nature portfolio

Peer Review File

Surface-displayed cholesterol enhances penetration and anti-tumor activity of a platinum nanodrug by targeting NPC1L1-expressing cancer associated fibroblasts

Corresponding Author: Professor Xiaorui Wang

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)
This manuscript by He et al. reports the development of a cholesterol-displayed dendrimeric cisplatin nanodrug (PtD-Chol) designed to target NPC1L1 and facilitate nanodrug transport among CAFs and tumor cells in TNBC and PDAC. Although the authors present promising in vivo antitumor and anti-metastatic effects, the mechanistic basis of the proposed delivery pathway remains insufficiently resolved. Several key aspects substantially weaken the overall interpretation of the study, including the clarity of the transcytosis mechanism, internal inconsistencies across datasets, and the lack of direct evidence supporting NPC1L1-mediated vesicular trafficking. Taken together, these concerns indicate that the manuscript is not suitable for publication in Nature Communications. Detailed comments are provided below:

1. The authors should reconsider their use of the term “transcytosis”, particularly the distinction between transcytosis and exocytosis. Transcytosis classically refers to a directional, vectorial transport process occurring in polarized cells, whereas CAFs and tumor cells are usually non-polarized and therefore not appropriate systems for applying this terminology. Moreover, the data presented in Fig. 3a-h, Fig. 4h-i, and Extended Fig. 3a-d do not demonstrate any directional transport characteristic of true transcytosis.

2. The central concept of this study is that NPC1L1-mediated transcytosis across CAFs enables deep tumor penetration. However, intravenously administered nanodrugs must first traverse the tumor endothelial barrier, which is a critical and rate-limiting step that precedes any interaction with CAFs or tumor cells. The authors provide no mechanistic or quantitative data demonstrating how PtD-Chol or PtD crosses tumor vasculature in vivo. Without comparing the transvascular efficacy of PtD-Chol and PtD, it is difficult to attribute the improved intratumoral distribution to NPC1L1-mediated transcytosis across CAFs or tumor cells.

3. The authors propose that PtD-Chol undergoes transcytosis through an ERC-ER-Golgi axis in CAFs and tumor cells; however, the supporting evidence is insufficient. No TEM images are provided to directly visualize PtD-Chol within the ER, Golgi, or ER-Golgi trafficking intermediates. Moreover, the confocal co-localization data in Extended Fig. 2e-f lack both high-resolution imaging and quantitative analyses. Therefore, the involvement of the ERC-ER-Golgi pathway in PtD-Chol transport cannot be convincingly established.

4. Several datasets in the manuscript present internal inconsistencies that weaken the interpretation.
a) In Fig. 2k, PtD-Chol appears diffusely distributed in 4T1 or Panc-02 cells. In Fig. 2l, using the same drug concentration and incubation time, the same cell line shows a punctate vesicular pattern.
b) Many confocal images (e.g., Fig. 3b, 3e, 3h "uptake", Fig. 3k, Fig. 3m) show PtD-Chol as diffuse cytosolic signal rather than punctate vesicles expected for vesicle-mediated transcytosis (authors' proposed ERC-ER-Golgi transcytosis route).
c) In Fig. 5c, intravascular signal of PtD-Chol appears significantly higher than that of PtD. However, the PK profiles (Extended Fig. 5a) do not support this difference.
d) Even at identical time points, intracellular PtD-Chol distribution patterns of Extended Fig. 2e and Extended Fig. 2f vary substantially. This might suggest imaging or sample preparation issues.

5. Given the high abundance of tumor-associated macrophages (TAMs) in breast cancer (~10-20%), the authors should address whether the macrophages may intercept or sequester the nanodrug its proposed transfer between CAFs and tumor cells. Such macrophage-mediated uptake could significantly alter the proposed NPC1L1-dependent nanodrug transport between CAFs and tumor cells.

6. It is reported that NPC1L1 is predominantly expressed in intestine and highly expressed in human and non-human primate liver. In Fig. 5b, PtD-Chol exhibits strong fluorescence signals not only in tumors but also in non-tumoral tissues. However, the authors do not provide biodistribution data for major organs (including the intestine) where NPC1L1 expression could significantly influence off-target uptake.

7. The manuscript lacks characterization of the stability of the fluorescent labels (BODIPY, Cy5) conjugated to PtD-Chol and PtD. Without verification of the stability under experimental conditions, the observed intercellular transport may reflect dye dissociation rather than true nanodrug transfer.

8. The manuscript lacks essential analyses of Pt distribution. Drug quantification across major organs is not provided, nor is the intratumoral micro-distribution distinguishing free Pt from nanodrug-bound Pt. These data are crucial to validate the proposed delivery mechanism and therapeutic advantage of PtD-Chol.

9. The clinical relevance of the study remains limited and unclear. Although the authors have demonstrated that PtD-Chol provides a therapeutic advantage in TNBC and PDAC, the study does not address key translational barriers, including cross-species differences in NPC1L1 expression, potential off-target uptake in NPC1L1-rich organs, pharmacokinetics, and validation in more clinically predictive systems such as PDX models.

