

Generation of GPC-3-Targeted Radicals for Precise Therapy of Hepatocellular Carcinoma

Corresponding Author: Professor Dalong Ni

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Hao et al. established a nanosystem of lipid nanoparticles (LNPs) containing tannic acid (TA) and hydrophobic DOSS-calcium peroxide (dCPO) nanoparticles. Upon cellular uptake of TA/dCPO LNPs, dCPO radicalizes TA and generates radical-conjugated gallic acid species (GA*) at the cellular membrane. GA* specifically binds to glypican-3 (GPC3), which is selectively expressed on the surface of hepatocellular carcinoma (HCC) cells and is involved in Wnt/ β -catenin signaling, leading to GPC3 degradation and then cell growth inhibition. Because the current data insufficiently indicate that GA* induces GPC3 degradation and subsequently inactivates the Wnt/ β -catenin signaling pathway, additional experiments and more detailed explanations are required. Point-by-point comments are provided below.

Comments:

1. There are concerns regarding the selectivity and reactivity of GA*. Although GA exhibits some affinity for GPC3, it does not bind with high specificity. The authors should verify that TA/dCPO LNPs are ineffective in GPC3-negative HCC cell lines such as JHH4 and HLF, and become effective when GPC3 is ectopically expressed in these cells. Additionally, they should confirm that knockout of GPC3 in GPC3-positive cell lines attenuates the inhibitory effects of GA*. Given that GA* has a lifetime on the order of nanoseconds, it is likely to be rapidly quenched in culture media or in vivo, in contrast to in vitro settings. Following the same approach as in Fig. 2d, they should demonstrate whether free radical generation by TA/dCPO LNPs persists in culture media containing FBS or in mouse and human serum.
2. GPC3 dimerization and degradation should be quantified in Fig. 3d and 3g. In addition to Coomassie staining, Western blot analysis should be performed using internal controls to semi-quantify the protein expression level of GPC3. Depending on the epitope recognized by the anti-GPC3 antibody, low molecular weight bands may appear if GPC3 is cleaved at specific sites, as suggested in Fig. 3i.
3. According to the results from Western blot analysis (Fig. 3d and 3g), TA/dCPO LNP treatment reduces GPC3 expression by approximately 50%. Therefore, it seems inconsistent that GPC3 expression is undetectable in immunocytochemical analyses (Fig. 4a–c).
4. As noted in Comment 1, the authors should perform colony formation and cell viability assays to determine whether TA/dCPO LNPs suppress proliferation in GPC3-negative HCC cell lines and GPC3-knockout subclones, as in Fig. 5a–c.
5. In many contexts, Wnt signaling inhibition increases CTNNB1 transcription through feedback regulation. The authors should evaluate whether the expression of downstream targets of the Wnt/ β -catenin signaling pathway such as LGR5, AXIN2, and RNF43 is altered following treatment with TA/dCPO LNPs.
6. Because β -catenin nuclear accumulation is not observed in cells cultured in vitro or tumors subcutaneously transplanted (Fig. 6k), Hep3B cells appear to be relatively independent of the Wnt/ β -catenin signaling pathway. To more accurately assess β -catenin activity, the authors should performed Western blot analysis of nuclear β -catenin using an anti- β -catenin antibody, not the antibody against non-phosphorylated β -catenin employed in this study, and should also conduct TOPFlash reporter assays. In contrast, since HepG2 cells express a constitutively active form of β -catenin due to CTNNB1 exon 3 deletion, GPC3-mediated regulation of Wnt/ β -catenin signaling exerts minimal effects on gene expression and biological behavior in these cells. This limitation should be discussed.

7. It is well known that LNPs tend to accumulate in the liver. Therefore, it is puzzling that a greater amount of LNPs remains in tumors than in the liver, even at 36 hours after intravenous injection (Fig. 6b). The authors should provide an explanation for this unexpected distribution.

Reviewer #2

(Remarks to the Author)

Thank you very much for inviting me to review "Generation of GPC-3-Targeted Radicals for Precise Therapy of Hepatocellular Carcinoma" by Hao et al. Authors developed a new nanosystem capable of generating radical-conjugated species with predefined oncogenic protein-targeting in-situ. It was engineered with lipid nanoparticles (LNP) that encapsulated polyphenolic tannic acid (TA) and hydrophobic dose-calcium peroxide nanoparticles (dCPO NPs). Using this, they specifically targeted GPC3. This inhibited the activation of downstream signaling pathways such as the canonical Wnt/ β -catenin pathway, and finally suppressed HCC proliferation. They report an approach for precise free radical-mediated antitumor therapy. New therapies are urgently needed against HCC and this approach does appear novel. However, several concerns regarding how the data is presented and lack of adequate control groups reduces my enthusiasm for this manuscript.

Authors did not use GPC-3 knockout to confirm the specificity of the TA/dCPO nanosystem. While they performed experiments using recombinant GPC3 in the in vitro setting, the researchers did not use GPC-3 recombinant in the in vivo setting.

The nanosystem is designed to generate "stabilized phenoxyl radicals" which are intended to overcome the limitations of short-lived hydroxyl radicals. While the presence of phenoxyl radicals is confirmed by ESR, quantitative data on their stability or longevity in situ compared to other free radicals would be beneficial.

The paper emphasizes overcoming the "non-specific activity" and "collateral damage to adjacent normal tissues" of conventional free radical therapies. While the TA/dCPO nanosystem demonstrates targeted action on GPC-3, a direct comparison showing reduced off-target effects compared to a generic, non-targeted free radical generating system is needed to confirm the "precision" claim.

Many figures lack adequate legends, making them difficult to interpret. Quantification is not provided for most IF or IHC images. Scale bars are absent in the majority of IF and IHC panels, and where present, they are inconsistently applied.

The free GPC-3 content in the culture medium was quantified using ELISA. As shown in Fig.4k-m, the introduction of TA/dCPO LNPs increased the free GPC-3 content in the culture medium, indicating that they could break off the lipid moiety. However, the fold change in soluble GPC3 appears negligible in two of the three cell lines shown.

The study relies on a single cell line derived subcutaneous model, which limits the generalizability of the findings. Tumor biology, immune interactions, and treatment responses can vary substantially between cell lines and may not reflect the heterogeneity of human tumors.

The toxicity assessment is insufficient. Only a single H&E section per organ is presented in the main figure, which does not allow for evaluation of variability or reproducibility. A more comprehensive analysis, including multiple sections per organ and quantitative pathology scoring, would be necessary to draw reliable conclusions about safety.

Reviewer #3

(Remarks to the Author)

This manuscript proposes a protein-targeted, free radical-mediated antitumor strategy that effectively addresses the issue of nonspecific oxidative damage inherent in traditional free radical-based therapeutic approaches. Notably, the authors integrate the oxidative damaging effects of phenoxyl radicals on proteins with the specific binding capability of small-molecule GA to GPC-3, thereby establishing a GPC-3-targeted phenoxyl radical-based therapeutic strategy. By promoting the targeted binding of GA• to GPC-3 and inducing the selective disruption of its peptide segments and lipid structures, the system effectively leads to the structural and functional disruption of GPC-3, subsequently suppressing activation of the downstream Wnt signaling pathway and ultimately eliciting a cascade-like anti-tumor response at the molecular level through targeted GPC-3 protein intervention. This work represents a significant integration of free radical chemistry, nanomedicine, and tumor protein-targeted therapy, offering a novel molecular intervention paradigm for precision oncology. Its academic significance and innovative value support its suitability for publication in Nature Communications. However, there are some concerns needed to be addressed:

1. How about the dispersibility and stability of the synthesized TA/dCPO LNP? Please give relevant characterization results.
2. Display a local magnified view of the fig.1g image.
3. Please supplement the size distribution of TA LNP by dynamic light scattering.
4. The manuscript suggests that the generation of hydroxyl radicals may originate from the degradation of dCPO. What specific factors could potentially contribute to the production of hydrogen peroxide?
5. In Fig. 5c, the statistical comparison of cell viability between the eLNP group and the TA/dCPO LNP group is missing.

What factors may account for the observed discrepancies in therapeutic efficacy among the three cell lines?

6. In vivo fluorescence imaging demonstrated that TA/dCPO LNPs predominantly accumulate in the liver. How do normal hepatocytes counteract the oxidative effects of dCPO within this system? Furthermore, if possible, it is recommended to provide additional experimental validation to elucidate the differential cytotoxic effects of dCPO on hepatocellular carcinoma cells and normal hepatocytes at the cellular level.

7. In practical applications, what measures can be taken to effectively ensure the efficiency of GA• generation at the cell membrane by the TA/dCPO LNP system?

Reviewer #4

(Remarks to the Author)

This manuscript reports the development of TA/dCPO-LNP capable of generating radical conjugated species in situ, with proposed oncogenic GpC-3 targeting capabilities. This system is based on LNP encapsulating TA and dCPO. While the study is interesting, in its current form, the manuscript lacks sufficient novelty and broad significance to meet the high standards of Nature Communications. Moreover, several aspects require clarification and further experimental validation, as outlined below:

1. The authors state that the system is based on lipid nanoparticles. Liposomes typically consist of a lipid bilayer enclosing an aqueous core, while lipid nanoparticles generally possess a solid or amorphous lipid core. For the LNPs in this study, what is the structure of the particle? Do dCPO and TA reside in a solid lipid core surrounded by a bilayer, or in a more liquid-like core? From Figures 1g and 1i, the nanoparticles appear highly heterogeneous, with some exhibiting dense cores and others appearing lighter. Please clarify the structural model and provide polydispersity index (PDI) values, along with more representative Cryo-TEM images.

2. What is the total loading of dCPO and TA, and what is their respective loading ratio? Please also report the encapsulation efficiency of the nanoparticles. Furthermore, please provide the experimental evidence demonstrating the co-localisation of TA and dCPO within a single LNP to support the hypothesis, as well as their spatial arrangement.

3. The manuscript mentions that TA and dCPO are spatially segregated and compartmentalised. However, TA is expected to contact dCPO aggregates unless there is a coating or stabilising layer. Figure 1d shows dCPO aggregates, which were dispersed in aqueous buffer or organic solvent. Please clarify and provide supporting evidence.

4. The synthesis procedure in the supplementary information indicates that TA and dCPO were premixed in ethanol as the organic phase for microfluidic production. Given that TA and dCPO can interact to form GA, please provide evidence that no premature reaction occurs during the preparation or storage of the stock solutions. Or, from my understanding, TA is more likely to be dissolved in the aqueous phase rather than the organic phase. Please clarify this point.

5. The authors propose that the nanoparticles undergo fusion with the cell membrane. However, nanoparticles larger than 100 nm are generally internalised through endocytosis, particularly when PEGylated. More direct experimental evidence is required to support membrane fusion as opposed to endocytosis. In addition, the description of dCPO-mediated membrane fusion is confusing. In Figure 1g of the main manuscript, LNPs are shown, whereas in the Supplementary Information, synthetic cell membranes are used. Please clarify the rationale for testing dCPO with synthetic cell membranes in this context and explain how these experiments relate to the proposed mechanism of LNP–cell interactions. Experimental validation is needed to substantiate the membrane fusion hypothesis.

6. The LNPs described are plain PEGylated nanoparticles with no specific tumour-targeting ligands. Upon intravenous injection, such nanoparticles are expected to accumulate predominantly in the liver. Data on nanoparticle stability, circulation behaviour, and release kinetics of dCPO and TA are needed. Do the particles prematurely release radicals in normal tissues, particularly in healthy liver cells? If so, how is systemic toxicity mitigated? Please also provide the immunostaining picture of healthy normal liver tissues.

7. For the claimed GPC-3 targeting, comparative studies with GPC-3–positive versus GPC-3–negative cells are required to quantify uptake and radical release. Antibody blocking experiments could further confirm whether GPC-3 binding drives the observed targeting.

8. The cellular studies only compared e-LNPs and TA/dCPO LNPs, without demonstrating the importance of co-loading TA and dCPO. It is recommended to include comparative data for e-LNPs, dCPO-LNPs, and TA/dCPO LNPs to establish a logical stepwise evaluation of formulations. In vivo studies included multiple groups (e-LNPs, TA LNPs, dCPO LNPs, and TA/dCPO LNPs), but the corresponding in vitro comparisons were not presented. Please ensure consistency between in vitro and in vivo studies or clearly explain the rationale.

9. Figure 6g lacks sufficient description in the legend. Biodistribution data should be presented with actual quantitative signal values, not only colour scale bars with the control group. Figures 6b and 6c present only one group, whereas Figure 6f shows all treatment groups, leading to inconsistency. Please include images for all groups, with each mouse represented individually, in the Supplementary Information. Add and standardise scale bars in Figure 6l.

10. The summary and discussion are overly brief. The debate should be expanded to provide a more comprehensive and insightful treatment of the work, clearly articulating its broader significance, limitations, and potential clinical relevance. The writing quality also requires improvement, particularly in the Supplementary Information. Several procedures are described

inaccurately or ambiguously—for example, “Mice were injected with TA/dCPO LNPs (Ca: 4.5 mg kg⁻¹) by tail vein twice,” which lacks essential details (e.g., dosing schedule, number of animals) and shows inconsistencies in verb tense/usage relative to the main text. Methodological detail is uneven, with some sections overly specific and others cursory. The authors should thoroughly revise the whole manuscript, including supplementary information, for accuracy, internal consistency, and reproducibility.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The rebuttal provided by Hao et al. is profoundly inadequate, particularly regarding my previous comments #6 and #7. The authors have failed to engage with the core scientific concerns raised; instead, they have provided data that lack transparency and mechanistic explanations that are fundamentally inconsistent. These unresolved issues, combined with serious concerns regarding the integrity of the newly presented data, render the manuscript unsuitable for publication in Nature Communications.

1. Regarding the rebuttal to my previous Comment #6, the Western blot analysis in Figure S37 is highly problematic and scientifically unacceptable. The authors have failed to include essential nuclear markers (e.g., Lamin B1 or Histone H3) to verify the purity of the nuclear fraction. More critically, the beta-actin bands in the nuclear extract appear suspiciously identical to those in the cytoplasmic fraction, yet there is a striking absence of expected cross-contamination markers. This lack of transparency and the inconsistent appearance of the blots raise grave concerns regarding the authenticity and integrity of the data. In the absence of rigorous, verifiable, and raw blot images using appropriate internal controls for each fraction, the claim of nuclear translocation cannot be validated.

2. Furthermore, in the second part of their response to Comment #6, the authors' explanation regarding the beta-catenin pathway in HepG2 cells remains fundamentally inconsistent and dismissive of the original critique. While acknowledging that HepG2 cells harbor a CTNNB1 mutation (deletion of exon 3) leading to constitutive activation, the authors fail to provide a convincing mechanism for how their treatment overrides this genetic driver. The cited work by Li et al. (2020) actually underscores the necessity for direct evidence; yet, the authors have still not performed the requested Western blot analysis of total and nuclear beta-catenin in HepG2 cells specifically treated with TA/dCPO LNPs. Furthermore, the suggestion that GPC3 might act through alternative pathways (FGF, HGF/c-Met, or TGF-beta) is purely speculative and lacks any direct experimental evidence in the present study.

3. The rebuttal to Comment #7 regarding IVIS imaging orientation (dorsal vs. ventral) does not sufficiently address the original concern. Given the high hepatic accumulation characteristic of LNPs, a strong liver signal is expected regardless of positioning, as demonstrated in the authors' own response figure (Fig. R1). The near-total absence of liver signal at 36 hours in the manuscript figure, contrasted with the lack of tumor signal in Fig. R1 at the same time point, strongly indicates inconsistent imaging parameters or selective data reporting. Without a side-by-side, quantitative comparison under identical acquisition settings, the claim of "sustained tumor retention" remains unsubstantiated and misleading.

Reviewer #2

(Remarks to the Author)

Authors have adequately addressed my concerns, thanks for making the revisions.

Reviewer #3

(Remarks to the Author)

The authors have addressed most of the concerns.

Reviewer #4

(Remarks to the Author)

The authors have addressed majority of my questions, however, a few remain:

(1) Drug loading is often defined as the mass % of drug by the total weight of the drug-loaded NPs. Please provide the drug loading.

(2) Fig. S10, size distribution in FBS shows multiple peaks and big PDI(>0.3) indicating aggregation. This means the TA/dCPO LNPs are not stable upon injection in the presence of proteins in serum.

(3) Fig.S40, it seems that the blood half life of TA/dCPO LNPs is so short. How does it compare with free drugs? What is the $t_{1/2}$?

(4) It is still unclear whether the proposed core-shell localization can be confidently resolved at this spatial scale. The authors may consider clarifying this limitation and moderating the structural interpretation accordingly.

(5) The manuscript emphasizes reduced off-target radical activity in normal tissues. Additional clarification of this claim in the discussion would further improve rigor.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

After carefully evaluating the latest rebuttal and newly provided data, I appreciate that the authors made substantial efforts to address the previous concerns. However, the most serious concern relates to the response to Comment #6. The authors now acknowledge that the originally presented Figure S37 was not derived from nuclear/cytoplasmic fractionation as described, but instead from whole-cell lysates. This inconsistency raises critical concerns regarding data handling and figure presentation rigor. Moreover, the absence of raw uncropped data (with markers) for all presented images prevents full restoration of confidence. For these reasons, I cannot recommend publication in Nature Communications.

Reviewer #4

(Remarks to the Author)

I appreciate the efforts of the authors in addressing the questions raised by the reviewers. However, based on the response and the data provided, several key limitations remain, as acknowledged by the authors as well.

For example, the unstable nature of the TA/dCPO LNPs and the very short blood half-life (Fig. S40) suggest that the in vivo stability of the system may be limited. These findings raise concerns regarding the robustness and usefulness of the current formulation in its present form.

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RESPONSE TO REFEREES' COMMENTS

Submission ID: NCOMMS-25-53490

Title: Generation of GPC-3-Targeted Radicals for Precise Therapy of Hepatocellular Carcinoma

Authors: Wanting Hao, Meng Zhang, Zi Fu, Cui Xinyuan, Hongjing Jiang, Ruokun Li, Fuhua Yan, Dalong Ni

We sincerely thank the editor and the reviewers for their time and valuable insights. Their constructive comments and suggestions have been instrumental in improving the quality of our manuscript. We have carefully considered all the feedback and have revised the manuscript accordingly.

Below, we provide a point-by-point response to each comment. The reviewers' original comments are presented in black text, and our responses follow in blue. All corresponding changes made to the manuscript text have been highlighted in yellow for easy reference.

We believe the manuscript has been significantly strengthened through this revision process and hope that it now meets the journal's high standards for publication.

Reviewer #1:

Hao et al. established a nanosystem of lipid nanoparticles (LNPs) containing tannic acid (TA) and hydrophobic DOSS-calcium peroxide (dCPO) nanoparticles. Upon cellular uptake of TA/dCPO LNPs, dCPO radicalizes TA and generates radical-conjugated gallic acid species (GA•) at the cellular membrane. GA• specifically binds to glypican-3 (GPC-3), which is selectively expressed on the surface of hepatocellular carcinoma (HCC) cells and is involved in Wnt/ β -catenin signaling, leading to GPC-3 degradation and then cell growth inhibition. Because the current data insufficiently indicate that GA• induces GPC-3 degradation and subsequently inactivates the Wnt/ β -catenin signaling pathway, additional experiments and more detailed explanations are required. Point-by-point comments are provided below.

Response: Thank you for your positive comments on our work.

Comments:

1. There are concerns regarding the selectivity and reactivity of GA•. Although GA exhibits some affinity for GPC-3, it does not bind with high specificity. The authors should verify that TA/dCPO LNPs are ineffective in GPC-3-negative HCC cell lines such as JHH4 and HLF, and become effective when GPC-3 is ectopically expressed in

these cells. Additionally, they should confirm that knockout of GPC-3 in GPC-3-positive cell lines attenuates the inhibitory effects of GA•.

Response: We sincerely appreciate the insightful and constructive comments. We fully concur that rigorous experimental validation of GPC-3 targeting specificity is essential to substantiating the central conclusions of our study.

