenotypic profiling of CAR T cells activated by GD2 in the presence or absence of LIN and DMSO (CTR). Data are shown as mean ± SD of 6 donors and values reported in supplementary Table1 Statistical significance was determined using paired t test. $^{*}P < 0.05$, $^{**}P < 0.01$ (**D**) Westernblot analysis of the cellular thermal shift assay at 52.3°C demonstrating direct engagement of ferrochelatase (FECH) by LIN in NB cells and CAR T cells. (**E,F**) Bar graphs illustrating the quantification of (**E**) heme levels in NB cells and CAR T cells following treatment with LIN or NMPP, and (**F**) protoporphyrin IX levels following LIN treatment. Data are shown as mean ± SD of n3. Statistical significance was determined using (**E**) Ordinary one-way ANOVA and Turkey's multiple comparison test and (**F**) Unpaired t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}$P < 0.001, $^{****}P < 0.0001$, ns: non significative; (**G**,**H**) Growth curves (**G**) and cell proliferation (**H**) assays for SH-SY5Y and CAR T cells incubated with LIN, NMPP or DMSO (CTR). (**G**) Data are shown as mean ± SD of n=3. (**H**) Data are representative of n ≥3 technical repeats. (**I**,**J**) Bar graphs showing CAR T-cell cytotoxicity in the presence of (**I**) NMPP added simultaneously with CAR T cells, and (**J**) CAR T cells pre-incubated with NMPP and LIN for 24 hours before co-culture with SH-SY5Y cells. Data are shown as mean ± SD of n=3, representative on 3 technical repeats. Statistical significance was determined using Ordinary one-way ANOVA and Turkey's multiple comparison test. $^{**}P < 0.01$, $^{****}P < 0.0001$; ns: non significative. (**K**) Bar graphs illustrating the quantification of granzyme B, TNF-α, and IFN-γ released by GD2-activated CAR T cells in presence of LIN, NMPP or DMSO as vehicle control. Basal levels of the cytokines in non activated CAR T cells is indicated as CTR. Data are shown as mean ± SD of 8 donors. Statistical significance was determined using Wilcoxon matched-pairs signed rank test. $^{*}P < 0.05$, $^{**}P < 0.01$

while consistently enriching central memory populations and reducing effector memory subsets in tumor-activated CAR T cells (Fig. 2A-C; Fig. S1G). Metabolic flux analyses further revealed that LIN and FECH inhibition by NMPP reshaped CAR T-cell bioenergetics, reducing ATP production, oxygen consumption and extracellular acidification rates, reflecting a slight attenuation of mitochondrial respiration and reduced glycolysis in antigen-activated CAR T cells (Fig. 2D-F). Such metabolic remodeling has been associated with enhanced memory differentiation and improved in vivo CAR T-cell persistence [13].

In summary, we confirm the therapeutic benefit of combining LIN with CAR T-cell therapy and show that heme metabolism contributes to CAR T-cell functional states. In NB, LIN enhances GD2.CAR T-cell activity through both direct tumor targeting and modulation of CAR T-cell metabolic and phenotypic programs. LIN and NMPP promote the enrichment of memory CAR T cells with reduced exhaustion and a less inflammatory phenotype, supporting the involvement of the FECH-associated heme biosynthesis pathway. However, potential additional off-target effects of compounds preclude definitive attribution solely to FECH inhibition alone. Further studies, including genetic approaches, are required to clarify the role of FECH and the underlying mechanisms. Overall, our findings identify heme metabolism as a candidate pathway influencing CAR T-cell fitness and function in NB, warranting further investigation in adoptive cell therapy.