Reviewer #2

(Remarks to the Author)
The manuscript leverages NPC1L1 as a “shared entry portal” for tumor cells and CAFs, and constructs a cholesterol surface–displayed dendrimeric cisplatin nanodrug (PtD-Chol) with the goal of traversing the CAF barrier via NPC1L1-mediated transcytosis to restore antitumor efficacy in TNBC and PDAC models. The Abstract and Introduction clearly articulate the challenge (the CAF barrier and the transcytotic route) and the central premise, maintaining a coherent narrative. In vitro, uptake/inhibitor/co-localization assays together with time-resolved tracking establish an evidence chain of “NPC1L1–ERC–ER/Golgi–exocytosis–re-uptake.” In vivo, conventional-volume, large-volume, and orthotopic tumor models are included, with dosing regimens and predefined endpoints, indicating translational potential. Overall, the innovation is clear and the evidentiary chain is largely complete, but several technical details still require improvement, as detailed below.

1.“Transcytosis” must be demonstrated more directly rather than inferred. The current evidence shows that the nanodrug first enters donor cells, then undergoes ERC/ER/Golgi-mediated exocytosis, followed by re-uptake by neighboring cells; BFA inhibits exocytosis and EZE inhibits re-uptake. While these are strongly correlative data, they still fall short of providing direct experimental proof of the authentic transcytotic route across the CAF barrier. It is recommended to add, in the main text and Methods, relevant experiments such as CAF–tumor cell co-culture systems and dynamic co-localization with vesicular markers.

2.In the Results and Methods sections, please add details of Western blot and immunostaining quantification (normalized to Tubulin or GAPDH), and report the number of replicates and statistical tests in the figure legends to avoid relying solely on workflows and representative bands.

3.Based on the data presented, briefly discuss potential differences in NPC1L1 expression between myCAFs and iCAFs.

4.For the in vivo studies, please provide sample-size calculations based on statistical power and expected effect sizes; regarding safety, beyond organ H&E and body-weight monitoring, include baseline hematology/serum biochemistry (e.g., ALT/AST, Cr/BUN, etc.), or acknowledge this limitation in the Discussion, given that NPC1L1 has baseline expression in the liver/intestine and NPC1L1 pharmacological intervention (EZE) is used in this study.

5.Multiple places use 0.02–0.04 g/L to report concentrations; please standardize to μg/mL or μM and indicate this in the figure legends.

6.The Methods already detail raw materials, polymerization, and probes (RAFT, FRET, antibodies, etc.). In the characterization section, please add a statement on between-batch consistency of the materials, and provide curves or tables demonstrating batch-to-batch reproducibility either in the main text or the Supplementary Information.

7.Because NPC1L1 is a clinical drug target (EZE is an approved medicine), please highlight in the Discussion potential

drug–drug interactions, the impact of dosing sequence or co-administration on efficacy, and briefly outline tissue-specific risks arising from intestinal/hepatic expression together with mitigation strategies.

8.Many figure legends state “representative images, repeated three times, mean ± SD, t-test.” For key conclusions, please add bar plots overlaid with individual data points and report exact P values.

9.Although the figure legends define abbreviations (EZE, BFA, MON, REC/PM, etc.), please also spell out the full terms at their first occurrence in the main text to improve readability.

10.For the animal experiments, please add standard elements such as the ethics approval ID and housing/environmental conditions.

11.A hemolysis assay has been performed; in the Supplementary Information, please provide positive/negative controls and thresholds, and include coagulation/hemolysis parameters—or acknowledge this limitation in the Discussion.

Reviewer #3

(Remarks to the Author)
This study addresses the issue of poor nanodrug penetration in stroma-rich tumor tissues by developing a cholesterol surface-displayed dendrimeric cisplatin nanodrug, PtD-Chol. This nanodrug simultaneously targets NPC1L1 on both tumor cells and cancer-associated fibroblasts. A series of experiments validated the transcytosis pathway and its deep penetration into tumors. Furthermore, the therapeutic efficacy and its ability to inhibit metastasis were demonstrated using in vivo breast cancer and pancreatic cancer models. Although the studies are well-designed and the results are impressive, there are still several major issues that need to be addressed.

1. NPC1L1 is highly expressed in the intestine (particularly the small intestine) for dietary cholesterol absorption. When analyzing the in vivo distribution of PtD-Chol, it is recommended to supplement data on its accumulation in intestinal tissues (ex vivo fluorescence imaging or histological staining) and discuss the potential intestinal toxicity or other side effects. This is crucial for evaluating the clinical safety profile of this targeting strategy.