As suggested, firstly, we evaluated the treatment efficiency of TA/dCPO LNPs in JHH4 and HLF cell lines, which exhibit low basal expression of GPC-3. No significant change of membrane GPC-3 protein expression was observed between e-LNP and TA/dCPO LNP group (Fig.S28, S32). CCK-8 and colony formation assays demonstrated that TA/dCPO LNPs did not induce significant cytotoxicity or anti-proliferative effects in these models (Fig.5d-g).

Then, we successfully established lentivirus-mediated GPC-3-stably overexpressing JHH4 and HLF cell lines. Compared to the empty vector control group, TA/dCPO LNPs significantly restored antiproliferative activity exclusively in GPC-3-overexpressing cells (Fig.5m-s). This functional rescue experiment directly confirmed that GPC-3 expression is a critical prerequisite for mediating the antitumor efficacy of TA/dCPO LNPs.

Moreover, we generated GPC-3-knockout (KO) Hep3B cells using CRISPR/Cas9 and evaluated their response to TA/dCPO LNP treatment. In the absence of GPC-3, TA/dCPO LNPs failed to inhibit tumor growth (Fig.5h-l). These results demonstrate that the anti-proliferative and cytotoxic effects of TA/dCPO LNPs are mechanistically dependent on the expression of GPC-3.

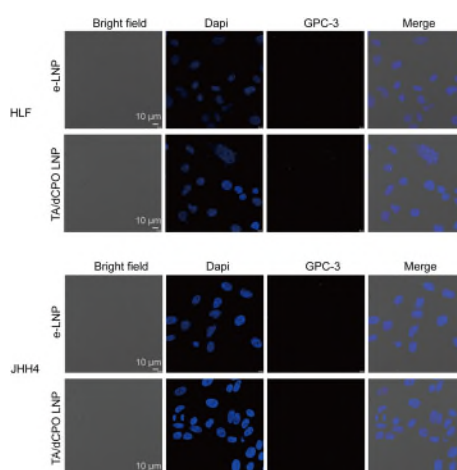


Fig.S28 Confocal images of HLF and JHH4 cells treated with empty LNPs or TA/dCPO LNPs, respectively, following by immunofluorescence staining of GPC-3.

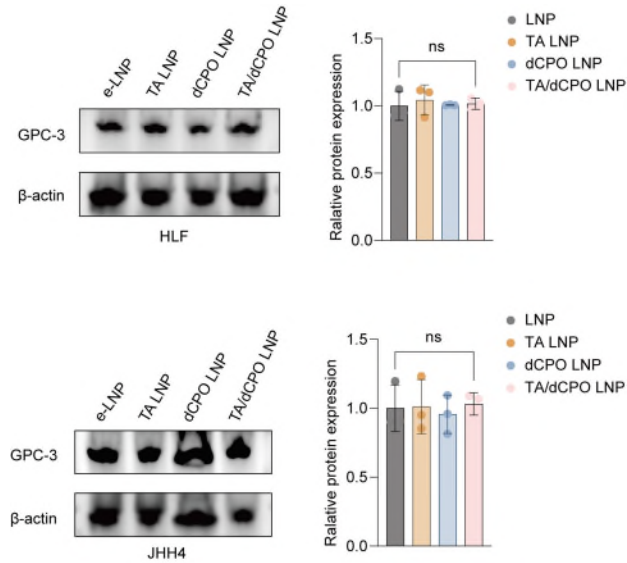


Fig.S32 Western blotting and quantification analysis in HLF and JHH4 cells after the varied treatments. Data are expressed as mean $\pm$ SD (n = 3, ns, $P \geq 0.05$).

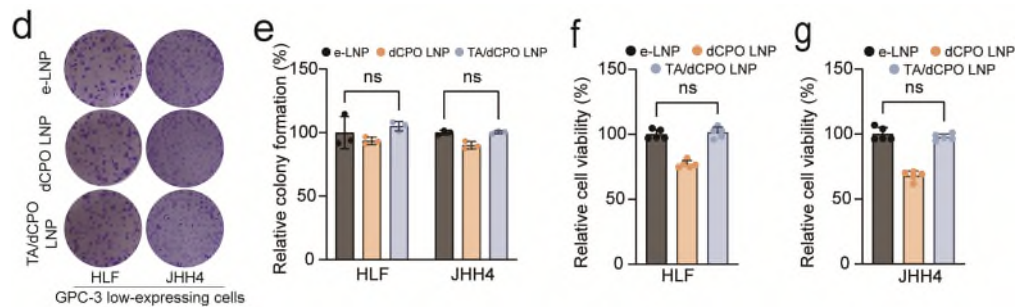


Fig.5 d,e) Clone assay and quantitative analysis of clone numbers in HLF and JHH4 cells following with varied treatment. Data are expressed as mean $\pm$ SD (n = 3, ns, $P \geq 0.05$). f,g) Cell viability of HLF and JHH4 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, $P \geq 0.05$).

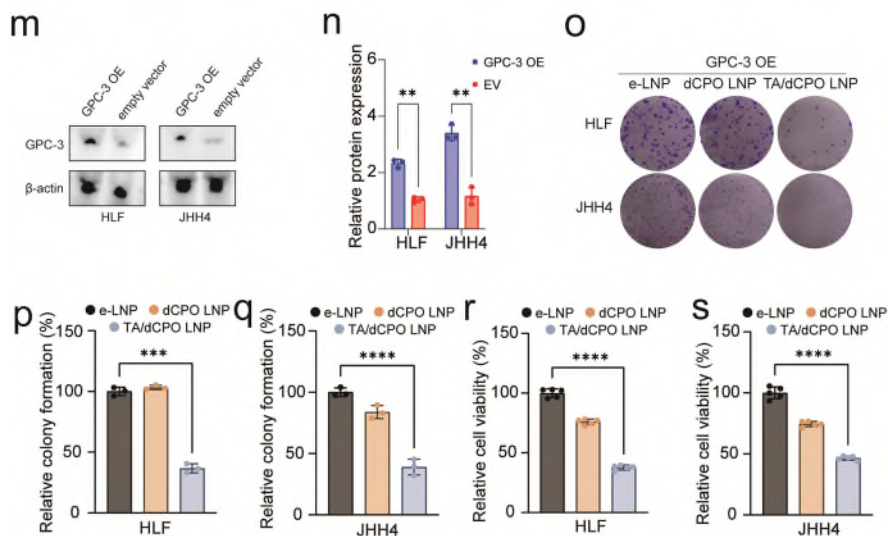


Fig.5 m,n) Immunoblot analysis of GPC-3 protein level in HLF and JHH4 cells following GPC-3 overexpression. Data are expressed as mean $\pm$ SD (n = 3, ** $P < 0.01$). o-q) Clone assay and

quantitative analysis of clone numbers in HLF and JHH4 cells after various LNP incubation following GPC-3 overexpression. Data are expressed as mean $\pm$ SD (n = 3, ***p < 0.001, ****P<0.0001). r,s) Cell viability of HLF and JHH4 cells with GPC-3 overexpression following incubation with e-LNPs, dCPO LNPs or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ****P<0.0001).

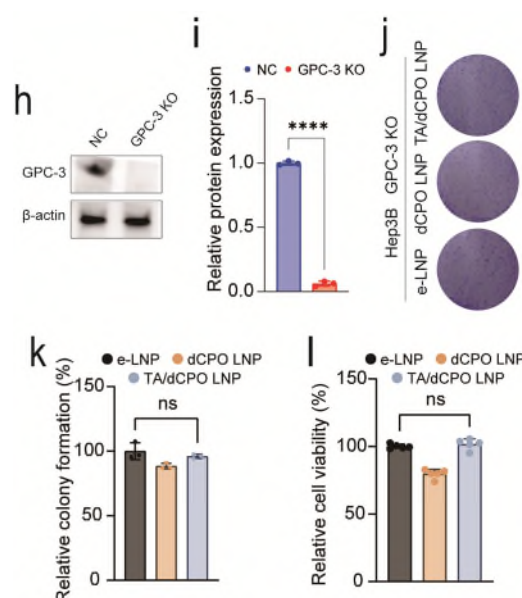


Fig. 5 h,l) immunoblot analysis of GPC-3 protein level in Hep3B cells following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ****P<0.0001). j,k) Clone assay and quantitative analysis of clone numbers in Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ns, P $\geq$ 0.05). l) Cell viability of Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

Given that $GA\bullet$ has a lifetime on the order of nanoseconds, it is likely to be rapidly quenched in culture media or in vivo, in contrast to in vitro settings. Following the same approach as in Fig. 2d, they should demonstrate whether free radical generation by TA/dCPO LNPs persists in culture media containing FBS or in mouse and human serum.

Response: Thank you for this insightful comment. We agree that demonstrating the persistence of free radical generation in serum-containing environments is crucial for validating the potential of the system *in vivo*. In response, we have conducted additional experiments to evaluate the free radical activity of TA/dCPO LNPs in culture media supplemented with FBS and mouse serum, following a similar approach to that in Fig. 2d. The results confirmed that even in the presence of serum proteins, significant MB degradation was observed, indicating that $GA\bullet$ remain active under these conditions.

This can be attributed to the fact that phenoxyl radicals ($GA\bullet$) exhibit longer lifetimes and greater selectivity compared to highly reactive oxygen species like hydroxyl radicals [1, 2]. Their relative stability and targeted reactivity allow them to resist rapid

quenching in complex biological milieus.

We have included these new data in the revised manuscript (Fig.S13) to better substantiate the remained GA• functionality in environments that more closely mimic physiological settings.

Reference:

1. Neta P, Grodkowski J. Rate constants for reactions of phenoxy radicals in solution[J]. Journal of physical and chemical reference data, 2005, 34(1): 109-199.
2. Dol C, Gerbaud G, Guigliarelli B, et al. Modulating lifetimes and relaxation times of phenoxy radicals through their incorporation into different hybrid nanostructures[J]. Physical Chemistry Chemical Physics, 2019, 21(29): 16337-16344.

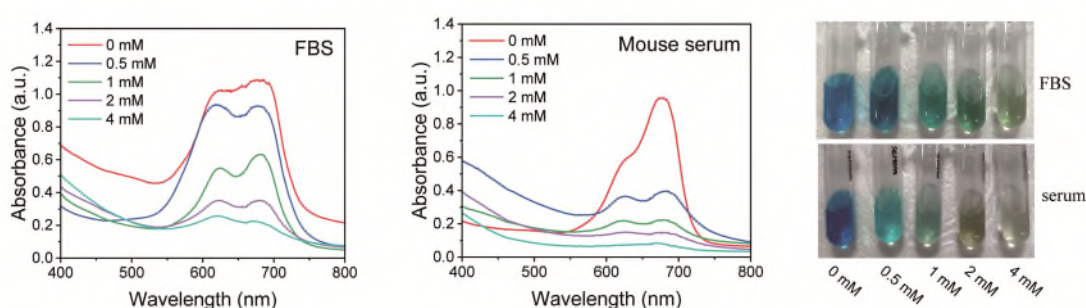


Fig.S13 UV-vis spectroscopy curves of MB solution in serum-containing environments, following reaction with dCPO NPs at different concentrations in the presence of TA.

2. GPC-3 dimerization and degradation should be quantified in Fig. 3d and 3g. In addition to Coomassie staining, Western blot analysis should be performed using internal controls to semi-quantify the protein expression level of GPC-3. Depending on the epitope recognized by the anti-GPC-3 antibody, low molecular weight bands may appear if GPC-3 is cleaved at specific sites, as suggested in Fig. 3i.

Response: Thank you for highlighting the need for quantitative analysis. The corresponding quantitative results have been incorporated into the revised Supplementary Materials as Figure S22 and S24.

Besides, we appreciate the reviewer's suggestion to further characterize the specific degradation fragments by Western blot. However, a relevant technical consideration is that the detection of such fragments depends on whether the epitope recognized by the antibody remains within the cleaved product, fragments lacking this epitope would not be detected. In our experiments, we used an anti-GPC-3 antibody (Abcam, ab95363). As the Fig.S25 illustrated, free stain gel showed a pronounced decrease in the full-length protein band (68 kDa). Western blot analysis further revealed the exclusive appearance of a specific cleavage fragment at 35-40 kDa in the TA/dCPO LNP-treated

group, confirming the generation of GPC-3 degradation products. In addition, bands in the 15-20 kDa region displayed an upward electrophoretic mobility shift and enhanced signal intensity compared to the control. These findings are consistent with oxidative modification of GPC-3 mediated by TA/dCPO LNPs.

As for the “internal controls”, in this specific experiment, we employed purified, recombinant human GPC-3 protein in a defined *in vitro* system to directly assess its interaction with the TA/dCPO components. In such a purely biochemical setup, there are no endogenous cellular proteins present that could serve as a conventional loading control (e.g., β -Actin or GAPDH), as would be standard in cell lysate experiments. Therefore, internal controls in the traditional Western blot sense are not applicable for normalizing the recombinant protein input.

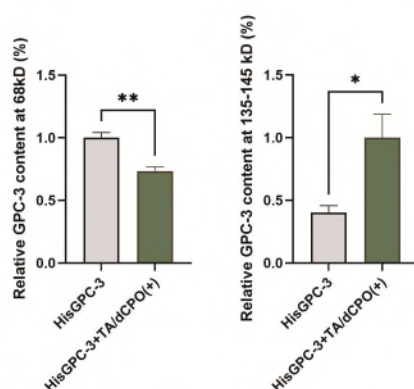


Fig.S22 Quantification of Coomassie blue staining of purified recombinant proteins His GPC-3 with or without treatment with a low-dose TA/dCPO complex.

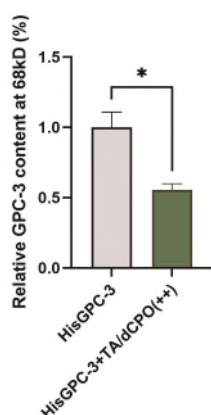


Fig.S24 Quantification of Coomassie blue staining of purified recombinant proteins His GPC-3 with or without treatment with a high-dose TA/dCPO complex.

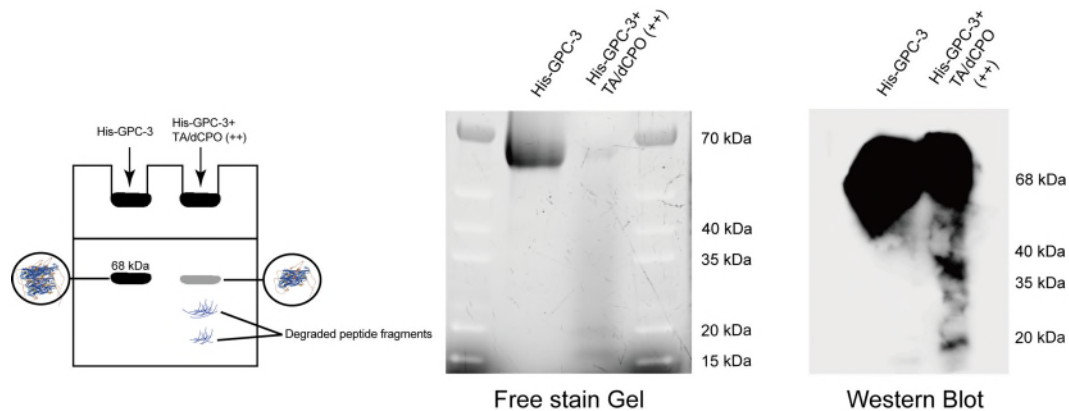


Fig.S25 Free stain gel and Western blot analysis of the recombinant protein His-GPC-3 following electrophoresis in the presence of TA/dCPO complex.

3. According to the results from Western blot analysis (Fig. 3d and 3g), TA/dCPO LNP treatment reduces GPC-3 expression by approximately 50%. Therefore, it seems inconsistent that GPC-3 expression is undetectable in immunocytochemical analyses (Fig. 4a–c).

Response: We sincerely appreciate you raising this important and insightful question. The data in Fig. 3d and 3g represent the results of native polyacrylamide gel electrophoresis (Native-PAGE) followed by Coomassie Brilliant Blue staining, conducted after *in vitro* co-incubation of purified human recombinant GPC-3 protein with TA/dCPO components. This experiment was designed to directly assess, in a cell-free system, the impact of the TA/dCPO system on GPC-3 conformation, its propensity to oligomerize (e.g., form dimers), and early signs of proteolytic degradation. Coomassie Brilliant Blue staining detects the total amount of recombinant GPC-3 protein and its degraded fragments in the gel system. In contrast, IF detects GPC-3 protein localized on the plasma membrane. Our IF staining protocol was performed without cell membrane permeabilization; thus, the primary antibody predominantly bound to membrane-surface GPC-3, enabling the specific visualization of expression and abundance changes in this membrane-associated protein pool.

Mechanistically, $GA\bullet$ generated *in situ* by the TA/dCPO system attacks and disrupts both the functional protein domains and GPI lipid anchor of membrane-anchored GPC-3, directly inducing its degradation and shedding from the plasma membrane. Consequently, compared with the untreated group, $GA\bullet$ -induced structural disruption of plasma membrane-localized GPC-3 results in a substantial reduction in the level of this protein on the cell membrane. The marked reduction observed via IF staining

validates this aforementioned mechanistic action. More importantly, the oxidative damage induced by $GA\bullet$ is likely to disrupt the exposed antigenic epitopes of membrane-localized GPC-3 and alter its overall protein conformation, which destroys the epitopes recognized by the primary antibody used in IF and consequently abrogates specific signal detection.

4. As noted in Comment 1, the authors should perform colony formation and cell viability assays to determine whether TA/dCPO LNPs suppress proliferation in GPC-3-negative HCC cell lines and GPC-3-knockout subclones, as in Fig. 5a–c.

Response: Thanks for your kind comments. As recommended, we have performed colony formation and cell viability assays in models with low or absent GPC-3 expression. As a result, CCK-8 and colony formation assays demonstrated that TA/dCPO LNPs did not induce significant cytotoxicity or anti-proliferative effects in these models (Fig.5d-g and Fig.5h-l)).

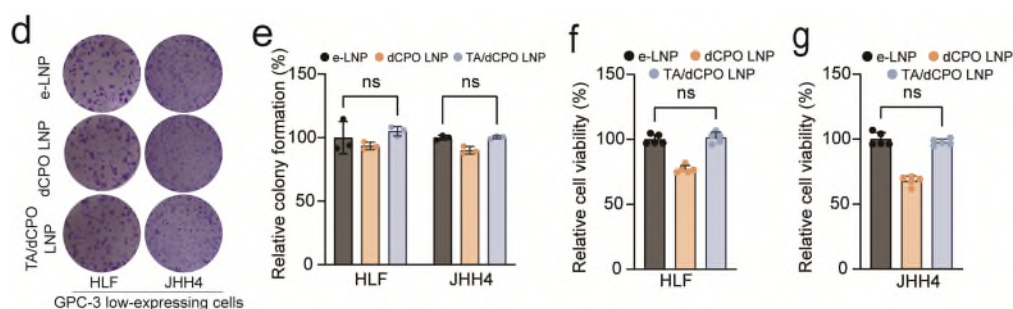


Fig.5 d,e) Clone assay and quantitative analysis of clone numbers in HLF and JHH4 cells following with varied treatment. Data are expressed as mean $\pm$ SD (n = 3, ns, $P \geq 0.05$). f,g) Cell viability of HLF and JHH4 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, $P \geq 0.05$).