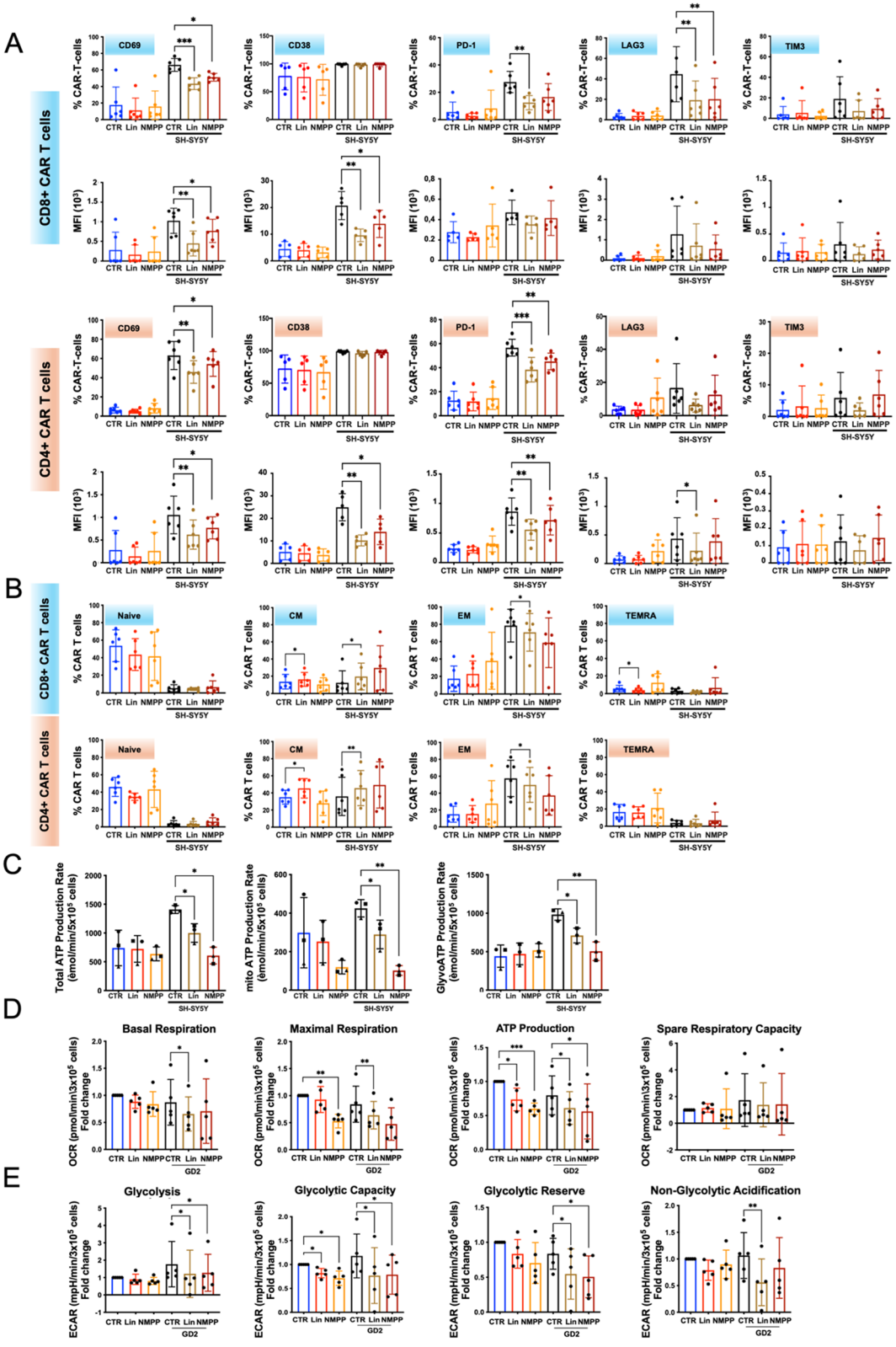


**Fig. 2** (See legend on next page.)

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**Fig. 2** FECH inhibition remodels CAR T-cell phenotype and metabolism. (**A**-**B**) Bar graphs representing phenotypic profiling of CD8+ and CD4+ CAR T cells with or without LIN, NMPP or DMSO (CTR) and in co-culture with SH-SY5Y NB cells for the markers associated to (**A**) activation and exhaustion and (**B**) T cell memory. Data are shown as mean ± SD of 6 donors and values reported in supplementary Tables 2 and 3. Statistical significance was determined using paired t test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$. (**C**-**E**) Bar graphs depicting the metabolic flux analysis, quantifying (**C**) the mitochondrial and glycolytic ATP production, (**D**) oxygen consumption (OCR) and (**E**) extracellular acidification rate (ECAR) in GD2-activated and non-activated CAR T cells and in the presence of DMSO (CTR), LIN and NMPP. Data are shown as mean ± SD of (**C**) 3 donors and (**D**-**E**) 5 donors. Statistical significance was determined using either t test, ordinary or 2ndway ANOVA and Turkey's multiple comparison test. *$P < 0.05$, **$P < 0.01$

**Abbreviations**

| | |
|---|---|
| IGF1R | Insulin-like growth factor 1 receptor |
| IR | Insulin receptor |
| LIN | Linsitinib |
| NMPP | N-methyl protoporphyrin IX |
| PpIX | Protoporphyrin IX |
| FECH | Ferrochelatase |
| CAR | Chimeric antigen receptor |
| PD-1 | Programmed cell death protein 1 |
| LAG3 | Lymphocyte-activation gene 3 |
| CD | Cluster of differentiation |
| TIM3 | T-cell immunoglobulin and mucin-domain containing-3 |
| NB | Neuroblastoma |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40364-026-00979-z.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

**Author contributions**

Data collection: MP, SV, IS, GDM, VS, SaC, MC, VF, LWS, EIV, EC, MS, EG, MG. Data analysis & interpretation: MP, CV, BMG, FeC, FN, AF, FrC, SiC, EnV, IC, EdB. Statistics: MP, IC, EdB. Manuscript drafting: IC, EdB. Manuscript revising: MP, IS, BMG, MC, VF, EG, FeC, FN, AF, FrC, SiC, EnV, IC, EdB. Study concept & design: IC, EdB.

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**Data availability**

No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**

Etical approval 250/20, AU-740.134-01/21.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

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## Publisher's note