2. Regarding the NPC1L1-mediated transcytosis pathway (via the ERC-ER-Golgi pathway) that shuttles PtD-Chol across the barrier in both tumor cells and fibroblasts: the schematic diagram (Fig. 1c) depicts all internalized PtD-Chol being transcytosed. However, in reality, a portion of the internalized PtD-Chol is likely retained within the cell. It is necessary to determine the proportion of cell-internalized PtD-Chol that is retained intracellularly versus the fraction that is released via transcytosis.

3. In Figures 3d-f, after the removal of the PtD-Chol-containing medium, transcytosis requires secretion into the medium before uptake by other cells. This implies that a portion of PtD-Chol should be present in the medium. However, the intracellular fluorescence intensity shown in the figure (0-4h) does not change. Please explain this observation.

4. In Fig.4h-i, the labels (i, ii, iii) should be clearly marked to correspond with the numbering used in the manuscript.

5. Lines 454-456 describing the inhibition of spheroid growth by PtD-Chol, the phrasing "resulting in a final volume increase of only 55%" could be ambiguous. For greater accuracy and clarity, this should be more precisely described as "decreasing to 55% of its initial volume" or "resulting in a final volume that was only 55% of the initial volume." The current phrasing is ambiguous.

Reviewer #4

(Remarks to the Author)
The manuscript by Peiyi He and colleagues entitled “NPC1L1-mediated transcytosis enables nanodrug delivery across CAF barrier to restore antitumor efficacy in triple-negative breast cancer and pancreatic cancer” reports on the development of PtD-Chol, a cholesterol-surface displayed dendrimeric cisplatin nanodrug engineered to exploit transcytosis for deep tumor penetration. Authors show that the strategy facilitates efficient tissue penetration through NPC1L1-mediated cellular uptake coupled with multicellular transcytosis. This improved tumor-penetration capacity translates into potent anti-cancer effects in both in vitro and in vivo models of breast and pancreatic cancer.

This manuscript describes very relevant and robust findings that can set the stage for original treatment improving cancer treatment. However, authors should provide few additional clarifications to this study.

In the Introduction section, please avoid referencing data/results that are described later in the Results. Instead, expand on the problem and the current state of the art prior to this study, including existing knowledge about NPC1L1 in the clinical and

oncological settings.

Line 104/Line 160: The current structure of the two first sections of Results is confusing, as the first section describes the development of the drug targeting NPC1L1, but the relevance of targeting NPC1L1 appears only in the following section. A reorganization would improve clarity.

Line 105: “Given the highly hydrophobic nature of cholesterol, it tends to localize within the hydrophobic core of conventional nanoparticles, thereby restricting surface display and impairing molecular recognition functions.” Please provide references supporting this statement.

Line 151: “The cisplatin drug release profiles from the nanodrugs were evaluated under a reducing condition that mimics the tumor microenvironment (5 mM ascorbic acid, pH 7.4).” Please provide data supporting that this condition mimics the tumor microenvironment and to what extent.

Figures 2 and 3 demonstrate extensive accumulation of PtD-Chol in the cytoplasm of target cells. However, given that platinum-based drugs act on DNA, it is important to clarify whether PtD-Chol is able to penetrate the nucleus. Additionally, the authors should provide quantitative data on how much platinum is retained within the cells relative to the fraction of the compound undergoing exocytosis.

Line 271: “Transcytosis has held considerable promise for enhancing nanodrug penetration in solid tumors. However, its efficacy is predominantly confined to cell-to-cell interactions between tumor cells.” Please provide references supporting this statement.

Line 346: “To evaluate penetration through CAF-rich barriers….”. In Figure 5d, α-SMA staining indicates only minor CAF infiltration within the tumor, making this specific breast cancer model unsuitable for the authors’ claim. Could the authors provide data from a breast cancer model with more substantial CAF accumulation in the tumor (similar to the pancreatic cancer model presented later (ie. extended Figure 8)?

On another note, Figure 5c suggests colocalization of CD31 and PtD-Chol (overlap of red and green staining produces yellow). In contrast, α-SMA and PtD-Chol colocalization is not evident in Figure 5d. Does PtD-Chol penetrate tumor endothelial cells in vivo more effectively? Could this enhance treatment penetration? Please provide data comparing PtD-Chol uptake in endothelial cells, cancer cells, and CAFs in the tumor in vivo.

Line 366: “The survival benefit was equally remarkable…”. Was the survival endpoint based solely on tumor size, as described in the Methods? The authors should clarify whether humane endpoints, such as body weight loss, reduced mobility, or other clinical signs, were also considered. Integrating these measures ensures both ethical treatment of animals and accurate interpretation of survival data.

Line 369: “the treatment regimens across all groups showed no adverse effects on the functions of the heart, liver, spleen, lungs, or kidneys indicating negligible systemic toxicity.” Of note, NPC1L1 is highly expressed in the intestine, making it potentially susceptible to adverse effects of PtD-Chol. However, this organ was not examined. Could the authors provide data on PtD-Chol uptake and effect (eg. apoptosis) in the intestine?

Line 400: “PtD-Chol potently inhibited both migration and invasion….”. Please discuss the potential reasons for the anti-migratory/invasion effect observed with this Pt-based compound.