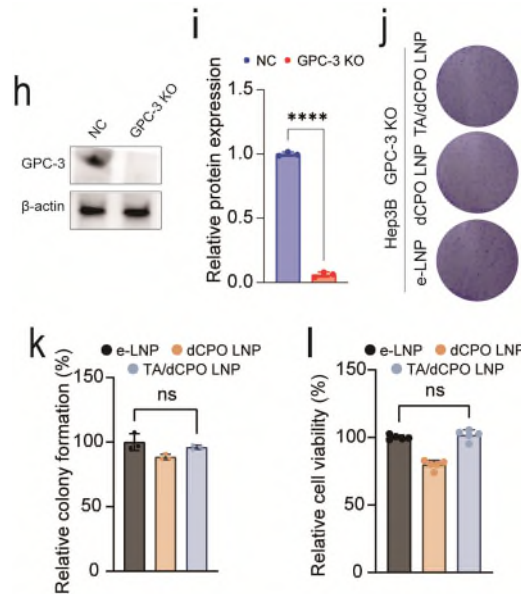


Fig. 5 h,l) immunoblot analysis of GPC-3 protein level in Hep3B cells following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ****P<0.0001). j,k) Clone assay and quantitative analysis of clone numbers in Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ns, P $\geq$ 0.05). l) Cell viability of Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

5. In many contexts, Wnt signaling inhibition increases CTNNB1 transcription through feedback regulation. The authors should evaluate whether the expression of downstream targets of the Wnt/ β -catenin signaling pathway such as *LGR5*, *AXIN2*, and *RNF43* is altered following treatment with TA/dCPO LNPs.

Response: We sincerely thank you for your insightful comments. To evaluate the functional suppression of the Wnt/ β -catenin pathway by TA/dCPO LNPs, we quantified the expression levels of three canonical downstream target genes, *LGR5*, *AXIN2* and *RNF43*, at both the mRNA and protein levels using qRT-PCR and Western blot analysis. As shown in our supplementary data (Fig. S38), TA/dCPO LNP treatment consistently induced significant downregulation in the expression of these targets at both levels. These findings provide direct evidence that TA/dCPO LNPs, via the selective targeting of GPC-3, effectively suppress the activity of the Wnt/ β -catenin signaling pathway and its downstream transcriptional output.

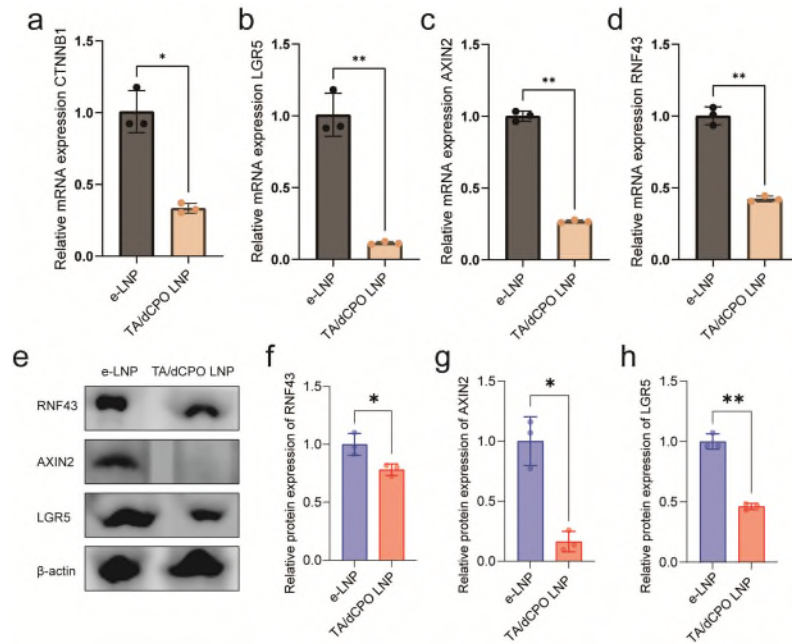


Fig.S38 a-d) qRT-PCR analysis of CTNNB1, LGR5, AXIN2 and RNF43 mRNA levels in Hep3B cells after TA/dCPO LNP treatment. Data are expressed as mean $\pm$ SD (n = 3, *p < 0.05, **p < 0.01) e-h) Western Blot and quantification analysis of LGR5, AXIN2, and RNF43 after TA/dCPO LNP treatment. Data are expressed as mean $\pm$ SD (n = 3, *p < 0.05, **p < 0.01)

6. Because β -catenin nuclear accumulation is not observed in cells cultured in vitro or tumors subcutaneously transplanted (Fig. 6k), Hep3B cells appear to be relatively independent of the Wnt/ β -catenin signaling pathway. To more accurately assess β -catenin activity, the authors should performed Western blot analysis of nuclear β -catenin using an anti- β -catenin antibody, not the antibody against non-phosphorylated β -catenin employed in this study, and should also conduct TOPFlash reporter assays.

Response: Thank you for your valuable comment regarding the precise assessment of β -catenin activity. Following your advice, we performed Western blot analysis of nuclear β -catenin using an anti-total β -catenin antibody, and the results demonstrated a concentration-dependent downregulation of nuclear β -catenin protein following TA/dCPO LNP treatment, demonstrating impaired nuclear translocation (Fig. S37). Furthermore, we performed TOPFlash/FOPFlash reporter assay to quantify canonical Wnt/ β -catenin transcriptional activity, As a result, TA/dCPO LNPs significantly reduced TOPFlash luciferase activity relative to the e-LNP control (Fig. 5w). Collectively, these data demonstrate that TA/dCPO LNPs effectively inhibit the canonical Wnt/ β -catenin signaling pathway.

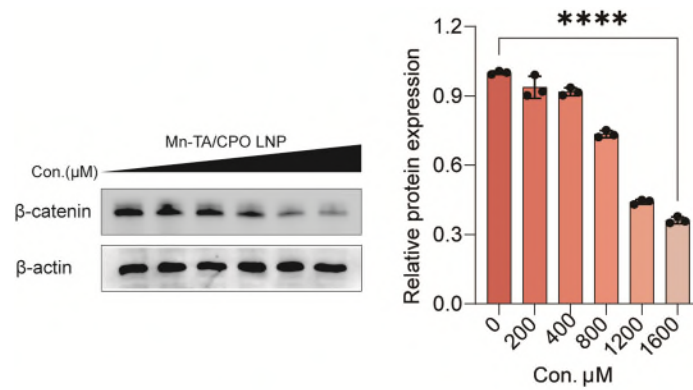


Fig.S37 Western blot analysis of nuclear β -catenin expression in Hep3B cells following treatment with various concentrations of TA/dCPO LNPs. Data are expressed as mean $\pm$ SD ($n = 3$, **** $p < 0.0001$).

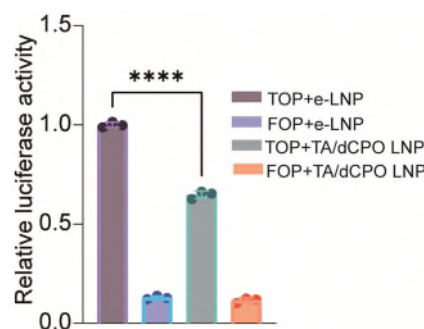


Fig.5 w) TOPFlash/FOPFlash luciferase reporter assay in Hep3B cells treated with TA/dCPO LNPs or e-LNPs. Luciferase activity was measured 48 h post-treatment and normalized to Renilla luciferase internal control. Data are expressed as mean $\pm$ SD ($n = 3$, **** $P < 0.0001$).

In contrast, since HepG2 cells express a constitutively active form of β -catenin due to CTNNB1 exon 3 deletion, GPC-3-mediated regulation of Wnt/ β -catenin signaling exerts minimal effects on gene expression and biological behavior in these cells. This limitation should be discussed.

Response: We sincerely thank you for raising these insightful and exploratory questions. The HepG2 cell line carries a deletion in exon 3 of CTNNB1, which confers constitutive activation of β -catenin, leading to its nuclear accumulation and persistent transactivation of downstream targets. This sustained activation of the Wnt/ β -catenin axis may inherently dampen the inhibitory efficacy of interventions that target upstream regulators. In our study, TA/dCPO LNPs disrupt the structural integrity of GPC-3, resulting in its functional ablation and consequent inhibition of GPC-3-dependent signaling. As a multifunctional signaling regulator, GPC3 acts not only as a co-receptor for the Wnt pathway but also engages with several key growth factor cascades, driving hepatocellular carcinoma progression, including the fibroblast growth factor (FGF), hepatocyte growth factor (HGF)/c-Met, and transforming growth factor- β (TGF- β) superfamily pathways [3]. Consistent with previous findings that targeting GPC-3 can

modulate Wnt signaling even in cells with constitutively active β -catenin [4], we observed partial suppression of the Wnt/ β -catenin pathway in HepG2 cells. This suggests that the anti-proliferative effects of TA/dCPO LNPs may arise, at least in part, from the coordinated inhibition of these alternative GPC-3-driven pathways, rather than from Wnt/ β -catenin blockade alone.

Taken together, while the constitutive activation of β -catenin in HepG2 cells may attenuate the direct impact of GPC-3 targeting on Wnt-driven transcription, the broad functional role of GPC-3 as a pivotal signaling hub means that interventions against it remain an effective strategy for inhibiting tumor growth. We sincerely appreciate your valuable insights, which have prompted us to more deeply investigate how TA/dCPO LNPs exert their therapeutic effects across a spectrum of genetic profiles in HCC.

Reference:

3. Zhou F, Shang W, Yu X, Tian J. Glypican-3: A promising biomarker for hepatocellular carcinoma diagnosis and treatment. *Med Res Rev.* 2018;38(2):741-767
4. Li D, Li N, Zhang YF, et al. Persistent Polyfunctional Chimeric Antigen Receptor T Cells That Target Glypican 3 Eliminate Orthotopic Hepatocellular Carcinomas in Mice. *Gastroenterology.* 2020;158(8):2250-2265.e20

7. It is well known that LNPs tend to accumulate in the liver. Therefore, it is puzzling that a greater amount of LNPs remains in tumors than in the liver, even at 36 hours after intravenous injection (Fig. 6b). The authors should provide an explanation for this unexpected distribution.

Response: Thank you for raising this point. In this study, the *ex vivo* organ imaging results (Fig. 6c) do confirm that the liver exhibits the highest fluorescence signal, which is consistent with the known biodistribution pattern of lipid nanoparticles (LNPs). However, to demonstrate the pronounced retention of LNPs within the tumor, Fig. 6b was acquired with the mouse in a dorsal position, which optimally visualizes the subcutaneous tumor located on the mouse's back. Actually, we also captured images in the ventral position, which more clearly reveals the liver signal and thus partly obscures the tumor signal, as shown in the Fig.R1 below.

Therefore, the *in vivo* and *ex vivo* data are consistent: while significant hepatic accumulation occurred, the LNPs also demonstrated a pronounced and sustained retention within the tumor, which is crucial for the observed therapeutic effect.

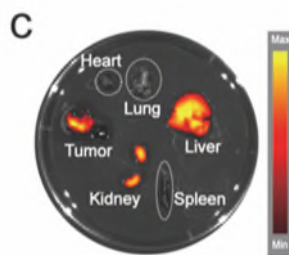


Fig.6 c) Fluorescence imaging of various organs at 8 h after the TA/dCPO LNPs treatment.

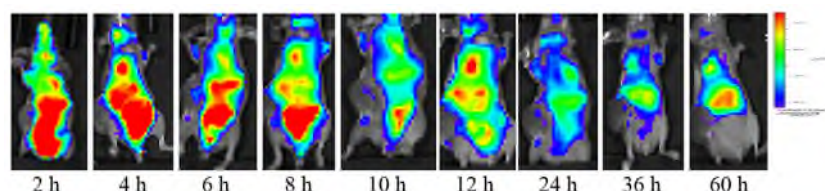


Fig.R1 IVIS imaging of tumor-bearing mice (ventral position) at different time point after the TA/dCPO LNPs injection.

Reviewer #2:

Thank you very much for inviting me to review “Generation of GPC-3-Targeted Radicals for Precise Therapy of Hepatocellular Carcinoma” by Hao et al. Authors developed a new nanosystem capable of generating radical-conjugated species with predefined oncogenic protein-targeting in-situ. It was engineered with lipid nanoparticles (LNP) that encapsulated polyphenolic tannic acid (TA) and hydrophobic dose-calcium peroxide nanoparticles (dCPO NPs). Using this, they specifically targeted GPC-3. This inhibited the activation of downstream signaling pathways such as the canonical Wnt/ β -catenin pathway, and finally suppressed HCC proliferation. They report an approach for precise free radical-mediated antitumor therapy. New therapies are urgently needed against HCC and this approach does appear novel. However, several concerns regarding how the data is presented and lack of adequate control groups reduces my enthusiasm for this manuscript.

Response: Thank you for your positive comments on our work.

Authors did not use GPC-3 knockout to confirm the specificity of the TA/dCPO nanosystem.

Response: Thank you for your comment. To further validate the GPC-3 target specificity of the TA/dCPO nanosystem, we generated a GPC-3-knockout (KO) Hep3B cell model using CRISPR-Cas9 technology. As shown in Fig.5h-l, TA/dCPO LNP showed no significant effect on the viability or proliferation of GPC-3 KO cells, indicating that its treatment efficiency depends on the presence of GPC-3. Furthermore,

we evaluated the effect of TA/dCPO LNP on hepatocellular carcinoma cell lines (JHH4 and HLF) with endogenous low expression of GPC-3. Consequently, TA/dCPO LNP exhibited minimal cytotoxicity and proliferation inhibition against these cells (Fig.5d-g). To assess the functional recovery upon GPC-3 restoration in low-GPC-3-expressing cells, we generated stable lentivirus-transduced GPC-3-overexpressing JHH4 and HLF cell lines. Consequently, the cytotoxic activity of TA/dCPO LNP was significantly restored (Fig.5m-s). Collectively, the integrated loss-of-function (GPC-3-KO and low-GPC-3-expression models) and gain-of-function (GPC-3-overexpression rescue) experiments indicated that the antitumor efficacy of TA/dCPO LNPs is specifically mediated by GPC-3.

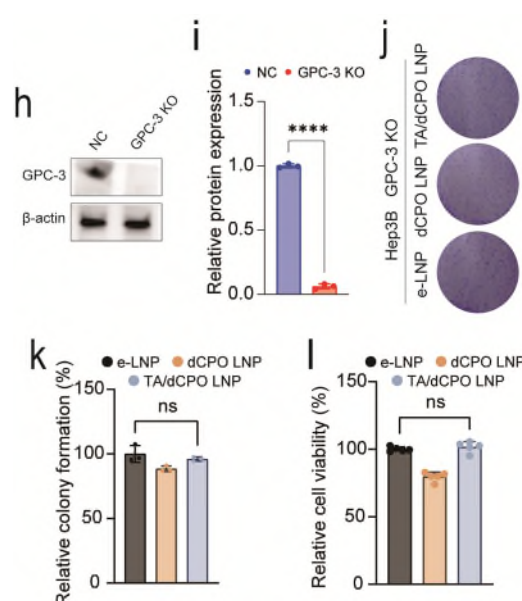


Fig. 5 h,i) immunoblot analysis of GPC-3 protein level in Hep3B cells following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ****P<0.0001). j,k) Clone assay and quantitative analysis of clone numbers in Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ns, P $\geq$ 0.05). l) Cell viability of Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

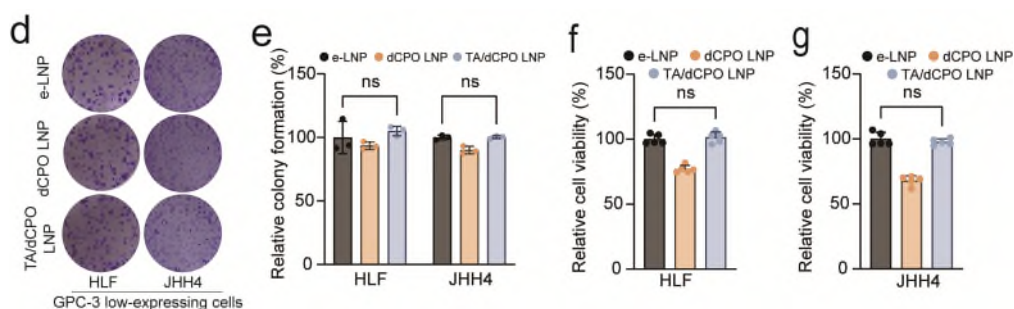


Fig.5 d,e) Clone assay and quantitative analysis of clone numbers in HLF and JHH4 cells following with varied treatment. Data are expressed as mean $\pm$ SD (n = 3, ns, P $\geq$ 0.05). f,g) Cell viability of

HLF and JHH4 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

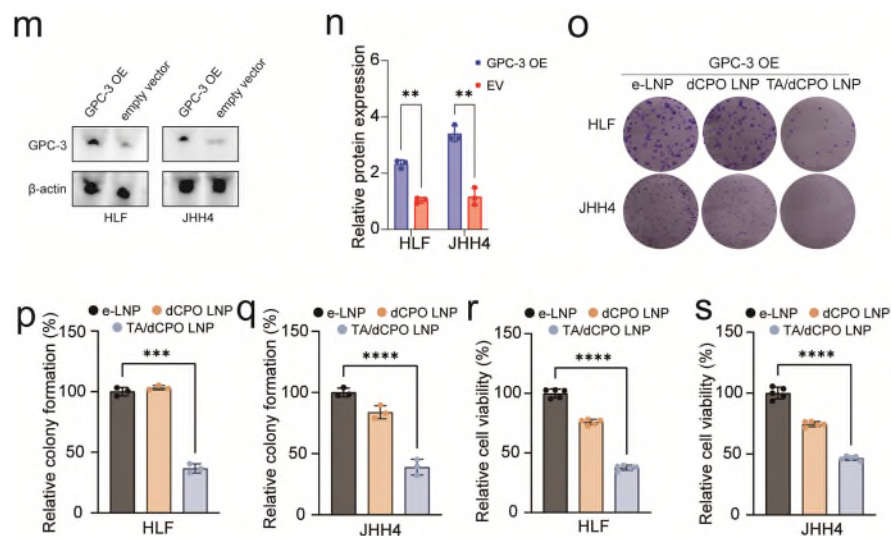


Fig. 5 m,n) Immunoblot analysis of GPC-3 protein level in HLF and JHH4 cells following GPC-3 overexpression. Data are expressed as mean $\pm$ SD (n = 3, **P<0.01). o-q) Clone assay and quantitative analysis of clone numbers in HLF and JHH4 cells after various LNP incubation following GPC-3 overexpression. Data are expressed as mean $\pm$ SD (n = 3, ***p < 0.001, ****P<0.0001). r,s) Cell viability of HLF and JHH4 cells with GPC-3 overexpression following incubation with e-LNPs, dCPO LNPs or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ****P<0.0001).

While they performed experiments using recombinant GPC-3 in the *in vitro* setting, the researchers did not use GPC-3 recombinant in the *in vivo* setting.

Response: Thank you for your comment. *In vitro*, we used recombinant GPC-3 to directly characterize its interaction with the TA/dCPO complex and analyze associated structural changes through proteomic approaches. This experimental setup isolates the specific binding event, eliminating interference from endogenous microenvironmental factors and ensuring results that are specific to GPC-3 engagement.

In contrast, our *in vivo* experiments were designed to recapitulate the molecular expression profile of GPC-3 in tumor lesions of clinically relevant hepatocellular carcinoma (HCC) [5]. Given this context, the implanted Hep3B tumor cells, which endogenously overexpress GPC-3, allowed us to evaluate the therapeutic efficacy and mechanism of our intervention relying on this endogenous GPC-3. This approach aligns with the design rationale employed in previous GPC-3-targeted intervention studies [6, 7]. Furthermore, this design circumvents potential biosafety risks that may arise from the introduction of exogenous recombinant proteins.

Reference:

5. Yu B, Ma W. Biomarker discovery in hepatocellular carcinoma (HCC) for personalized treatment

and enhanced prognosis. *Cytokine Growth Factor Rev.* 2024;79:29-38.

6. Sun L, Gao F, Gao Z, et al. Shed antigen-induced blocking effect on CAR-T cells targeting Glypican-3 in Hepatocellular Carcinoma. *J Immunother Cancer.* 2021;9(4):e001875.

7. Gao W, Kim H, Feng M, et al. Inactivation of Wnt signaling by a human antibody that recognizes the heparan sulfate chains of glypican-3 for liver cancer therapy. *Hepatology.* 2014;60(2):576-587.

The nanosystem is designed to generate "stabilized phenoxyl radicals" which are intended to overcome the limitations of short-lived hydroxyl radicals. While the presence of phenoxyl radicals is confirmed by ESR, quantitative data on their stability or longevity *in situ* compared to other free radicals would be beneficial.

Response: Thank you for this valuable suggestion. We have performed additional experiments using TMB (3,3',5,5'-Tetramethylbenzidine) as a chromogenic probe to monitor radical-mediated oxidation kinetics. TMB could be oxidized by reactive species, producing a blue-colored product that can be quantitatively measured. We compared the oxidation kinetics of TMB induced by hydroxyl radicals ($\bullet\text{OH}$, generated from Fenton-like reaction between dCPO and Fe^{2+}) versus the phenoxyl radicals ($\text{GA}\bullet$) produced by the TA+dCPO system.

As expected, $\bullet\text{OH}$ drove rapid, intense TMB oxidation within 3 minutes, while $\text{GA}\bullet$ mediated a far more gradual and sustained oxidation pattern, with an oxidation intensity around 50% that of $\bullet\text{OH}$ at the same time point (Fig S13). This distinct kinetic profile reflected the reduced reactivity of $\text{GA}\bullet$, which conferred enhanced stability and an extended *in situ* lifetime. Such steady and durable reactivity demonstrates that $\text{GA}\bullet$ possess improved longevity and better temporal persistence relative to the highly transient, short-lived $\bullet\text{OH}$. These quantitative data have been added to the revised manuscript to further validate the favorable stability profile of our radical-based strategy.

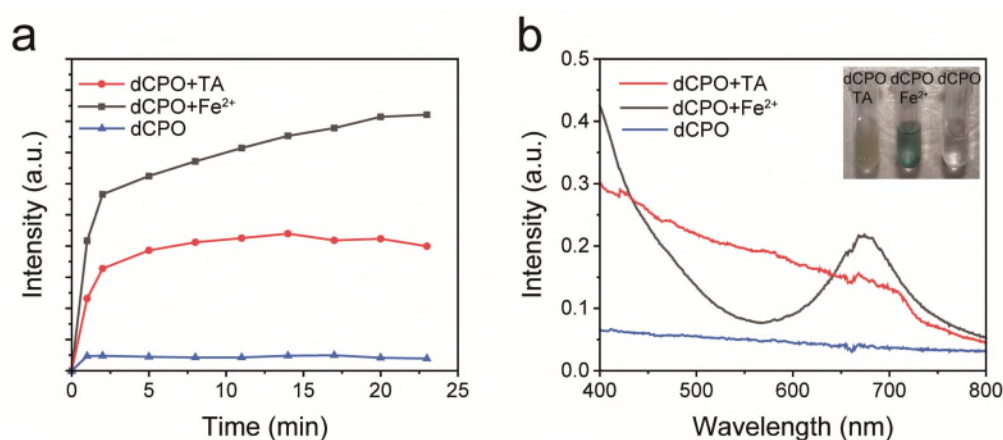


Fig.S13 a) The oxidation kinetics of TMB induced by hydroxyl radicals (generated from dCPO and Fe^{2+}), phenoxyl radicals ($\text{GA}\bullet$) (produced by the dCPO and TA) or dCPO alone. b) UV-vis spectra

of TMB oxidation induced by dCPO+TA, dCPO+Fe²⁺ or dCPO alone at 23 min.

The paper emphasizes overcoming the "non-specific activity" and "collateral damage to adjacent normal tissues" of conventional free radical therapies. While the TA/dCPO nanosystem demonstrates targeted action on GPC-3, a direct comparison showing reduced off-target effects compared to a generic, non-targeted free radical generating system is needed to confirm the "precision" claim.

Response: We greatly appreciate your valuable comment. To address the request for direct evidence of reduced off-target effects, we employed hydroxyl radicals generated by the classic Fenton reaction system as a non-targeted free radical control. Both the Fenton system and TA/dCPO LNPs were co-incubated in parallel with three cell types: normal hepatocytes (AML12), wild-type hepatocellular carcinoma cells (Hep3B), and GPC-3-knockout (GPC-3-KO) Hep3B cells.

As shown in Fig. S35, hydroxyl radicals induced significant cytotoxicity across all three cell types, confirming their non-specific, broad-spectrum damaging effect. In contrast, TA/dCPO LNPs exhibited potent cytotoxicity specifically toward wild-type Hep3B cells (GPC-3-positive), while showing no detectable toxicity to normal AML12 hepatocytes or GPC-3-KO Hep3B cells (GPC-3-negative).

These results clearly demonstrate that the TA/dCPO nanosystem exerts targeted antitumor activity via GPC-3-dependent recognition, rather than a non-specific, broad-spectrum mechanism. This direct head-to-head comparison substantiates our claim of enhanced precision and minimized off-target effects relative to conventional free radical therapies, and the data have been incorporated into the revised manuscript.

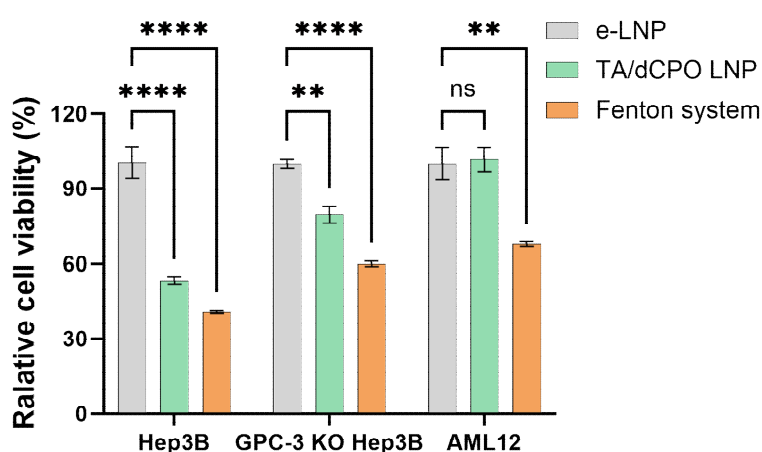


Fig.S34 Cell viability of Hep3B, GPC-3 KO Hep3B and AML12 cells after incubation with e-LNP, TA/dCPO LNP or Fenton system. Data are expressed as mean $\pm$ SD (n = 5, **P<0.01, ****P<0.0001).

Many figures lack adequate legends, making them difficult to interpret. Quantification is not provided for most IF or IHC images. Scale bars are absent in the majority of IF and IHC panels, and where present, they are inconsistently applied.

Response: We sincerely thank you for your thoughtful and meticulous comments. We have checked and revised the relevant content as required, supplementing and improving the legends of all figures, adding quantitative analysis for all IF and IHC images, and standardizing the application and labeling of scale bars for all IF and IHC panels. All revisions have been incorporated into the revised manuscript.

The free GPC-3 content in the culture medium was quantified using ELISA. As shown in Fig.4k-m, the introduction of TA/dCPO LNPs increased the free GPC-3 content in the culture medium, indicating that they could break off the lipid moiety. However, the fold change in soluble GPC-3 appears negligible in two of the three cell lines shown.

Response: We appreciate your insightful observation. The fold-change in soluble GPC-3 (sGPC-3) after TA/dCPO LNP treatment indeed varies across hepatocellular carcinoma cell lines, reflecting inherent biological heterogeneity such as differences in basal membrane-GPC-3 levels, oxidative susceptibility, and post-cleavage processing kinetics. For example, at the same TA/dCPO LNP dose, cell lines with higher membrane-GPC-3 show more pronounced sGPC-3 increases, consistent with greater substrate availability. In contrast, lines where oxidative degradation predominates or ectodomain shedding is kinetically constrained show attenuated or even dose-dependent declines in detectable sGPC-3, particularly at higher nanosystem concentrations. Notably, the biphasic concentration-response relationship, where sGPC-3 initially increase but decline at higher doses, reflects the dynamic interplay between GPC-3 shedding, oxidative and degradation. This profile underscores the dose-dependent mechanistic complexity of GPC-3 modulation by TA/dCPO LNP.

The study relies on a single cell line derived subcutaneous model, which limits the generalizability of the findings. Tumor biology, immune interactions, and treatment responses can vary substantially between cell lines and may not reflect the heterogeneity of human tumors.

Response: Thanks for your suggestion. We fully agree that relying solely on a single cell line-derived subcutaneous xenograft model limits the generalizability of our findings. To address this concern and enhance the robustness of our conclusions, we have expanded our *in vivo* studies to include a second GPC-3-high human

hepatocellular carcinoma (HCC) cell line, Huh7, which has distinct genetic characteristics from the original cell line. We replicated the TA/dCPO LNP treatment experiment using a subcutaneous Huh7 xenograft mouse model. As shown in Supplementary Fig. S47, TA/dCPO LNPs similarly exerted significant tumor growth inhibition and promoted tumor cell apoptosis in this model. These results demonstrate that the nanosystem is effective against GPC-3-positive HCC tumors across multiple models with distinct genetic backgrounds.

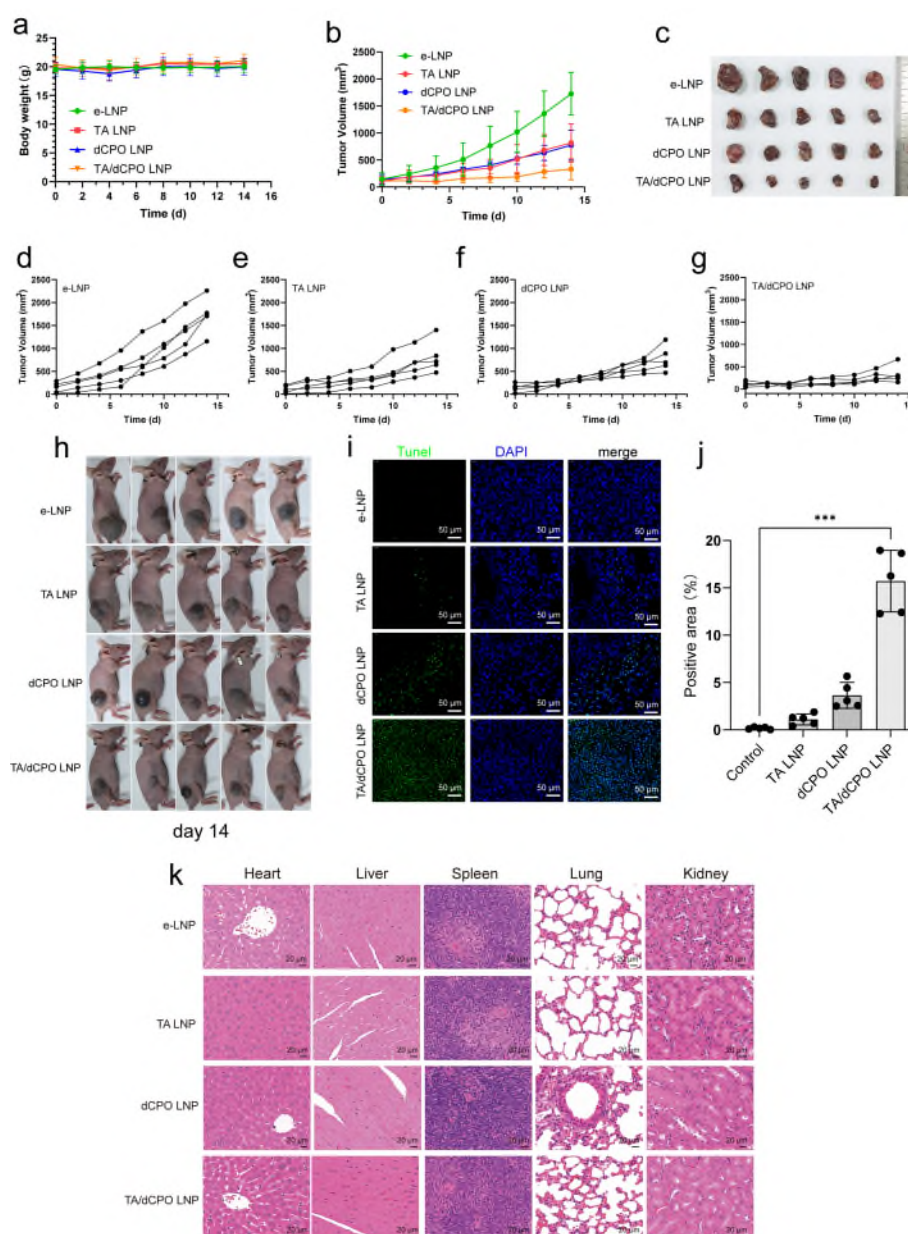


Fig.S47. a) Mice body weight changes during the treatment period. b) Tumor volume monitoring at different time intervals. c) Representative photos of tumor tissue on day 14 after different treatments. d-g) Relative tumor volumes plot with different treatment during the therapeutic process (n=5; Ca: 4.5 mg kg⁻¹ for i.v. injection). h) Representative photos of mice treated with various LNPs at day 14. i) TUNEL staining images on excised tumor tissues with various LNPs treatments and j)

Quantification analysis of mean fluorescent intensities. Data are expressed as mean $\pm$ SD (n = 5, ***P<0.001). k) H&E staining of major organs treated with varied LNPs agents.

The toxicity assessment is insufficient. Only a single H&E section per organ is presented in the main figure, which does not allow for evaluation of variability or reproducibility. A more comprehensive analysis, including multiple sections per organ and quantitative pathology scoring, would be necessary to draw reliable conclusions about safety.

Response: Thank you for raising this important point regarding toxicity assessment. Following the suggestion, we have performed a more comprehensive histopathological evaluation. Specifically, we examined additional H&E-stained sections from two distinct tissue regions per organ (Fig. S45) and conducted quantitative pathological scoring for all key tissues by three board-certified pathologists blinded to the treatment groups. These detailed scoring data are summarized in Table 2. No treatment-related organ damage was detectable in TA/dCPO LNP-treated animals. Collectively, these quantitative findings validate the favorable systemic safety profile of TA/dCPO LNPs at the administered doses.

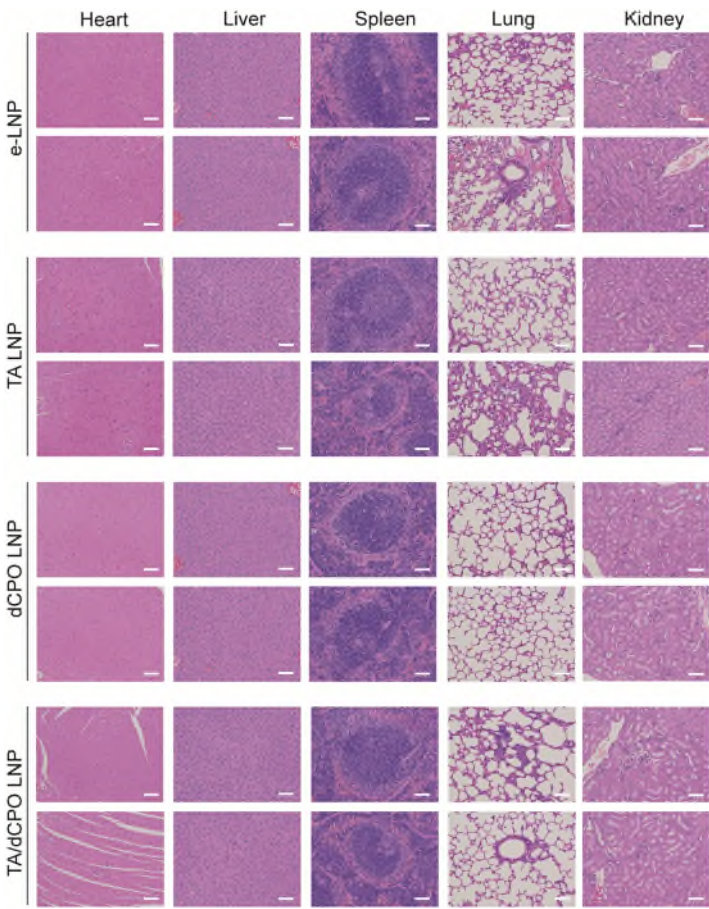


Fig.S45 H&E staining of major organs from different tissue sections treated with varied LNPs agents. Scale bar, 100 μ m.

Organ	e-LNP			TA LNP			dCPO LNP			TA/dCPO LNP		
Heart	0	0	0	1	0	0	1	1	1	0	0	0
Liver	1	0	0	0	0	0	1	2	1	0	0	0
Spleen	0	0	0	1	1	0	1	0	0	0	1	0
Lung	0	1	0	0	0	1	0	0	0	0	0	0
Kidney	1	0	0	0	0	0	0	1	0	0	0	0

Table.2 Semi-quantitative histopathology scoring of major organs by three blinded pathologists following treatment.

Reviewer #3:

This manuscript proposes a protein-targeted, free radical-mediated antitumor strategy that effectively addresses the issue of nonspecific oxidative damage inherent in traditional free radical-based therapeutic approaches. Notably, the authors integrate the oxidative damaging effects of phenoxyl radicals on proteins with the specific binding capability of small-molecule GA to GPC-3, thereby establishing a GPC-3-targeted phenoxyl radical-based therapeutic strategy. By promoting the targeted binding of GA

- to GPC-3 and inducing the selective disruption of its peptide segments and lipid structures, the system effectively leads to the structural and functional disruption of GPC-3, subsequently suppressing activation of the downstream Wnt signaling pathway and ultimately eliciting a cascade-like anti-tumor response at the molecular level through targeted GPC-3 protein intervention. This work represents a significant integration of free radical chemistry, nanomedicine, and tumor protein-targeted therapy, offering a novel molecular intervention paradigm for precision oncology. Its academic significance and innovative value support its suitability for publication in Nature Communications. However, there are some concerns needed to be addressed:

Response: Thank you for your positive comments on our work.

1. How about the dispersibility and stability of the synthesized TA/dCPO LNP? Please give relevant characterization results.

Response: Thank you for your valuable suggestions. The stability of the LNPs in physiological saline was investigated over a 15-day period by DLS (Fig.S10). The

results demonstrated that the particle size remained stable with no significant changes, indicating good dispersibility and stability of the nanoparticles. Besides, we also evaluated their stability in FBS-containing buffers over 4 hours – a timeframe reflecting typical blood circulation duration for nanoparticles prior to tissue accumulation. Compared to the hydrodynamic diameter of LNPs in saline solution, their diameter in FBS decreased from 120 nm to 90 nm, likely due to transient protein adsorption forming a dynamic "soft corona" that compressed the PEG layer [8]. And the increased PDI value further reflected the variability of protein-LNP interactions during adsorption/desorption equilibration. The results demonstrated that LNPs maintain colloidal stability in serum for at least 4 hours, with minimal size variation and no aggregation. The dispersibility and stability of TA/dCPO LNPs were supplied in Fig. S10.

References:

8. Berger, M., Degey, M., Leblond Chain, J., Maquoi, E., Evrard, B., Lechanteur, A., & Piel, G. (2023). Effect of PEG anchor and serum on lipid nanoparticles: development of a nanoparticles tracking method. *Pharmaceutics*, 15(2), 597.

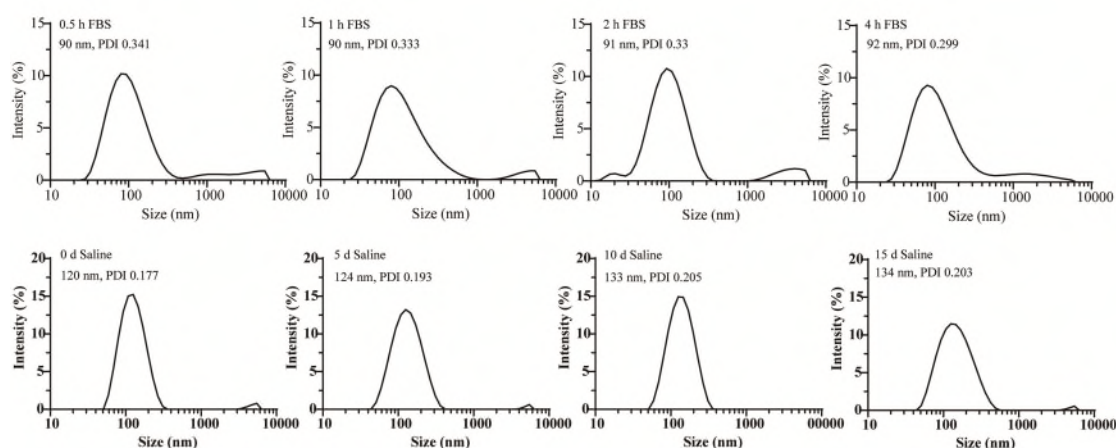


Fig.S10 Size distribution of the TA/dCPO LNP in physiological saline over a 15-day period by DLS (down), and in FBS-containing physiological fluids within 4 h (up), a duration representative of typical systemic circulation time for nanoparticles prior to tissue accumulation.