Line 411: “The phenotypic reversion of CAFs induced by PtD-Chol ….”. Please discuss the potential mechanisms underlying CAF phenotypic reversion driven by PtD-Chol.

Line 432: “The marked reduction in α-SMA and NPC1L1 levels following PtD-Chol treatment indicates decreased CAF infiltration and phenotypic reversion toward normal fibroblasts (Fig. 6j).” In view of the data provided, it is unclear whether the reduction in α-SMA correlates with CAF reversion to NAF, CAF clearance, or both. Please provide an objective analysis of positive cell counts to support the statement.

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 reviewer appreciates the additional experiments and substantial revisions provided by the authors. However, several major conceptual concerns remain regarding the proposed delivery mechanism.

1. Concern regarding active nanoparticle transport by dying cells. It remains unclear whether the proposed active transport mechanism can operate efficiently during therapy. As treatment progresses, many donor cells are expected to undergo apoptosis or become severely damaged. It therefore remains unclear whether these dying cells retain the capacity to efficiently transport nanoparticles to neighboring cells.

2. The authors claimed a novel cell-to-cell transport pathway (Fig. 1c; 4h), yet all of the presented experiments remain consistent with a much simpler explanation: PtD-Chol is exocytosed into the extracellular space, diffuses locally, and is subsequently internalized by neighboring NPC1L1-positive cells. The current data do not establish, in reviewer's opinion, that nanoparticles are "actively" transported from one cell to another as a "continuous" multicellular transport process (i.e., from peri-endothelial tumor cells to deep tumor cells).

3. The authors assumed that NPC1L1 expression alone is sufficient to enable cross-multicellular transport. However, receptor expression does not necessarily confer transport competence. It remains unclear whether CAFs and tumor cells possess comparable NPC1L1-mediated endocytic recycling kinetics, intracellular trafficking, exocytic efficiency, and membrane recycling capacity. Therefore, the conclusion that both cell types contribute equivalently to the proposed transport cascade is insufficiently supported.

4. The TEM images presented in Fig. 4j are difficult to interpret due to their limited resolution. How did the authors confirm that the observed electron-dense black dots indeed represent PtD-Chol nanoparticles?

Reviewer #2

(Remarks to the Author)
I am pleased to have the opportunity to review this manuscript again. The authors have adequately addressed my previous concerns, which has substantially improved the completeness and overall value of the manuscript. I therefore recommend its acceptance.

Reviewer #4

(Remarks to the Author)
The authors have addressed my previous comments and concerns. I appreciate their responses and the substantial revisions made, which have improved the quality and clarity of the manuscript.

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)
The reviewer appreciates the authors' efforts to address the concerns by incorporating additional experimental data, which have strengthened the manuscript. The new in vitro results provide further support for the occurrence of the observed transcytosis phenomenon among apoptotic cells under cell culture conditions. However, an important question is the robustness and relevance of this effect in a therapeutic setting. Moreover, the proposed mechanism appears to serve as a central premise throughout the manuscript (e.g., Fig. 1) - whether this mechanism is indeed the dominant contributor to the observed effects.

Reviewer #2

(Remarks to the Author)
The authors have addressed all the comments and concerns. I recommend publication.

RESPONSES TO REFEREES:
(Reviewer comments in black, our response in blue)

Reviewer #1 (Remarks to the Author):

This manuscript by He et al. reports the development of a cholesterol-displayed dendrimeric cisplatin nanodrug (PtD-Chol) designed to target NPC1L1 and facilitate nanodrug transport among CAFs and tumor cells in TNBC and PDAC. Although the authors present promising in vivo antitumor and anti-metastatic effects, the mechanistic basis of the proposed delivery pathway remains insufficiently resolved. Several key aspects substantially weaken the overall interpretation of the study, including the clarity of the transcytosis mechanism, internal inconsistencies across datasets, and the lack of direct evidence supporting NPC1L1-mediated vesicular trafficking. Taken together, these concerns indicate that the manuscript is not suitable for publication in Nature Communications. Detailed comments are provided below:

We sincerely appreciate the time you have dedicated to reviewing our manuscript. We sincerely thank you for the constructive suggestions you have provided, which have enhanced the completeness and scientific nature of our work. All comments and suggestions have been thoroughly considered and incorporated into this revised manuscript.

1. The authors should reconsider their use of the term “transcytosis”, particularly the distinction between transcytosis and exocytosis. Transcytosis classically refers to a directional, vectorial transport process occurring in polarized cells, whereas CAFs and tumor cells are usually non-polarized and therefore not appropriate systems for applying this terminology. Moreover, the data presented in Fig. 3a-h, Fig. 4h-i, and Extended Fig. 3a-d do not demonstrate any directional transport characteristic of true transcytosis.