2. Display a local magnified view of the fig.1g image.

Response: Thanks for your comment. A locally magnified inset of the image in Fig.1g has been displayed for clearer observation.

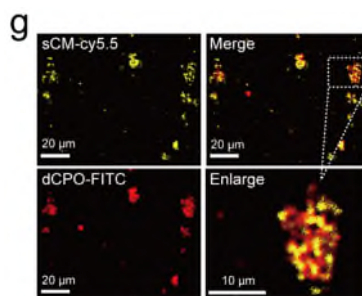


Fig.1 g) Fluorescence colocalization of dCPO-FITC and the cy5.5 labeled synthetic cell membranes (sCM-cy5.5).

3. Please supplement the size distribution of TA LNP by dynamic light scattering.

Response: We greatly appreciate your valuable comment. We have now supplemented the size distribution profile of the TA-encapsulated LNPs (TA LNP) as measured by dynamic light scattering (DLS).

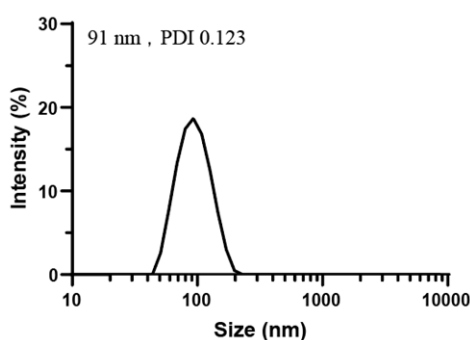


Fig.S6. Size distribution of the TA LNPs by DLS.

4. The manuscript suggests that the generation of hydroxyl radicals may originate from the degradation of dCPO. What specific factors could potentially contribute to the production of hydrogen peroxide?

Response: Thank you for this insightful question. In our TA/dCPO LNP system, the $\bullet\text{OH}$ detected by ESR is derived from H_2O_2 generated during the degradation of dCPO nanoparticles. The formation of H_2O_2 can be explained by several key features inherent to the material design and redox chemistry of our platform.

First, the core component CaO_2 contains labile peroxide bonds ($-\text{O}-\text{O}-$) that undergo spontaneous hydrolysis in aqueous or physiological environments, releasing H_2O_2 via the reaction: $\text{CaO}_2 + 2\text{H}_2\text{O} \rightarrow \text{Ca}(\text{OH})_2 + \text{H}_2\text{O}_2$. Although dCPO was hydrophobically modified to improve membrane interaction, its underlying peroxide chemistry remains preserved, allowing sustained H_2O_2 generation in the cellular microenvironment.

Second, as shown in Supplementary Fig. S18, dCPO acts as an oxidant that drives tannic acid (TA) radicalization through single-electron transfer and hydrogen atom transfer. This redox process is accompanied by oxygen consumption and intermediate H_2O_2 formation. Third, the compartmentalized LNP structure spatially separates TA and dCPO to avoid premature reaction. Upon membrane fusion, dCPO is released into the lipid-rich environment where it reacts with TA. The rate and extent of H_2O_2 release are further modulated by local lipid composition, cellular redox potential, and the surface properties of hydrophobically modified dCPO.

In summary, H_2O_2 is produced through the intrinsic hydrolysis of CaO_2 , its redox activity during TA radicalization, and its controlled release within the membrane microenvironment. The generated H_2O_2 can then undergo photolysis or metal-mediated Fenton-like conversion to form $\bullet\text{OH}$, consistent with our ESR observations.

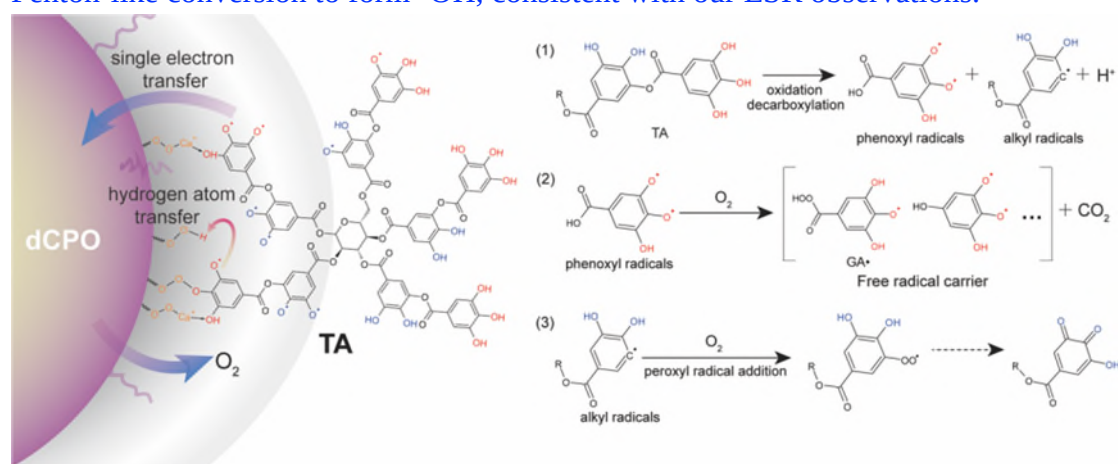


Fig.S18 Chemical reaction mechanism of the TA/dCPO system.

5. In Fig. 5c, the statistical comparison of cell viability between the eLNP group and the TA/dCPO LNP group is missing.

Response: We sincerely thank the reviewer for the observation. In the revised manuscript, the comparison has been incorporated directly into the updated figure. This revision ensures a more complete, transparent, and rigorously supported presentation of the experimental results.

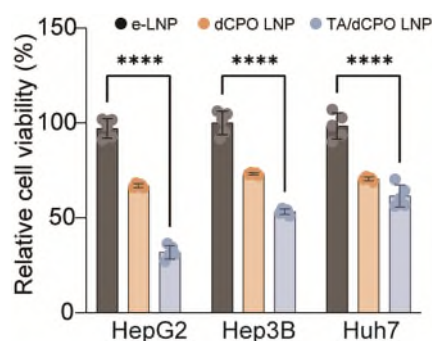


Fig.5 c) Cell viability of HepG2, Hep3B and Huh7 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ****P<0.0001).

What factors may account for the observed discrepancies in therapeutic efficacy among the three cell lines?

Response: We appreciate for your insightful question regarding the variability in therapeutic efficacy across the three cell lines. In our opinion, GPC-3 expression level represents the primary determinants of response in our system. We specifically included cell lines with high GPC-3 membrane expression (HepG2, Hep3B, Huh7) and low GPC-3 membrane expression (HLF, JHH4) in our study. The results consistently demonstrate that significantly stronger antitumor effects were achieved in high-GPC-3 cell lines, whereas efficacy was markedly attenuated in low-GPC-3 lines, as illustrated by Fig.5c, 5f-g. These findings directly confirm that GPC-3 membrane expression level is a key regulator of the therapeutic efficacy of our nanosystem.

Among GPC3-high HCC cell lines, genetic background heterogeneity can lead to distinct responses to downstream signaling events triggered by GPC3 modulation. Some cell lines depend heavily on GPC3-mediated Wnt signaling to maintain proliferation. In contrast, other lines concurrently activate strong alternative survival pathways, including PI3K/AKT and MAPK signaling [9]. Such differences can result in variable effects on proliferation inhibition, apoptosis induction, and migration suppression across tumor cell lines. Nevertheless, our TA/dCPO LNP system exerts its antitumor effect primarily by specifically disrupting the upstream GPC3 protein, thereby suppressing the activation of a broad range of downstream pro-tumorigenic signaling pathways.

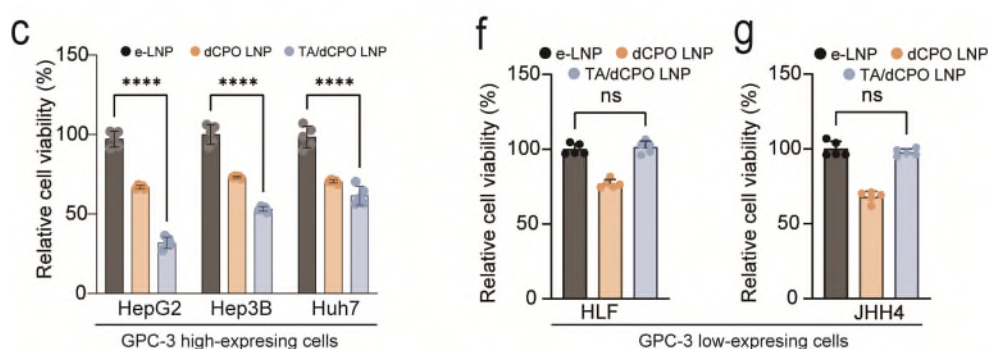


Fig.5 c) Cell viability of HepG2, Hep3B and Huh7 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ****P<0.0001). f,g) Cell viability of HLF and JHH4 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

Reference:

9. Zhou F, Shang W, Yu X, Tian J. Glypican-3: A promising biomarker for hepatocellular carcinoma

6. In vivo fluorescence imaging demonstrated that TA/dCPO LNPs predominantly accumulate in the liver. How do normal hepatocytes counteract the oxidative effects of dCPO within this system? Furthermore, if possible, it is recommended to provide additional experimental validation to elucidate the differential cytotoxic effects of dCPO on hepatocellular carcinoma cells and normal hepatocytes at the cellular level.

Response: We thank the reviewer for raising this critical point. Our system mediates targeted cytotoxicity in a GPC3-dependent manner. Since normal hepatocytes completely lack GPC3 expression [10], the molecular trigger required for GA• radical binding and subsequent nanosystem activation is absent, thereby precluding cytotoxic or oxidative damage in these cells. Furthermore, normal cells exhibit significantly higher catalase activity than tumor cells [11, 12]; this endogenous antioxidant enzyme efficiently scavenges GA• radicals and mitigates oxidative stress. Consistent with this mechanistic rationale, our experiments in the non-tumorigenic murine hepatocyte line AML12 revealed no significant cytotoxicity or oxidative damage following TA/dCPO LNP treatment (Fig. S34).

Reference:

10. Dhungel, Bijay et al. "Evaluation of the Glypican 3 promoter for transcriptional targeting of hepatocellular carcinoma." *Gene therapy* vol. 25,2 (2018): 115-128.
11. Cecerska-Heryć, E., Surowska, O., Heryć, R., Serwin, N., Napiontek-Balińska, S., & Dołęgowska, B. (2021). Are antioxidant enzymes essential markers in the diagnosis and monitoring of cancer patients—a review. *Clinical biochemistry*, 93, 1-8.
12. Asaduzzaman Khan, M., Tania, M., Zhang, D. Z., & Chen, H. C. (2010). Antioxidant enzymes and cancer. *Chinese Journal of Cancer Research*, 22(2), 87-92.

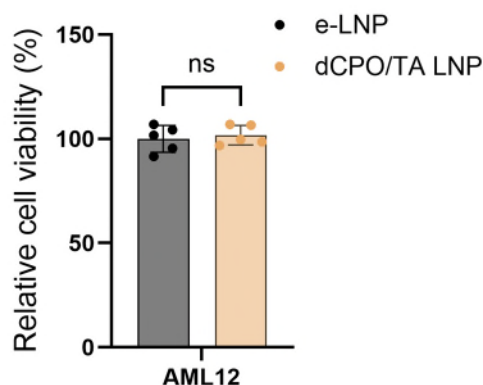


Fig.S33 Cell viability of AML12 cells after incubation with empty LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, $P \geq 0.05$).

7. In practical applications, what measures can be taken to effectively ensure the efficiency of GA• generation at the cell membrane by the TA/dCPO LNP system?

Response: Thank you for raising this question. To ensure the efficient generation of GA• on the cell membrane through the TA/dCPO LNP system in practical applications, the following targeted strategies are crucial. i) Designing LNP membranes to promote surface retention: The LNP formulation can adopt specific lipid compositions, for example, adding cholesterol analogues or lipids with high phase transition temperatures to facilitate stable adsorption and fusion ability on the membrane rather than immediate internalization [13-15]. The optimized LNPs feature a rationally engineered lipid composition that actively destabilizes the plasma membrane and facilitates membrane fusion, thereby enabling rapid, localized release of TA and dCPO and providing a critical time window for the interaction of TA and dCPO on the extracellular or membrane interface to generate GA•. ii) Optimizing molar ratio and co-localization: The molar ratio of TA to dCPO within each LNP must be systematically optimized *in vitro* to achieve the highest yield of GA•, minimizing the accumulation of competing by-products not only suppresses their formation but also thermodynamically and kinetically favors the selective conversion of intermediates toward GA [15]. iii) Active targeting of membrane microdomains: In addition to the general targeting of GPC3, it is also possible to explore the connection with cell membrane microdomains, such as lipid raft, recruiting or stabilizing lipid nanoparticles in specific membrane microdomains, so that LNPs can further precisely target the location of GPC-3 protein [16]. This precise localization may help to more concentrated and efficiently release the payload locally.

Reference:

13. Semple, S., Akinc, A., Chen, J. *et al.* Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* **28**, 172–176 (2010).
14. Cheng X, Lee RJ. The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery. *Adv Drug Deliv Rev.* 2016;99(Pt A):129-137.
15. Patel S, Ashwanikumar N, Robinson E, et al. Boosting Intracellular Delivery of Lipid Nanoparticle-Encapsulated mRNA. *Nano Lett.* 2017;17(9):5711-5718.
16. Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. *Science.* 2010;327(5961):46-50.

Reviewer #4:

This manuscript reports the development of TA/dCPO-LNP capable of generating radical conjugated species in situ, with proposed oncogenic GpC-3 targeting capabilities. This system is based on LNP encapsulating TA and dCPO. While the study

is interesting, in its current form, the manuscript lacks sufficient novelty and broad significance to meet the high standards of Nature Communications. Moreover, several aspects require clarification and further experimental validation, as outlined below:

Response: Thank you for your comments on our work.

1. The authors state that the system is based on lipid nanoparticles. Liposomes typically consist of a lipid bilayer enclosing an aqueous core, while lipid nanoparticles generally possess a solid or amorphous lipid core. For the LNPs in this study, what is the structure of the particle? Do dCPO and TA reside in a solid lipid core surrounded by a bilayer, or in a more liquid-like core? From Figures 1g and 1i, the nanoparticles appear highly heterogeneous, with some exhibiting dense cores and others appearing lighter. Please clarify the structural model and provide polydispersity index (PDI) values, along with more representative Cryo-TEM images.

Response: Thank you for your insightful comment. We have carefully re-examined our samples using Cryo-TEM to provide a clearer structural model. The images reveal that the particles exhibit a concentric multilamellar vesicle architecture, often referred to as an "onion-like" structure, composed of multiple lipid bilayers. Within this arrangement, the core appears as a dense region due to the presence of hydrophobic dCPO nanoparticles, which contain metal 'Ca' element and exhibit high electron density. This core is surrounded by lower-density, liquid-like shell regions where the hydrophilic TA is localized, consistent with its molecular composition primarily of carbon and hydrogen. The multilamellar nature likely results from the self-assembly behavior of the lipid components during formulation, which facilitates the encapsulation of both hydrophobic and hydrophilic agents. DLS analysis indicates an average particle size of 120 nm with a PDI of 0.210, reflecting moderate size distribution. The representative Cryo-TEM images were included in the revised manuscript to better illustrate this structural homogeneity.

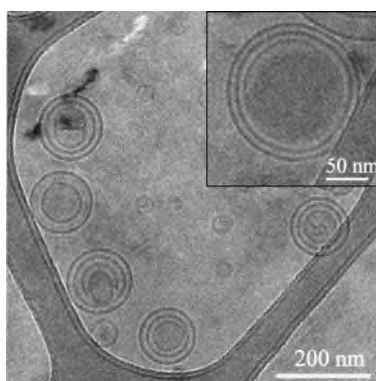


Fig.1 i) Cryo-TEM images of TA/dCPO LNPs.

2. What is the total loading of dCPO and TA, and what is their respective loading ratio? Please also report the encapsulation efficiency of the nanoparticles.

Response: Thank you for the valuable comment. We have conducted detailed measurements to determine the precise loading content and encapsulation efficiency for both dCPO and TA. The dCPO loading was quantified by measuring calcium content using ICP-MS, while TA loading was determined via UV-vis spectroscopy. The loading ratio and the encapsulation efficiency (EE) were calculated as:

Loading ratio = encapsulated payload / total lipid $\times$ 100% (mol/mol)

EE (%) = encapsulated payload / total payload added $\times$ 100%

The results indicate that the dCPO loading ratio is 0.36 mol/mol, and the TA loading ratio is 0.057 mol/mol. The encapsulation efficiency for dCPO was 84.3%, and for TA 74.5%. These values confirm the successful and efficient co-encapsulation of both components within the lipid nanoparticles.

The quantitative data was included in the revised manuscript (Table. 1).

Samples	1	2	3	Average
Encapsulated dCPO / μ mol	12.56	13.42	11.96	/
Total dCPO for production / μ mol	15	15	15	/
Encapsulation efficiency (dCPO) %	83.7%	89.5%	79.7%	84.3%
Encapsulated TA / μ mol	1.85	1.76	2.04	/
Total TA for production / μ mol	2.53	2.53	2.53	/
Encapsulation efficiency (TA) %	73.3%	69.4%	80.7%	74.5%
Total lipids for production / μ mol	33	33	33	/
Loading ratio (dCPO) mol/mol	0.38	0.34	0.36	0.36
Loading ratio (TA) mol/mol	0.056	0.053	0.062	0.057

Table.1 The loading ratio and encapsulation efficiency of dCPO and TA.

Furthermore, please provide the experimental evidence demonstrating the co-localisation of TA and dCPO within a single LNP to support the hypothesis, as well as their spatial arrangement.

Response: Thank you for this valuable comment. In response, we conducted experiments using high-resolution confocal fluorescence microscopy to investigate the co-localization and spatial arrangement of dCPO and TA within a single nanoparticle.

We prepared TA/dCPO LNPs using sulforhodamine B-labeled dCPO NPs and Cy5-labeled TA. Imaging of individual LNP revealed distinct fluorescent signals with

minimal overlap: the sulforhodamine signal (dCPO) was concentrated in a central, core-like region, while the Cy5 signal (TA) was distributed in a surrounding, shell-like area (Fig S9). This spatial segregation is consistent with the layered structure observed in cryo-TEM images, which show electron-dense cores enveloped by a lower-density, multilamellar lipid membrane. Together, these data support a structural model where the hydrophobic dCPO NPs reside within the core region, while the TA molecules are associated with the hydrophilic domains of the concentric lipid layers in the shell.

We have included these new confocal microscopy images alongside the cryo-EM data in the revised manuscript to provide direct experimental support for the proposed structure of the TA/dCPO LNPs.

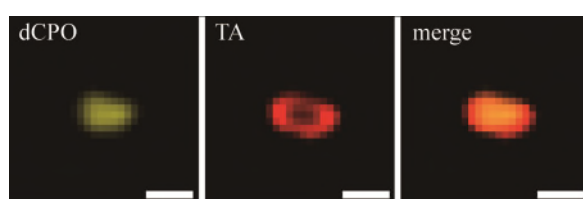


Fig.S9 High-resolution confocal fluorescence images of the double labeled TA/dCPO LNP (yellow - sulforhodamine B-labeled dCPO; red - Cy5-labeled TA). Scale bar: 500 nm.

3. The manuscript mentions that TA and dCPO are spatially segregated and compartmentalised. However, TA is expected to contact dCPO aggregates unless there is a coating or stabilising layer. Figure 1d shows dCPO aggregates, which were dispersed in aqueous buffer or organic solvent. Please clarify and provide supporting evidence.

Response: Thank you for your insightful comment. In this study, a single-chain phospholipid MAPK was strategically incorporated into the LNP formulation to prevent direct contact between dCPO and TA. The MAPK molecule acted as a stabilizing layer by preferentially interacting with the C12 alkyl chains (DOSS) modified on the surface of the dCPO nanoparticles. This interaction effectively coated the dCPO to create a hydrophobic shield, facilitating its integration into the lipid matrix and preventing uncontrolled interaction with TA.

To directly confirm this, a comparative TEM analysis was performed (Fig.S5). When dCPO was mixed directly with TA, TEM images showed dCPO cores coated with a uniform organic layer, indicating direct interaction. In contrast, when dCPO was first pre-coated with MAPK before adding TA, the resulting images showed only discrete dCPO aggregates without the surrounding organic layer. This visual evidence confirms that the MAPK layer effectively prevents contact between dCPO and TA, ensuring their

compartmentalization.

Furthermore, we acknowledge that the aggregates visible in Figure 1d were a transient artifact formed during the specific ethanol-based sample preparation for TEM. We have now updated this with images from samples prepared using dichloromethane, which reveal monodisperse dCPO particles without aggregation. these data were updated in the manuscript.

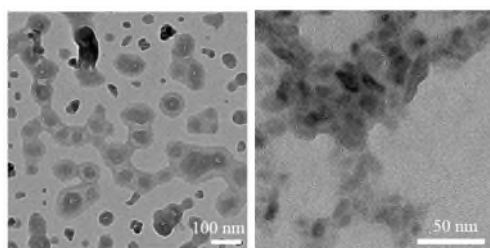


Fig.S5 TEM images of the mixture of dCPO +TA (left) and MAPK coated dCPO +TA (right).

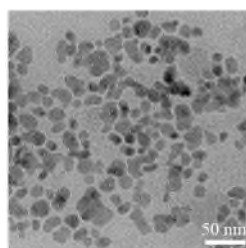


Fig.1 c) TEM images dCPO NPs.

4. The synthesis procedure in the supplementary information indicates that TA and dCPO were premixed in ethanol as the organic phase for microfluidic production. Given that TA and dCPO can interact to form GA, please provide evidence that no premature reaction occurs during the preparation or storage of the stock solutions.

Response: Thank you for the thoughtful comment. Due to the incorporation of the single-chain phospholipid MAPK as a stabilizer agent, direct interactions between TA and dCPO could be prevented in the organic phase mixture. During the premixing step in ethanol, MAPK was first combined with the dCPO nanoparticles to form a protective stabilizing layer around them before TA was introduced. This physical barrier effectively isolated dCPO from direct contact with TA in the organic solvent, thereby preventing premature radical generation or degradation. Furthermore, the organic phase mixture was prepared fresh for each experiment and used for microfluidic production within 10 minutes of preparation, minimizing any potential time-dependent interactions.

To experimentally validate this, the mixtures of dCPO + TA versus (MAPK + dCPO) + TA were applied to record the TA degradation over time (Fig.S4). The results clearly

showed a time-dependent degradation of TA (red shift) in the unprotected mixture, while the TA signature in the MAPK-stabilized mixture remained unchanged, confirming the absence of premature reaction.

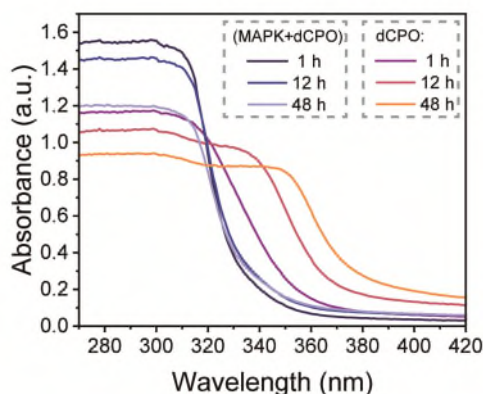


Fig. S4 TA degradation measurement over time when TA was mixed with dCPO or MAPK+dCPO.

Or, from my understanding, TA is more likely to be dissolved in the aqueous phase rather than the organic phase. Please clarify this point.

Response: Thank you for the insightful comment. We agree that TA would be favored by dissolving in an aqueous phase under this circumstance. Actually, during the formulation development, we initially explored this approach. However, we found that dissolving TA in the aqueous phase resulted in a significantly lower encapsulation efficiency within the LNPs (31.8%), which would undermine the therapeutic payload delivered to the target cells.

To achieve a therapeutically effective and stable nano-formulation with high drug loading, we therefore adopted the strategy of co-dissolving TA with the lipid components in the ethanol phase. This method substantially improved the encapsulation efficiency of TA into the LNPs during the microfluidic mixing process.

we also recognized that this approach created the potential for premature interaction with dCPO, as noted in your previous comment. This is precisely why we incorporated the specific MAPK agent. The combination of MAPK effectively mitigated the risk of premature reaction, allowing us to leverage the encapsulation without compromising the stability of the active components.

5. The authors propose that the nanoparticles undergo fusion with the cell membrane. However, nanoparticles larger than 100 nm are generally internalised through endocytosis, particularly when PEGylated. More direct experimental evidence is required to support membrane fusion as opposed to endocytosis.

Response: Thank you for the insightful comment. We agree that for LNPs larger than 100 nm and containing PEGylated lipids, endocytosis is indeed the expected cellular uptake pathway. In this study, we do not claim that membrane fusion becomes the primary mode of cellular entry for the developed LNP formulation. Instead, our hypothesis focused on the enhanced membrane interaction and facilitated content release enabled by the introduction of MAPK. Due to its conical shape and single alkyl chain, the presence of MAPK may induce structural defects within the LNP core and disrupt the tight packing of the lipid bilayer, thereby increasing membrane fluidity and reducing the stability of the lipid assembly.

These alterations are expected to induce local destabilization and heightened lipid exchange at the interface between the LNP and the cell membrane following initial endocytic uptake or contact. The perturbed membrane state is intended to accelerate the release of the encapsulated TA and dCPO into the cellular membrane environment, where the subsequent radical generation could occur more efficiently. Additional cryo-TEM images were provided to show the defected structures of LNPs (Fig. S11).

Despite the claim above, we acknowledge that enhanced membrane fusion capacity would substantially improve cytosolic delivery efficiency. Further investigations are on the way to enable more efficient membrane fusion-mediated internalization in subsequent formulations. In the revised manuscript, we have reframed the "fusion" to emphasize "enhanced membrane interaction and content release".

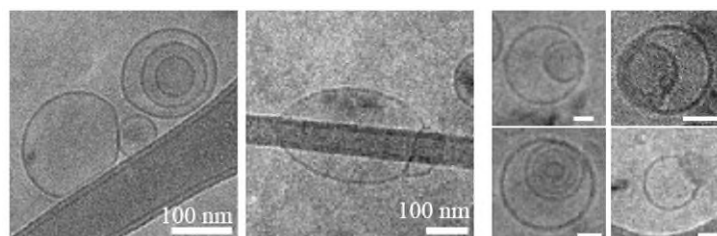


Fig.S11 Cryo-TEM images of dCPO/TA LNPs treated in PBS medium, showing oval or eccentric morphologies with structural defects. Scale bar: 50 nm (right).

In addition, the description of dCPO-mediated membrane fusion is confusing. In Figure 1g of the main manuscript, LNPs are shown, whereas in the Supplementary Information, synthetic cell membranes are used. Please clarify the rationale for testing dCPO with synthetic cell membranes in this context and explain how these experiments relate to the proposed mechanism of LNP-cell interactions. Experimental validation is needed to substantiate the membrane fusion hypothesis.

Response: Thank you for this valuable comment. We sincerely apologize for the confusion caused by the labeling in the original Figure 1g. The experiment was designed to probe the capability of dCPO-membrane interaction. To achieve this, we

utilized synthetic cell membranes (liposomes) labeled with Cy5.5. The label "LNP-Cy5.5" in the original figure was indeed misleading; it has been corrected to "Cy5.5-labeled synthetic cell membranes" in the revised manuscript to accurately reflect the experimental setup.

The rationale for using synthetic membranes was to establish an environment free from complex cellular machinery. This allows us to demonstrate the core physicochemical property of dCPO NPs, their ability to associate with and penetrate lipid bilayer structures. This evidence provides crucial preliminary support for the hypothesis that dCPO can facilitate membrane perturbation.

We agree that direct experimental validation of the interaction between TA/dCPO LNPs and live cell membranes will strengthen the proposed mechanism. We have visualized the processes of lipid exchange at the interface between the LNP and the cell membrane via confocal microscopy (Fig.S19). We sincerely thank the reviewer for highlighting this important point, which has prompted a more precise articulation of our observations.

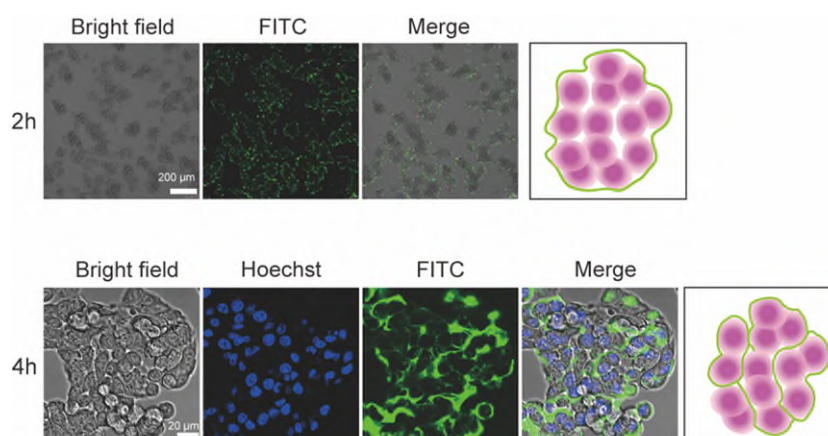


Fig.S19 The lipid exchange at the interface between the LNP and the cell membrane.

6. The LNPs described are plain PEGylated nanoparticles with no specific tumour-targeting ligands. Upon intravenous injection, such nanoparticles are expected to accumulate predominantly in the liver.

Response: Thank you for raising this point. In this study, the *ex vivo* organ imaging results (Fig. 6c) do confirm that the liver exhibits the highest fluorescence signal, which is consistent with the known biodistribution pattern of PEGylated nanoparticles. However, to demonstrate the pronounced retention of LNPs within the tumor, Fig. 6b was acquired with the mouse in a dorsal position, which optimally visualizes the subcutaneous tumor located on the mouse's back. Actually, we also captured images in the ventral position, which more clearly reveals the liver signal and thus partly obscures

the tumor signal, as shown in the Fig.R2 below.

Therefore, the *in vivo* and *ex vivo* data are consistent: while significant hepatic accumulation occurred, the LNPs also demonstrated a pronounced and sustained retention within the tumor, which is crucial for the observed therapeutic effect.

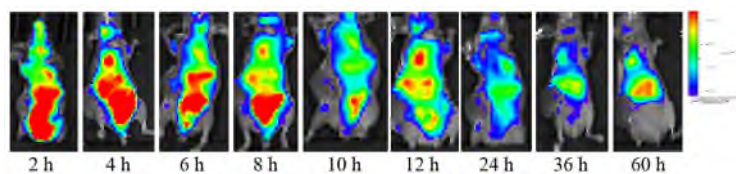


Fig.R2 IVIS imaging of tumor-bearing mice (ventral position) at different time point after the TA/dCPO LNPs injection.

Data on nanoparticle stability, circulation behaviour, and release kinetics of dCPO and TA are needed.

Response: Thank you for your valuable suggestions. As Fig.S10 illustrated, the stability of LNPs in physiological saline was monitored by DLS over 15 days, showing no significant change in particle size, indicative of good dispersibility and colloidal stability. Their stability in FBS-containing buffer was also assessed over 4 h, representing the typical blood circulation time before tissue accumulation. Compared to saline (120 nm), the hydrodynamic diameter decreased to 90 nm in FBS, likely due to transient protein adsorption forming a dynamic “soft corona” that compresses the PEG layer [8]. The slightly increased PDI reflects heterogeneity from ongoing protein–LNP adsorption/desorption equilibration. Overall, LNPs remained colloidally stable in serum for at least 4 h, with minimal size change and no aggregation.

The blood circulation half-life of the TA/dCPO LNPs was determined to be approximately 18.3 h (Fig.S40), which provides a favorable timeframe for tumor accumulation. Furthermore, as suggested, we performed *in vitro* release studies of both dCPO and TA in a simulated physiological buffer (containing 0.5% Tween 80). The results (Fig.R3) showed a limited release, with less than 20% of either component detected in the supernatant over 140 hours. We claim that the low cumulative release could not be interpreted as an indication of poor payload delivery *in vivo*, but rather as a limitation of the static *in vitro* model. The simplified buffering system cannot replicate the dynamic and complex biological triggers, such as the membrane interaction, cellular uptake, and subsequent endolysosomal processing. These biological processes are expected to facilitate the localized and responsive release of the active components within the target cellular environment, which cannot be mimicked by passive diffusion in conventional *in vitro* release assays.

References:

8. Berger, M., Degey, M., Leblond Chain, J., Maquoui, E., Evrard, B., Lechanteur, A., & Piel, G. (2023). Effect of PEG anchor and serum on lipid nanoparticles: development of a nanoparticles tracking method. *Pharmaceutics*, 15(2), 597.

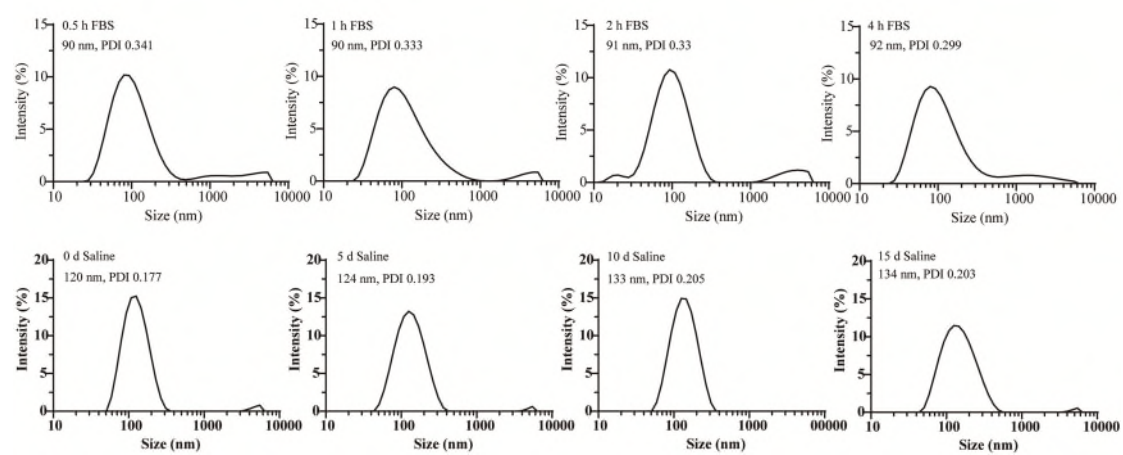


Fig.S10 Size distribution of the TA/dCPO LNP in physiological saline over a 15-day period by DLS (down), and in FBS-containing physiological fluids within 4 h (up), a duration representative of typical systemic circulation time for nanoparticles prior to tissue accumulation.

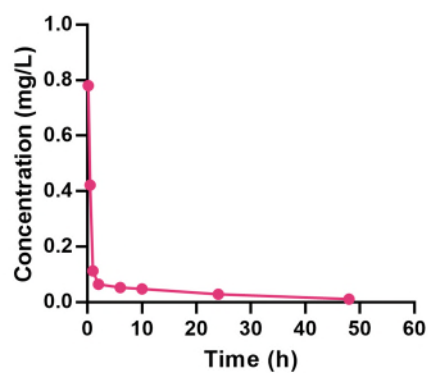


Fig.S40 Blood half-life of TA/dCPO LNPs in tumor-bearing mice based on Ca levels measured by ICP-OES.

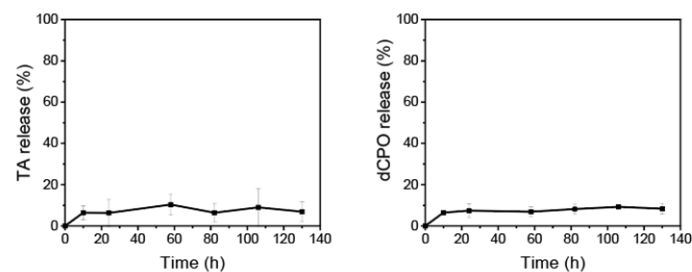


Fig.R3 *in vitro* release studies of TA and dCPO NPs in a simulated physiological buffer (containing 0.5% Tween 80).

Do the particles prematurely release radicals in normal tissues, particularly in healthy liver cells? If so, how is systemic toxicity mitigated? Please also provide the immunostaining picture of healthy normal liver tissues.

Response: We thank the reviewer for raising this critical point. Our system mediates targeted cytotoxicity in a GPC3-dependent manner. Since normal hepatocytes completely lack GPC3 expression [10], the molecular trigger required for GA• radical binding and subsequent nanosystem activation is absent—thereby precluding cytotoxic or oxidative damage in these cells. Furthermore, normal cells exhibit significantly higher catalase activity than tumor cells [11,12]; this endogenous antioxidant enzyme efficiently scavenges GA• radicals and mitigates oxidative stress. Consistent with this mechanistic rationale, our experiments in the non-tumorigenic murine hepatocyte line AML12 revealed no significant cytotoxicity or oxidative damage following TA/dCPO LNP treatment (Fig. S33).

To validate the safety in normal liver, mouse liver tissues were harvested and subjected to H&E staining after intravenous administration of TA/dCPO LNPs (Ca: 4.5 mg/kg), Histopathological evaluation revealed no discernible abnormalities in hepatic lobular architecture, hepatocyte morphology, or the structural integrity of hepatic sinusoids and bile ducts (Fig. S46). This result is consistent with the *in vitro* observation that it lacks significant cytotoxicity in normal liver cells, jointly supporting its good safety for normal liver tissue.

Reference:

10. Dhungel, Bijay et al. "Evaluation of the Glypican 3 promoter for transcriptional targeting of hepatocellular carcinoma." *Gene therapy* vol. 25,2 (2018): 115-128.
11. Cecerska-Heryć, E., Surowska, O., Heryć, R., Serwin, N., Napiontek-Balińska, S., & Dołęgowska, B. (2021). Are antioxidant enzymes essential markers in the diagnosis and monitoring of cancer patients—a review. *Clinical biochemistry*, 93, 1-8.
12. Asaduzzaman Khan, M., Tania, M., Zhang, D. Z., & Chen, H. C. (2010). Antioxidant enzymes and cancer. *Chinese Journal of Cancer Research*, 22(2), 87-92.

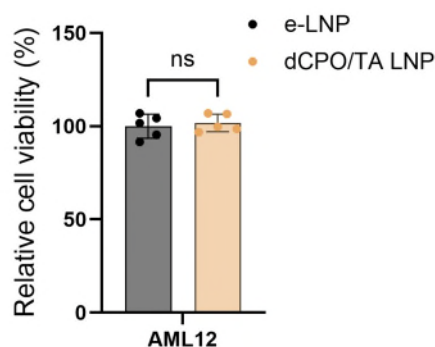


Fig.S33 Cell viability of AML12 cells after incubation with empty LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, $P \geq 0.05$).