We are grateful for your high professional comment. Through your guidance, we have realized and agreed that the term "transcytosis" classically refers to a directional, vectorial transport process that occurs in polarized cells, such as epithelial or endothelial cells. As you correctly point out, CAFs and tumor cells are generally non-polarized, and our data do not demonstrate directional transport across a polarized cell layer. Therefore, to avoid the conceptual ambiguity, we have now revised the terminology throughout the manuscript. Specifically, we replace "transcytosis" with the more descriptive and functionally neutral term "cross-multicellular transport (CMCT)" to better reflect the non-directional, intercellular vesicular transfer observed in our non-polarized cell system.

2. The central concept of this study is that NPC1L1-mediated transcytosis across CAFs enables deep tumor penetration. However, intravenously administered nanodrugs must first traverse the tumor endothelial barrier, which is a critical and rate-limiting step that precedes any interaction with CAFs or tumor cells. The authors provide no mechanistic or quantitative data demonstrating how PtD-Chol or PtD crosses tumor vasculature in vivo. Without comparing the transvascular efficacy of PtD-Chol and PtD, it is difficult to attribute the improved intratumoral distribution to NPC1L1-mediated transcytosis across CAFs or tumor cells.

Thank you for raising this important point regarding the transvascular efficacy of PtD-Chol and PtD. In our previous experiments, the primary focus was on overcoming the CAF barrier in tumors, and the research on the mechanism of transvascular transport was not comprehensive enough. Subsequently, we conducted the following supplementary experiments to further investigate the transvascular mechanism of PtD-Chol.

We constructed both in vitro and in vivo models to intuitively evaluate the transvascular efficacy of PtD-Chol and PtD. First, we used a C166 (mouse vascular endothelial cells)-transwell model to simulate the vascular barrier (Fig. 5c-f). The experimental results showed that both PtD-Chol and PtD could cross this vascular barrier, which were attribute to their small size (hydrodynamic size: ~8 nm). However, because PtD lacks surface-displayed cholesterol, it cannot be rapidly taken up by CAFs and tumor cells, nor can it further spread into deeper tumor regions; instead, it is gradually cleared from the tumor site. This was also confirmed by real-time fluorescence imaging in vivo (Fig. 5g). Therefore, the current results indicate that the main difference between the nanodrugs PtD-Chol and PtD used in this study lies in their tumor penetration performance after breaching the vascular barrier. The cholesterol surface-displayed nanodrug PtD-Chol, through specific binding to NPC1L1 and an advantage in the endocytosis pathway, triggers cross-multicellular transport between tumor cells and cancer-associated fibroblasts (CAFs).

3. The authors propose that PtD-Chol undergoes transcytosis through an ERC-ER-Golgi axis in CAFs and tumor cells; however, the supporting evidence is insufficient. No TEM images are provided to directly visualize PtD-Chol within the ER, Golgi, or ER-Golgi trafficking intermediates. Moreover, the confocal co-localization data in Extended Fig. 2e-f lack both high-resolution imaging and quantitative analyses. Therefore, the involvement of the ERC-ER-Golgi pathway in PtD-Chol transport cannot be convincingly established.

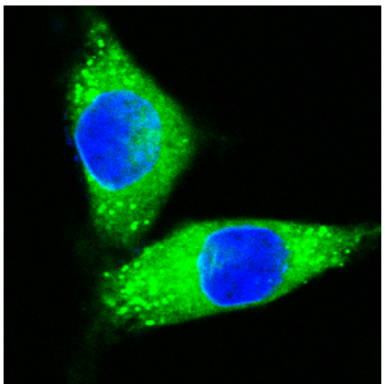
We appreciate your observation that the proposed mechanism of cross-multicellular transport is not yet adequately supported by evidence. In our previous manuscript, we mainly verified this mechanism through two lines of evidence: cell co-localization experiments and treatment with pathway inhibitors. We acknowledge that we did not conduct a more detailed observation and confirmation of this process using TEM, which was due to inadequate consideration on our part. According to your requirements, we have now performed TEM experiments and analyzed the co-localization data to determine the co-localization coefficient. Using TEM, we can observe the distribution of PtD-Chol in the ER, Golgi, and ER-Golgi trafficking intermediates (Extended Data Fig. 2k), as well as its transport between CAFs and tumor cells (Fig. 4j). Using co-localization coefficient analysis, we further verified the temporal changes in the nanomaterial's distribution across different cellular organelles (Extended Data Fig. 2g-j). Taken together, these additional experiments provide a better explanation of the transport process, in which PtD-Chol undergoes cross-multicellular transport through the ERC-ER-Golgi axis in CAFs and tumor cells.

4. Several datasets in the manuscript present internal inconsistencies that weaken the interpretation.

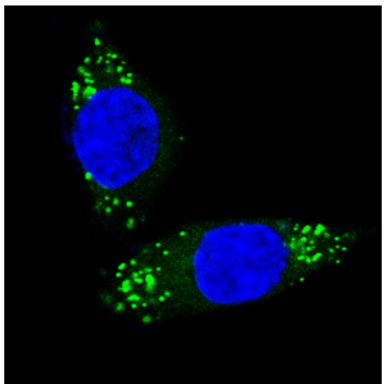
a) In Fig. 2k, PtD-Chol appears diffusely distributed in 4T1 or Panc-02 cells. In Fig. 2l, using the same drug concentration and incubation time, the same cell line shows a punctate vesicular pattern.