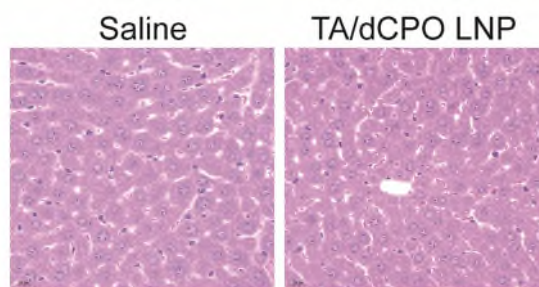


Fig.S46 HE staining of normal liver tissue after treated with saline and TA/dCPO LNP.

7. For the claimed GPC-3 targeting, comparative studies with GPC-3–positive versus GPC-3–negative cells are required to quantify uptake and radical release. Antibody blocking experiments could further confirm whether GPC-3 binding drives the observed targeting.

Response: Thank you for raising this important point. To address this, we systematically evaluated the efficacy of TA/dCPO LNPs across a panel of hepatocellular carcinoma cell lines with differential basal expression of membrane GPC-3. In cell lines with high GPC-3 membrane expression (HepG2, Hep3B and Huh7), TA/dCPO LNPs elicited potent anti-proliferative and cytotoxic effects. In contrast, in cell lines with low endogenous GPC-3 expression (HLF and JHH4), the nanosystem showed significantly attenuated activity, with no substantial cytotoxicity or reduction in colony formation ability (Fig.5d-g).

Furthermore, we employed genetic gain- and loss-of-function strategies. Using CRISPR/Cas9, we generated GPC-3 knockout Hep3B cells. In the absence of GPC-3, TA/dCPO LNPs completely lost their anti-proliferative efficacy, confirming that GPC-3 is indispensable for the observed therapeutic effect (Fig.5h-l). Conversely, we established stable GPC-3-overexpressing variants of the low-expression JHH4 and HLF cell lines. In these engineered models, TA/dCPO LNPs significantly restored potent anti-tumor activity specifically in the GPC-3-overexpressing populations, while the empty vector controls remained insensitive (Fig.5m-s). This functional rescue experiment provides direct evidence that GPC-3 expression is a critical mechanistic determinant for the radical mediated therapy.

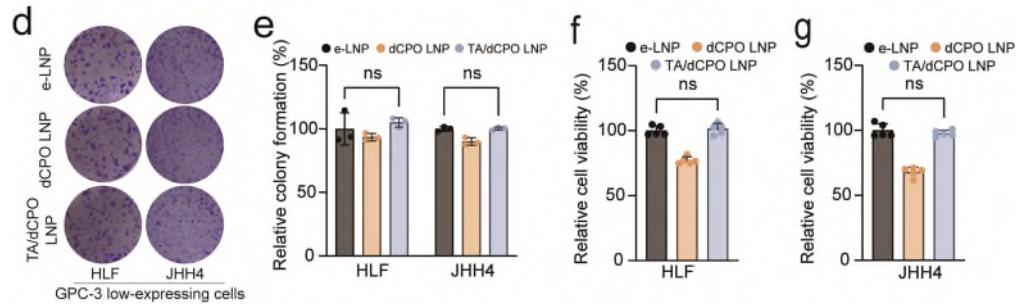


Fig. 5 d,e) Clone assay and quantitative analysis of clone numbers in HLF and JHH4 cells following with varied treatment. Data are expressed as mean $\pm$ SD (n = 3, ns, P $\geq$ 0.05). f,g) Cell viability of HLF and JHH4 cells after incubation with e-LNP, dCPO LNP or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

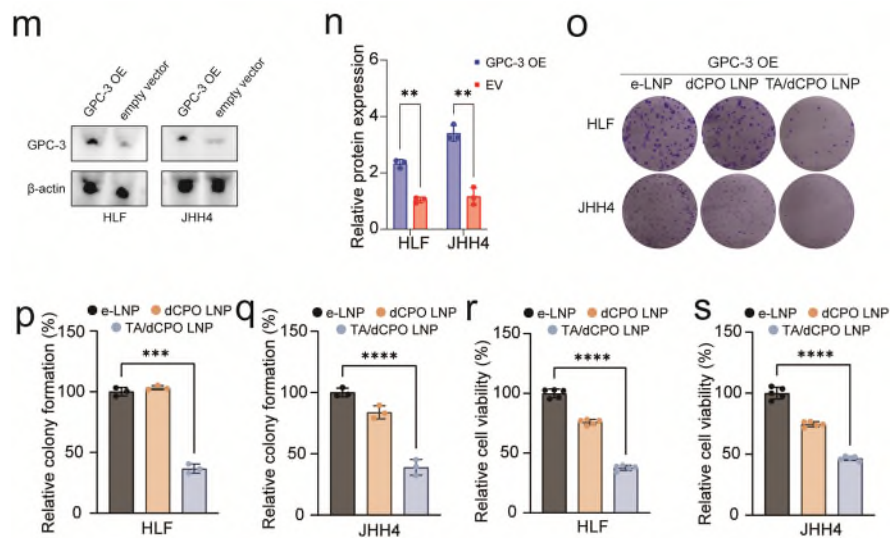


Fig. 5 m,n) Immunoblot analysis of GPC-3 protein level in HLF and JHH4 cells following GPC-3 overexpression. Data are expressed as mean $\pm$ SD (n = 3, **P < 0.01). o-q) Clone assay and quantitative analysis of clone numbers in HLF and JHH4 cells after various LNP incubation following GPC-3 overexpression. Data are expressed as mean $\pm$ SD (n = 3, ***p < 0.001, ****P < 0.0001). r,s) Cell viability of HLF and JHH4 cells with GPC-3 overexpression following incubation with e-LNPs, dCPO LNPs or TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 5, ****P < 0.0001).

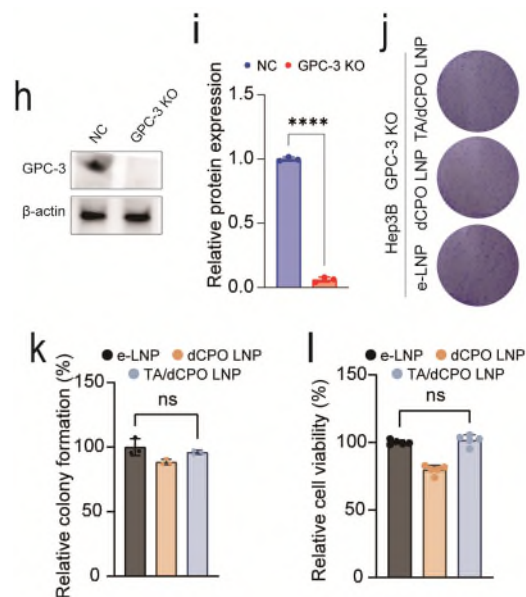


Fig. 5 h,i) immunoblot analysis of GPC-3 protein level in Hep3B cells following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ****P<0.0001). j,k) Clone assay and quantitative analysis of clone numbers in Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 3, ns, P $\geq$ 0.05). l) Cell viability of Hep3B cell after various LNP incubation following GPC-3 knockout. Data are expressed as mean $\pm$ SD (n = 5, ns, P $\geq$ 0.05).

8. The cellular studies only compared e-LNPs and TA/dCPO LNPs, without demonstrating the importance of co-loading TA and dCPO. It is recommended to include comparative data for e-LNPs, dCPO-LNPs, and TA/dCPO LNPs to establish a logical stepwise evaluation of formulations. In vivo studies included multiple groups (e-LNPs, TA LNPs, dCPO LNPs, and TA/dCPO LNPs), but the corresponding in vitro comparisons were not presented. Please ensure consistency between in vitro and in vivo studies or clearly explain the rationale.

Response: Thank you for this valuable suggestion. We have now included comprehensive *in vitro* data comparing the effects of e-LNPs, dCPO-LNPs and TA/dCPO LNPs across relevant cellular assays. These results have been integrated to the revised manuscript (Fig.5) and its supplementary information (Fig.S32), suggesting that the combination of TA and dCPO within the LNP is essential for generating the therapeutic phenoxy radical species and achieving efficient efficacy.

9. Figure 6g lacks sufficient description in the legend.

Response: We thank the reviewer for noting this. The figure legend has been comprehensively supplied in the revised manuscript.

Biodistribution data should be presented with actual quantitative signal values, not only colour scale bars with the control group.

Response: We sincerely thank the reviewer for this comment. The actual signal quantification values of Fig.6b were showed in Fig.6d, and the numerical values of Fig.6c was further detailed in Fig.S39. This adjustment addressed the concern regarding reliance on qualitative color scales alone.

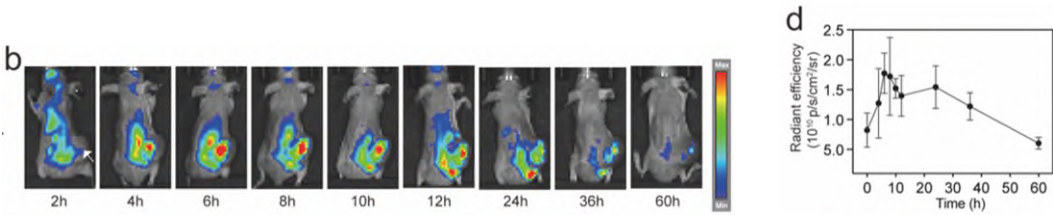


Fig.6 b) IVIS imaging of tumor-bearing mice at different time point after the TA/dCPO LNPs injection. d) Quantification analysis of the intensity in tumor region at different time points (n=3, biologically independent mice per group).

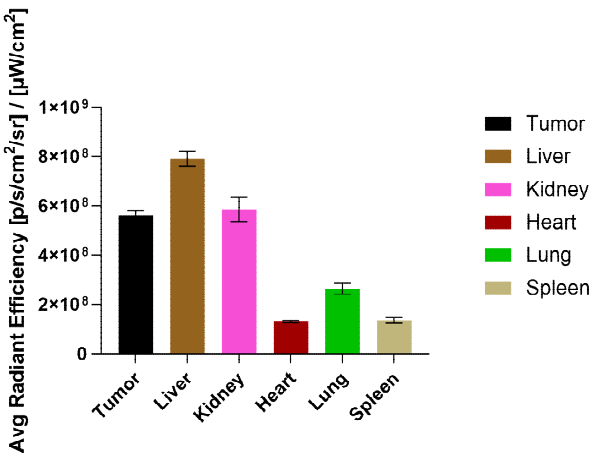


Fig.S39 Quantification of fluorescence signals from major organs harvested 8 hours after intravenous injection of TA/dCPO LNPs.

Figures 6b and 6c present only one group, whereas Figure 6f shows all treatment groups, leading to inconsistency. Please include images for all groups, with each mouse represented individually, in the Supplementary Information.

Response: Thank you for your comment. We sincerely apologize for the unclear description and appreciate the opportunity to clarify the distinct experimental purposes behind Figures 6b-c and Figure 6f. Figure 6b (*in vivo* fluorescence imaging) and 6c (*ex vivo* organ imaging) were designed to track the spatial and temporal biodistribution of the fluorescently labeled TA/dCPO LNPs following intravenous administration. The purpose of this experiment was to visualize the journey of the LNPs through the body

over time and its final organ accumulation. Therefore, including other formulated control groups for biodistribution was not relevant to our specific aim.

In contrast, Figure 6f presented the final therapeutic outcome, showing *ex vivo* tumors harvested from all treatment groups at the endpoint of the study. This experiment utilized a tumor model without fluorescence reporting, and tumor sizes were assessed by direct caliper measurement. The purpose here was to compare the anti-tumor efficacy across the various therapeutic formulations.

Thus, the two sets of figures report on separate experiments with different goals: one focused on the fate of the nanoparticle itself, and the other on the therapeutic effect. We hope this explanation resolves the apparent inconsistency.

Add and standardise scale bars in Figure 6l.

Response: Thank you for your kind suggestions. We have added scale bars in Figure 6l.

10. The summary and discussion are overly brief. The debate should be expanded to provide a more comprehensive and insightful treatment of the work, clearly articulating its broader significance, limitations, and potential clinical relevance.

Response: Thank you for your kind suggestions. We have made corresponding revisions to the Summary and Discussion sections.

The writing quality also requires improvement, particularly in the Supplementary Information. Several procedures are described inaccurately or ambiguously—for example, “Mice were injected with TA/dCPO LNPs (Ca: 4.5 mg kg⁻¹) by tail vein twice,” which lacks essential details (e.g., dosing schedule, number of animals) and shows inconsistencies in verb tense/usage relative to the main text. Methodological detail is uneven, with some sections overly specific and others cursory. The authors should thoroughly revise the whole manuscript, including supplementary information, for accuracy, internal consistency, and reproducibility.

Response: Thank you for your valuable comments. We have undertaken a thorough revision of the entire manuscript, including the Supplementary Information, with a focus on accuracy, internal consistency, and providing sufficient detail for experimental replication. Specifically regarding the example you highlighted, the sentence has been corrected to provide complete dosing information: “Healthy mice (n=5) were

administered with TA/dCPO LNPs (Ca: 4.5 mg/kg) by tail vein injection twice (on day 0 and 2)"

Beyond this specific correction, we have systematically reviewed all methodological descriptions. We have standardized verb tenses, ensured uniform formatting, and carefully balanced the level of detail, by expanding cursory sections and streamlining overly specific ones where appropriate.

We believe these comprehensive edits have significantly improved the manuscript's readability and rigor, and we are grateful for your feedback, which was essential to this improvement.

RESPONSE TO REFEREES' COMMENTS

Submission ID: NCOMMS-25-53490A

Title: Generation of GPC-3-Targeted Radicals for Precise Therapy of Hepatocellular Carcinoma

Authors: Wanting Hao, Meng Zhang, Zi Fu, Cui Xinyuan, Hongjing Jiang, Ruokun Li, Fuhua Yan, Dalong Ni

We would like to express our sincere gratitude to the reviewers for their helpful and constructive suggestions, which we believe have greatly improved the revised version. We have taken careful note of their comments and suggestions, carried out further experiments, and revised our manuscript accordingly.

A point-by-point response to all reviewers' comments is given in blue typescript alongside their comments below. We have highlighted the changes in our revised manuscript in blue typescript.

Reviewer #1:

The rebuttal provided by Hao et al. is profoundly inadequate, particularly regarding my previous comments #6 and #7. The authors have failed to engage with the core scientific concerns raised; instead, they have provided data that lack transparency and mechanistic explanations that are fundamentally inconsistent. These unresolved issues, combined with serious concerns regarding the integrity of the newly presented data, render the manuscript unsuitable for publication in Nature Communications.

Response: Thank you for acknowledging our responses.

1. Regarding the rebuttal to my previous Comment #6, the Western blot analysis in Figure S37 is highly problematic and scientifically unacceptable. The authors have failed to include essential nuclear markers (e.g., Lamin B1 or Histone H3) to verify the purity of the nuclear fraction. More critically, the beta-actin bands in the nuclear extract appear suspiciously identical to those in the cytoplasmic fraction, yet there is a striking absence of expected cross-contamination markers. This lack of transparency and the inconsistent appearance of the blots raise grave concerns regarding the authenticity and integrity of the data. In the absence of rigorous, verifiable, and raw blot images using appropriate internal controls for each fraction, the claim of nuclear translocation cannot be validated.

Response: We sincerely thank the reviewer for their meticulous examination of our data and for bringing this critical issue to our attention. Upon careful re-evaluation of our experimental records and previous response, we realize that a misunderstanding occurred in our interpretation of the requested analysis. We apologize for any confusion this may have caused.

In the original experiment presented in Fig. S37, the samples analyzed were total protein lysates (whole-cell extracts) probed with an anti- β -catenin antibody as suggested. Therefore, these blots do not represent fractionated nuclear or cytoplasmic

samples. We regret this inaccuracy and any resulting concern regarding data interpretation. We wish to clarify, however, that the presented Western blot data remain authentic and were acquired with appropriate loading controls.

We fully agree with you that nuclear β -catenin levels are a more direct and reliable indicator of its transcriptional activity and nuclear translocation. To address this point rigorously, we have now performed nuclear-cytoplasmic fractionation and assessed nuclear β -catenin expression with appropriate fraction-purity controls, including Lamin B1 as a nuclear marker and GAPDH as a cytoplasmic marker. As shown in the revised Fig. S37, TA/dCPO LNP treatment induced a concentration-dependent decrease in nuclear β -catenin protein, which confirms the impairment of its nuclear translocation.

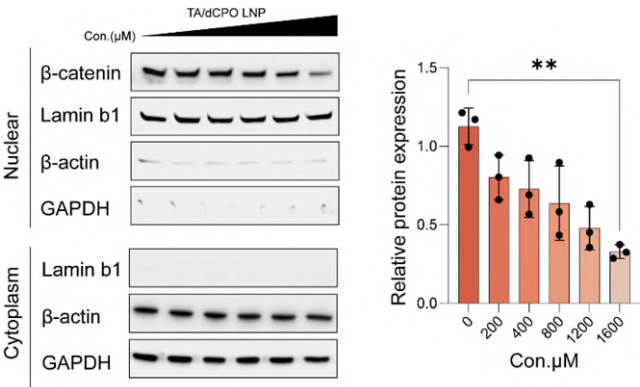


Fig.S37 Western blot analysis of nuclear β -catenin expression in Hep3B cells following treatment with various concentrations of TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 3, **p < 0.01)

We deeply appreciate the reviewer's rigorous scrutiny, which has helped us identify and correct this oversight, ultimately strengthening the scientific rigor of our work.

2. Furthermore, in the second part of their response to Comment #6, the authors' explanation regarding the beta-catenin pathway in HepG2 cells remains fundamentally inconsistent and dismissive of the original critique. While acknowledging that HepG2 cells harbor a CTNNB1 mutation (deletion of exon 3) leading to constitutive activation, the authors fail to provide a convincing mechanism for how their treatment overrides this genetic driver. The cited work by Li et al. (2020) actually underscores the necessity for direct evidence; yet, the authors have still not performed the requested Western blot analysis of total and nuclear beta-catenin in HepG2 cells specifically treated with TA/dCPO LNPs. Furthermore, the suggestion that GPC3 might act through alternative pathways (FGF, HGF/c-Met, or TGF-beta) is purely speculative and lacks any direct experimental evidence in the present study.

Response: Thank you for this rigorous and constructive comment. We fully agree with the reviewer that citing a previous report does not substitute for providing direct experimental evidence from our own system. We also accept the reviewer's point that, from a theoretical standpoint, the CTNNB1 exon 3 deletion in HepG2 cells should render β -catenin constitutively active and largely resistant to upstream regulation. The constitutive activation of β -catenin in HepG2 cells should theoretically make it resistant

to upstream regulation.

To address this issue directly, we have now performed the requested Western blot analysis of total and nuclear β -catenin in HepG2 cells treated with TA/dCPO LNPs. The results show that treatment with TA/dCPO LNPs leads to a partial reduction in nuclear β -catenin levels, while total β -catenin remains largely unchanged (Figure R1). This partial suppression is consistent with the constitutive activation caused by the CTNNB1 exon 3 deletion, which renders β -catenin resistant to GSK3 β -mediated degradation. Of note, this partial reduction demonstrates that TA/dCPO LNPs can still modulate β -catenin downstream of the degradation machinery. The precise mechanism (e.g., effects on nuclear translocation, retention, or TCF/LEF interaction) remains to be determined and is beyond the scope of the present study. Importantly, the anti-proliferative effect in HepG2 cells is likely achieved through a combination of this partial Wnt/ β -catenin modulation and the GPC-3-dependent AKT pathway inhibition supported by our experimental data (Figure R2). It is therefore also possible that the observed anti-proliferative effect in HepG2 cells results from a combination of partial Wnt modulation and other GPC-3-dependent but Wnt-independent pathways. Importantly, none of these mechanistic nuances alter the main conclusion of our study, which is that TA/dCPO LNPs exert antitumor effects through targeted GPC-3 radical-mediated disruption, leading to functional inhibition of the protein and suppression of downstream oncogenic signaling.