Thank you for pointing out the discrepancy in fluorescence distribution between Fig. 2k and Fig. 2l. Through additional experiments, we found that this difference arises from the different cellular sample preparation processes. In Fig. 2k, the sample was imaged without fixation or permeabilization, retaining both diffuse cytosolic and vesicular signals. In contrast, the sample in Fig. 2l underwent immunofluorescence processing, which involves permeabilization, blocking, and extensive washing. These steps might remove the diffuse cytosolic nanomedicine that is not encapsulated within vesicles, leaving predominantly a punctate vesicular pattern.

Before permeabilization and blocking



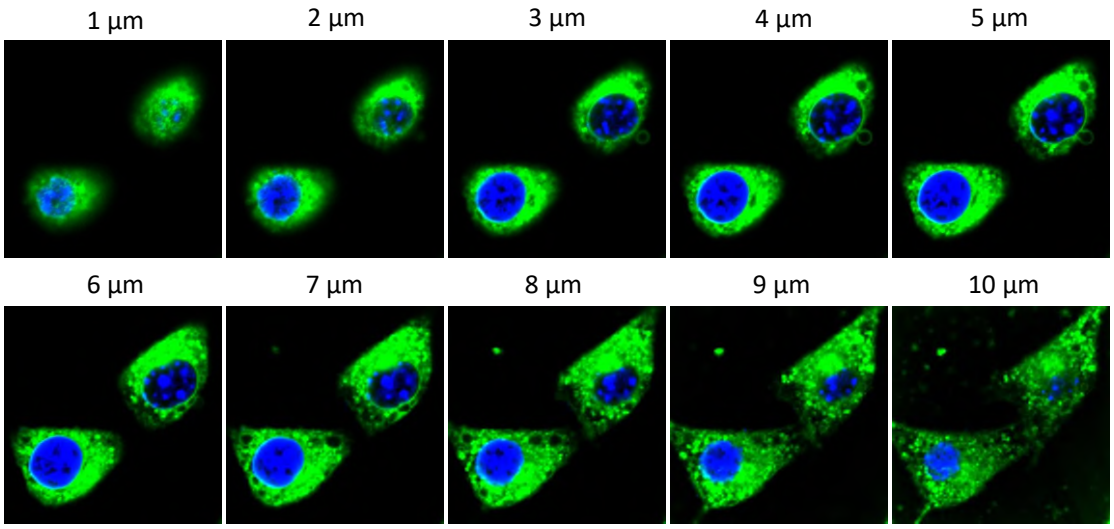
After permeabilization and blocking



b) Many confocal images (e.g., Fig. 3b, 3e, 3h "uptake", Fig. 3k, Fig. 3m) show PtD-Chol as diffuse cytosolic signal rather than punctate vesicles expected for vesicle-mediated transcytosis (authors' proposed ERC-ER-Golgi transcytosis route).

Thank you for raising this important issue. In the confocal images you mentioned (Fig. 3b, 3e, 3h "uptake", 3k, 3m), both diffuse cytosolic signals and punctate vesicular signals are actually present simultaneously. After PtD-Chol enters cells via NPC1L1-mediated uptake, not all PtD-Chol are transported through the ERC-ER-Golgi pathway and released extracellularly. A portion of the PtD-Chol is instead released into the cytoplasm, which gives rise to the diffuse distribution pattern. Meanwhile, the PtD-Chol that remains within vesicular transport structures appears as punctate signals. Additionally, for the "uptake" groups specifically, the overall fluorescence intensity is particularly strong, resulting in a predominantly diffuse cytosolic appearance across the entire cell. Through confocal z-stack analysis, we found that in these strongly fluorescent samples, the signals from multiple vesicular PtD-Chol particles superimpose within the same focal plane, creating the appearance of a diffuse high-intensity pattern. However, when examining other optical sections of the same cells, the vesicular distribution can be clearly resolved. Therefore, the observed diffuse signal does not contradict

the proposed vesicle-mediated transport; rather, it reflects both genuine cytosolic release and projection artifacts due to intense vesicular signals. We hope this clarifies your concern.



c) In Fig. 5c, intravascular signal of PtD-Chol appears significantly higher than that of PtD. However, the PK profiles (Extended Fig. 5a) do not support this difference.