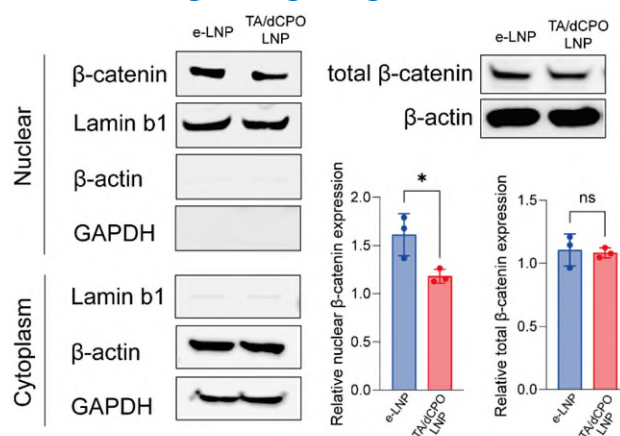


Fig.R1 Western blot analysis of total and nuclear β -catenin expression in HepG2 cells following treatment with e-LNP and TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 3, *p < 0.05)

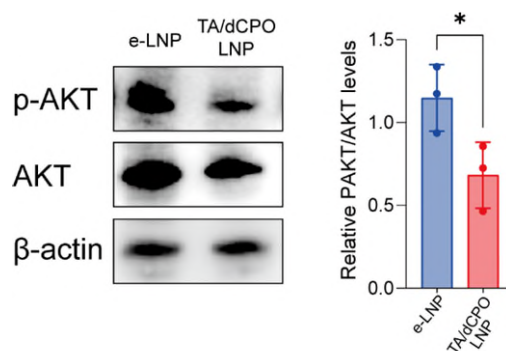


Fig.R2 Western blot analysis of pAKT/AKT expression in HepG2 cells following treatment with e-LNP and TA/dCPO LNPs. Data are expressed as mean $\pm$ SD (n = 3, *p < 0.05)

We sincerely thank the reviewer for prompting us to examine this issue more carefully.

3. The rebuttal to Comment #7 regarding IVIS imaging orientation (dorsal vs. ventral) does not sufficiently address the original concern. Given the high hepatic accumulation characteristic of LNPs, a strong liver signal is expected regardless of positioning, as demonstrated in the authors' own response figure (Fig. R1). The near-total absence of liver signal at 36 hours in the manuscript figure, contrasted with the lack of tumor signal in Fig. R1 at the same time point, strongly indicates inconsistent imaging parameters or selective data reporting. Without a side-by-side, quantitative comparison under identical acquisition settings, the claim of "sustained tumor retention" remains unsubstantiated and misleading.

Response: Thank you for your thoughtful comment. We sincerely appreciate the opportunity to clarify this issue. We confirm that all IVIS imaging (both dorsal and ventral) was performed using identical acquisition settings, including exposure time, binning, f/stop, and excitation/emission filters. The raw images were presented side-by-side to demonstrate the normalized fluorescence signals across time points clearly (Figure R3).

A key factor to consider is the limited tissue penetration depth of fluorescence imaging. For a subcutaneous tumor located on the back, the dorsal orientation places the tumor directly facing the camera with minimal overlying tissue, allowing efficient detection of its fluorescence signal. In contrast, the ventral orientation requires the emitted light to traverse multiple tissues before reaching the detector. Consequently, while the ventral view clearly captures the strong hepatic signal expected for LNPs (as shown in our previous Fig. R1 and the raw data), the same orientation significantly attenuates the tumor signal. Conversely, in the dorsal view, the liver lies deep within the abdomen and its signal is heavily obscured by overlying tissues, which explains why it appears nearly absent in Fig. 6b. This is not a result of selective reporting but a physical consequence of imaging geometry.

Regarding the 36-h time point specifically, we hypothesize that LNPs were predominantly cleared from the liver at this stage, resulting in relatively low residual fluorescence signal in hepatic tissue. In the ventral images at 36h (and 60h), the fluorescence observed in abdominal region of whole-animal images may represent artifact signals, potentially originating from autofluorescence of residual gastrointestinal contents, a common artifact encountered in *in vivo* imaging studies.

We hope this detailed explanation, supported by raw data and quantitative analysis, convincingly demonstrates that our conclusion of sustained tumor retention is well-founded and not misleading. Thank you again for pushing us to improve the rigor of our analysis.

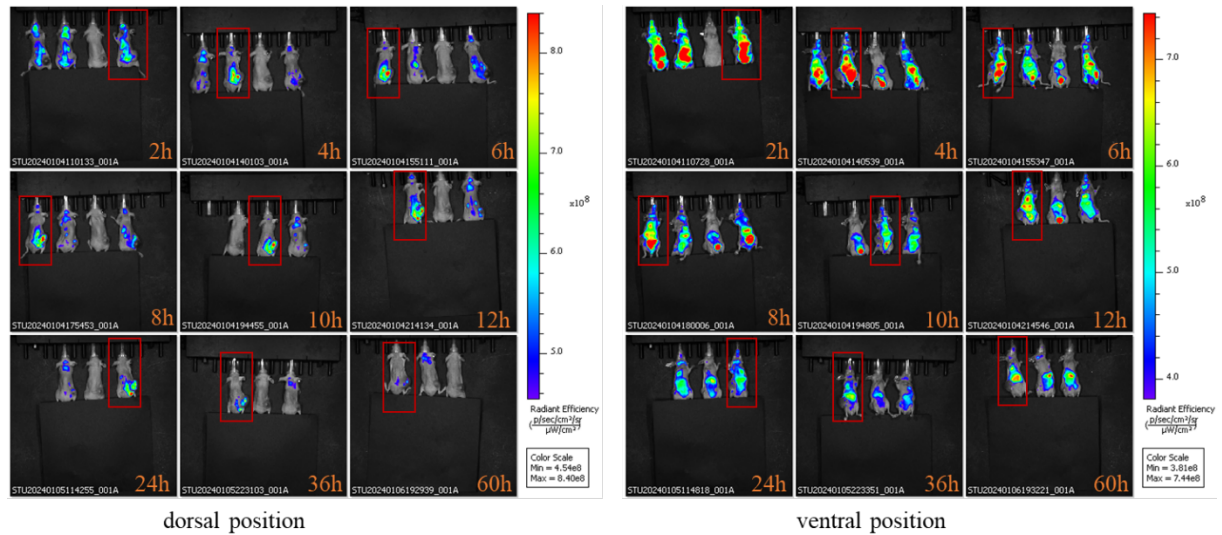


Figure R3: Raw images from the normalized time-course fluorescence intensity analysis at different anatomical positions. Left: dorsal view; right: ventral view. The exact imaging time is indicated in the filename (bottom-left corner of each image). Red boxes highlight representative images corresponding to Figure R4 at various time points. We note that during IVIS imaging at different time points, the mice were not positioned in the same order; individual mice were identified using ear tags. At the 8-hour time point, one mouse was randomly selected for euthanasia and subsequent *ex vivo* imaging of major organs.

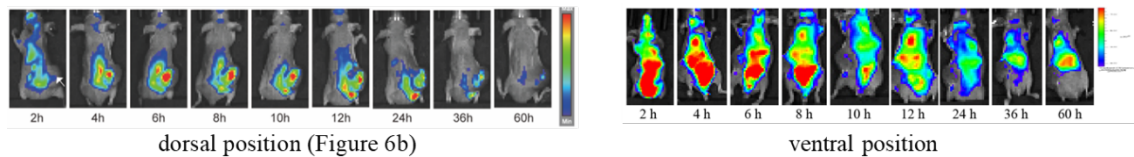


Figure R4: representative IVIS images of tumor-bearing mice at various time points. Left: dorsal view (Figure 6b); right: ventral view (Figure R5).

Reviewer #2:

Authors have adequately addressed my concerns, thanks for making the revisions.

Response: Thank you very much for your positive feedback.

Reviewer #3:

The authors have addressed most of the concerns.

Response: Thank you very much for your positive assessment of our responses.

Reviewer #4:

The authors have addressed majority of my questions, however, a few remain:

Response: Thank you very much for your positive evaluation and constructive suggestion.

(1) Drug loading is often defined as the mass % of drug by the total weight of the drug-loaded NPs. Please provide the drug loading.

Response: We appreciate the reviewer's valuable comment regarding the reporting of drug loading. The drug loading ratios have been recalculated using the formula:

Loading ratio = encapsulated payload / (total lipid + encapsulated payload) × 100% (w/w)

The revised calculations show that the dCPO loading ratio is 3.68% (w/w), and the TA loading ratio is 12.93% (w/w). These quantitative values have been incorporated into Table 1 of the revised manuscript.

Samples	1	2	3	Average
Encapsulated dCPO / μ mol	12.56	13.42	11.96	/
Total dCPO for production / μ mol	15	15	15	/
Encapsulation efficiency (dCPO) %	83.7%	89.5%	79.7%	84.3%
Encapsulated TA / μ mol	1.85	1.76	2.04	/
Total TA for production / μ mol	2.53	2.53	2.53	/
Encapsulation efficiency (TA) %	73.3%	69.4%	80.7%	74.5%
Total lipids for production / μ mol	33	33	33	/
Loading ratio (dCPO) (% , w/w)	3.66	3.92	3.45	3.68
Loading ratio (TA) (% , w/w)	12.73	12.16	13.89	12.93

Table.1 The loading ratio and encapsulation efficiency of dCPO and TA.

(2) Fig. S10, size distribution in FBS shows multiple peaks and big PDI (>0.3) indicating aggregation. This means the TA/dCPO LNPs are not stable upon injection in the presence of proteins in serum.

Response: We thank the reviewer for this insightful observation. We acknowledge that the presence of multiple peaks and big PDI value indeed indicates aggregation and reduced colloidal stability in protein-rich environments. While the current LNP formulation was specifically optimized for efficient encapsulation of dCPO and TA payloads, comprehensive optimization of LNP-protein interactions and protein corona formation was not the primary focus of this study. The observed aggregation behavior in serum highlights an important area for further development. This limitation has been explicitly acknowledged in the discussion section of the revised manuscript, and ongoing investigations are being conducted to engineer LNPs with enhanced serum stability through surface modification strategies and formulation refinements. We appreciate this valuable feedback which will guide our future research directions.

(3) Fig.S40, it seems that the blood half life of TA/dCPO LNPs is so short. How does it compare with free drugs? What is the $t_{1/2}$?

Response: We thank the reviewer for raising this important question. Based on the newly acquired pharmacokinetic data collected over a 24-hour period, the blood

circulation profile was found to follow a two-compartment model, with the initial distribution phase half-life ($t_{1/2}(\alpha)$) determined to be 0.68 h and the terminal elimination phase half-life ($t_{1/2}(\beta)$) determined to be approximately 18 h. These values have been updated in the revised manuscript (Fig. S40).

Direct comparison with free dCPO was not feasible, as the hydrophobic nature of dCPO NPs disqualifies its intravenous administration without a delivery vehicle. However, to provide context for the observed circulation kinetics, literature values for similar nanosystems were referenced: $\text{CaO}_2@\text{MnO}_2$ -PEG nanoparticles exhibited $t_{1/2}(\alpha)$ of 2.48 ± 1.0 h and $t_{1/2}(\beta)$ of 8.6 ± 2.7 h, while PGFCaCO_3 -PEG nanoparticles showed $t_{1/2}(\alpha)$ of 2.65 ± 1.71 h and $t_{1/2}(\beta)$ of 35.3 ± 5.80 h [1,2].

The relatively shorter initial half-life of TA/dCPO LNPs could be explained by the colloidal stability limitations observed in serum-containing environments (as discussed in Comment 2), where protein adsorption and aggregation likely accelerate clearance [3]. This limitation has been acknowledged in the revised manuscript (Discussion section), and efforts to enhance serum stability through surface engineering are currently underway to improve circulation persistence in future iterations.

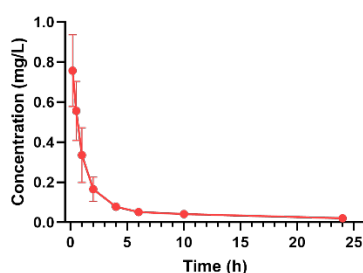


Fig.S40 Blood half-life of TA/dCPO LNPs in tumor-bearing mice based on Ca levels measured by ICP-OES.

Reference:

1. Li W, Wang C, Zhu Y, et al. MnO_2 -coated CaO_2 nanoparticles as pH-responsive oxygen self-sufficient carriers to potentiate radioimmunotherapy[J]. *Chemical Engineering Journal*, 2025: 171501.
2. Han Y, Dong Z, Wang C, et al. Ferrous ions doped calcium carbonate nanoparticles potentiate chemotherapy by inducing ferroptosis[J]. *Journal of Controlled Release*, 2022, 348: 346-356.
3. Lai K, He P, He Y, et al. Pre-intervention with intravenous immunoglobulin reverses the immunogenic clearance of PEGylated nanomedicines[J]. *Nature Biomedical Engineering*, 2026: 1-19.

(4) It is still unclear whether the proposed core-shell localization can be confidently resolved at this spatial scale. The authors may consider clarifying this limitation and moderating the structural interpretation accordingly.

Response: We appreciate the reviewer's thoughtful comment regarding the spatial resolution limitations in confirming the core-shell architecture of TA/dCPO LNPs. Given this important consideration, high-resolution confocal fluorescence microscopy

was performed using intentionally enlarged LNP particles (approximately 500 nm in diameter) to facilitate clearer visualization of the internal structural arrangement (Fig.S9). While the observed spatial segregation of fluorescent signals provided preliminary support for the proposed structural model, we fully acknowledge that the inherent resolution constraints of optical microscopy preclude definitive confirmation of the core-shell localization at the nanoscale. This limitation has been explicitly clarified in the revised manuscript (Discussion section), where the interpretation of structural arrangement has been moderated accordingly.

We agree that comprehensive structural validation would benefit from complementary techniques such as super-resolution microscopy, small-angle X-ray scattering, or advanced cryo-electron tomography, which are currently being planned for future investigations to provide more rigorous evidence of the LNP architecture.

(5) The manuscript emphasizes reduced off-target radical activity in normal tissues. Additional clarification of this claim in the discussion would further improve rigor.

Response: We thank the reviewer for this constructive suggestion. The discussion section has been expanded to provide a more comprehensive mechanistic rationale for the observed selectivity. This clarification strengthens the manuscript's argument for the favorable safety profile of our nanosystem while maintaining scientific rigor.

Reviewer #5:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you very much for your positive evaluation and constructive suggestion.

REVIEWERS' COMMENTS

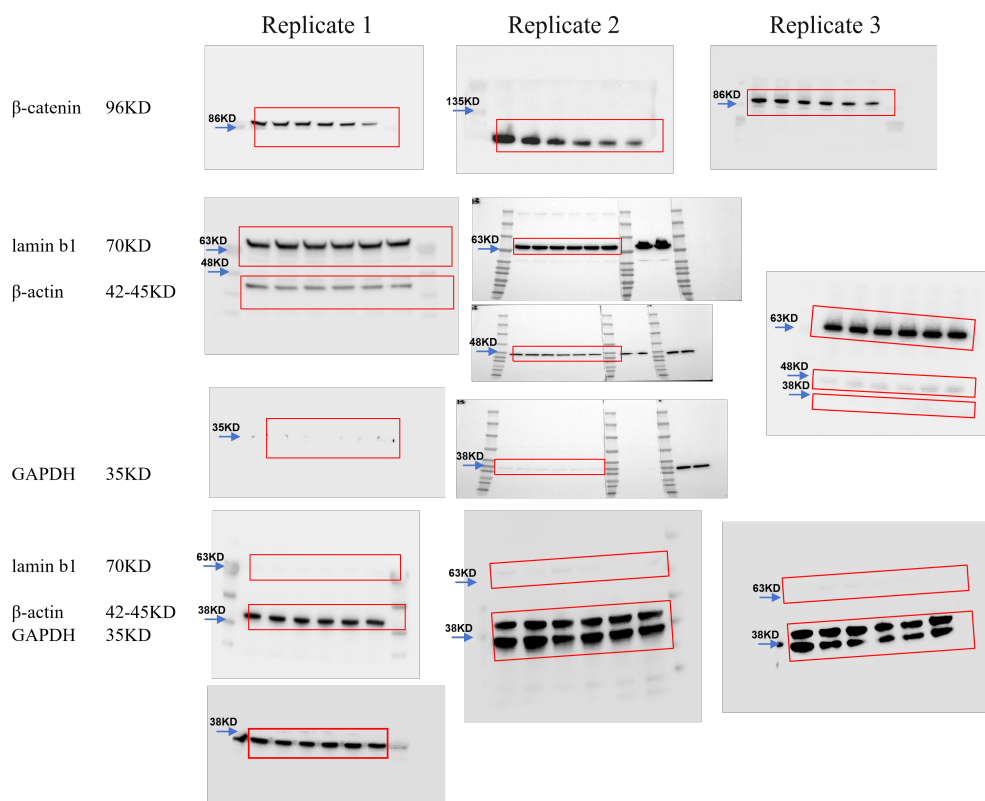
Reviewer #1 (Remarks to the Author):

After carefully evaluating the latest rebuttal and newly provided data, I appreciate that the authors made substantial efforts to address the previous concerns. However, the most serious concern relates to the response to Comment #6. The authors now acknowledge that the originally presented Figure S37 was not derived from nuclear/cytoplasmic fractionation as described, but instead from whole-cell lysates. This inconsistency raises critical concerns regarding data handling and figure presentation rigor. Moreover, the absence of raw uncropped data (with markers) for all presented images prevents full restoration of confidence. For these reasons, I cannot recommend publication in Nature Communications.

Response: We sincerely thank the reviewer for their careful evaluation and for acknowledging our efforts.

In the previous round of revision, we addressed the methodological concern regarding Figure S37 by performing the nuclear/cytoplasmic fractionation experiment de novo, followed by rigorous validation of fraction purity using compartment-specific markers. The results obtained from the correctly executed experiment were presented in the revised Figure S37.

In the current revision, we have further responded to the reviewer's concern regarding data transparency. Specifically, we now provide the full uncropped raw images for all blots shown in Figure S37, with both the corresponding molecular weight markers and internal loading controls clearly indicated.



Reviewer #4 (Remarks to the Author):

I appreciate the efforts of the authors in addressing the questions raised by the reviewers. However, based on the response and the data provided, several key limitations remain, as acknowledged by the authors as well.

For example, the unstable nature of the TA/dCPO LNPs and the very short blood half-life (Fig. S40) suggest that the *in vivo* stability of the system may be limited. These findings raise concerns regarding the robustness and usefulness of the current formulation in its present form.

Response: We thank the reviewer for this thoughtful comment. We agree that the current TA/dCPO LNP formulation exhibits limited circulation stability and a relatively short blood half-life, which may restrict its future translational optimization. However, we respectfully note that this formulation-related limitation does not affect the central conclusion of the present study. The primary objective of this work is to establish the feasibility of *in situ* generation of GPC-3-targeting radical-conjugated species for selective free-radical-mediated therapy against HCC, whereas the LNP serves as a delivery vehicle rather than the focus of the therapeutic innovation. Importantly, despite the relatively rapid systemic clearance, the observed tumor accumulation, mechanistic evidence, and therapeutic efficacy collectively demonstrate that sufficient TA/dCPO reached the tumor site to enable the proposed radical-targeting process *in vivo*.

Therefore, the mechanistic conclusions remain well supported by the experimental data.

We further note that circulation persistence of nanomedicines is an actively evolving area, and recent studies have shown that pharmacokinetic limitations associated with nanoparticle clearance can be substantially improved through modulation of mononuclear phagocyte system-mediated uptake, even without reformulating the nanoparticles themselves [1].

Therefore, we view the limited circulation stability of the current formulation as an engineering optimization opportunity rather than a limitation of the therapeutic concept demonstrated in this work.

Reference:

1. Lai K, He P, He Y, et al. Pre-intervention with intravenous immunoglobulin reverses the immunogenic clearance of PEGylated nanomedicines[J]. Nature Biomedical Engineering, 2026: 1-19.