We thank you for raising this question. We would like to clarify that the intravascular signal shown in Fig. 5h (originally Fig. 5c in the previous version) primarily reflects the signal intensity within tumor blood vessels, whereas the pharmacokinetic curve in Extended Data Fig. 6a (originally Extended Data Fig. 5a in the previous version) represents the systemic distribution of the nanomaterials in peripheral blood. After further quantitative analysis of the intravascular signal in the tumor sections, we found that the intravascular signal of PtD-Chol was slightly higher than that of PtD (Extended Data Fig. 6m), indicating that PtD-Chol possesses a better tumor-homing ability. In addition, real-time intravascular imaging results also showed that the fluorescence signal of PtD within blood vessels gradually diminished over time (Fig. 5g), further reflecting the better tumor enrichment performance of PtD-Chol.

d) Even at identical time points, intracellular PtD-Chol distribution patterns of Extended Fig. 2e and Extended Fig. 2f vary substantially. This might suggest imaging or sample preparation issues.

Thank you for your careful observation. We agree that the PtD-Chol distribution patterns in Extended Data Fig. 2e and Extended Data Fig. 2f appear different at identical time points, and we would like to clarify the reason.

Extended Data Fig. 2e was obtained using immunofluorescence stain, which involves a permeabilization step. During this process, a portion of the diffuse, non-vesicle-associated PtD-Chol in the cytoplasm might be lost upon washing, resulting in a more pronounced punctate/vesicular pattern. In contrast, Extended Data Fig. 2f was performed using

commercially available organelle dyes without the permeabilization process, thus preserving a relatively diffuse fluorescence distribution.

To ensure methodological consistency and comparability among experiments aimed at visualizing the same subcellular compartments, we have now repeated the ER/Golgi imaging in Extended Data Fig. 2f using immunofluorescence staining with anti-PDI (ER marker) and anti-GM130 (Golgi marker) antibodies, following the same fixation, permeabilization, and washing procedures as in Extended Data Fig. 2e. These new results are presented in the revised manuscript, and they show a distribution pattern consistent with that observed in Extended Data Fig. 2e.

We have updated the figure and the corresponding legend accordingly. We apologize for any confusion caused by the previous version and thank you again for your insightful suggestion.

5. Given the high abundance of tumor-associated macrophages (TAMs) in breast cancer (~10-20%), the authors should address whether the macrophages may intercept or sequester the nanodrug its proposed transfer between CAFs and tumor cells. Such macrophage-mediated uptake could significantly alter the proposed NPC1L1-dependent nanodrug transport between CAFs and tumor cells.

Thank you for your insightful comments. In previous studies, we focused solely on the interplay between CAFs and tumor cells within the tumor microenvironment, without considering the potential influence of tumor-associated macrophages (TAMs). To address this, we have performed additional experiments to evaluate the impact of TAMs.

Specifically, we dissected and digested tumors from mice treated with nanoparticles at different time points into single cells. We then used flow cytometry with relevant antibody labeling to measure nanoparticle uptake by different cell populations within the tumors. The results showed that for PtD-Chol, at 2 hours post-injection, TAMs exhibited a strong fluorescence signal. However, by 12 hours, the signal in TAMs remained largely unchanged, while it became mainly concentrated in tumor cells (Extended Data Fig. 6k). This indicates that although TAMs capture PtD-Chol to a certain extent, this does not prevent PtD-Chol from subsequently accumulating in tumor cells via cross-multicellular transport. In contrast, at 12 hours, PtD fluorescence was markedly reduced, while PtD-Chol exhibited a significant shift toward tumor cells, with the majority of PtD-Chol signal now concentrated within the tumor cell compartment (Extended Data Fig. 6l). The results demonstrate that while TAMs take up the nanomedicine to some degree, this does not hinder the nanoparticles from reaching and entering tumor cells to exert their function.

6. It is reported that NPC1L1 is predominantly expressed in intestine and highly expressed in human and non-human primate liver. In Fig. 5b, PtD-Chol exhibits strong fluorescence signals not only in tumors but also in non-tumoral tissues. However, the authors do not provide biodistribution data for major organs (including the intestine) where NPC1L1 expression could significantly influence off-target uptake.

We sincerely appreciate for you raising this important concern regarding intestinal and hepatic accumulation as well as their safety, an aspect we had not sufficiently considered in our previous work and which indeed merits further investigation.

To address this, we have performed additional experiments. First, we collected biodistribution profiles of the nanodrug in major organs (including the intestines and liver) at different time points. The results showed that due to the targeting effect of the cholesterol-displayed, PtD-Chol exhibited additional intestinal accumulation compared to PtD at 2 hours post-injection; this difference gradually diminished after 12 hours, and no significant extra accumulation was observed in the liver (Extended Data Fig. 6f-i). Second, to assess whether this transient intestinal accumulation was associated with pathological damage, we performed H&E staining on major organs, including the liver and intestine, from tumor-bearing mice that received six injections of PtD-Chol (at the end of the therapeutic regimen). The results demonstrated that the treatment strategy did not induce any significant lesions in the liver or intestine (Extended Data Fig. 10j).

Furthermore, we explored a strategy to prevent this extra-intestinal accumulation of PtD-Chol. Oral pre-administration of ezetimibe (EZE), an oral cholesterol absorption inhibitor that targets the NPC1L1 transporter at the brush border of the small intestine, specifically inhibits intestinal cholesterol uptake and subsequently reduces cholesterol transport from the intestine to the liver. This treatment effectively abolished the extra-intestinal accumulation of PtD-Chol without compromising its tumor accumulation or therapeutic efficacy (Fig. 6 and Extended Data Fig. 6f-i).

Taken together, these results indicate that both the cholesterol-mediated NPC1L1-targeting strategy and its combination with oral EZE hold promise for achieving highly tumor-targeted therapy while mitigating potential off-target safety concerns.

7. The manuscript lacks characterization of the stability of the fluorescent labels (BODIPY, Cy5) conjugated to PtD-Chol and PtD. Without verification of the stability under experimental conditions, the observed intercellular transport may reflect dye dissociation rather than true nanodrug transfer.

Thank you for raising this important concern regarding the stability of the fluorescent labels (BODIPY, Cy5) conjugated to PtD-Chol and PtD. To address this, we have performed additional stability studies. First, we evaluated the fluorescence stability of both BODIPY- and Cy5-labeled PtD-Chol and PtD in PBS at 37 °C over 0, 4, and 7 days. The results confirmed that both fluorophores remained stable on the nanodrugs throughout the tested period Extended Data Fig. 1e,f). Second, we constructed a FRET (Förster Resonance Energy Transfer) pair by co-labeling PtD-Chol with BODIPY and RhB (P1P1'/PtD-Chol, Supplementary Tables 1 and 2). We monitored the FRET signal of P1P1'/PtD-Chol in PBS at 37 °C over 0, 4, and 7 days. Stable FRET efficiency was observed (Extended Data Fig. 1k), indicating that the dyes did not leach or dissociate from the nanodrug during the experimental timeframe.

Furthermore, we performed cross-multicellular transport experiments using P1P1'/PtD-Chol. In recipient cells, the internalized nanodrug still clearly exhibited a FRET signal, demonstrating that intact nanodrugs-not free dissociated dyes-were transported across cells (Extended Data Fig. 3g,h). Collectively, these results confirm that the observed intercellular transport reflects nanodrug transfer rather than fluorescence dye dissociation. We have now included these data in the revised manuscript. Thank you for the very valuable question you raised.

8. The manuscript lacks essential analyses of Pt distribution. Drug quantification across major organs is not provided, nor is the intratumoral micro-distribution distinguishing free Pt from nanodrug-bound Pt. These data are crucial to validate the proposed delivery mechanism and therapeutic advantage of PtD-Chol.

We are grateful for your raising these important points regarding the lack of Pt distribution analysis. Therefore, we have now supplemented the drug quantification data across major organs at the 12-hour time point. The results showed that PtD-Chol predominantly accumulates in the tumor and is primarily cleared via the kidneys (Extended Data Fig. 6j).

Regarding the distinction between intratumoral free Pt and nanodrug-conjugated Pt, we acknowledge that we have not yet identified a suitable experimental approach to directly differentiate these two forms within the tumor microenvironment. Nevertheless, our other data support that PtD-Chol can release cisplatin in the tumor to exert its therapeutic effect. Specifically, during the cross-multicellular transport of PtD-Chol, a portion of the Pt species accumulates inside tumor cells (Extended Data Fig. 3b). At the cellular level, we have verified that Pt can enter the nucleus and bind to DNA, thereby triggering antitumor activity (Extended Data Fig. 5j-l). Collectively, these findings indicate that once PtD-Chol reaches the tumor tissue, it is capable of releasing the active cisplatin payload under the reducing tumor microenvironment to kill tumor cells. If a suitable characterization method becomes available in the future to distinguish between nanodrug-conjugated platinum prodrug and released platinum drug in the tumor, we would be happy to further investigate and refine this aspect of the study.

9. The clinical relevance of the study remains limited and unclear. Although the authors have demonstrated that PtD-Chol provides a therapeutic advantage in TNBC and PDAC, the study does not address key translational barriers, including cross-species differences in NPC1L1 expression, potential off-target uptake in NPC1L1-rich organs, pharmacokinetics, and validation in more clinically predictive systems such as PDX models.

We appreciate your constructive comments regarding the clinical relevance and translational barriers of our study. We fully acknowledge these limitations and have conducted additional experiments to address some of them, while recognizing that the remaining limitations represent valuable directions for future work.

About issues: cross-species differences and model systems.
We agree that PDX models would provide higher clinical predictability. Indeed, it is difficult for our laboratory to complete the acquisition of a sufficient number of patient-derived samples and the corresponding ethical review work in the short term. As a second-best

alternative, we instead used human cell lines and the more clinically relevant cell line-derived xenograft (CDX) model to evaluate whether the NPC1L1-mediated cross-multicellular transport mechanism translates to human cells. It has been reported that NPC1L1 is overexpressed in multiple human cancers, including TNBC and PDAC, and correlates with poor prognosis (Miao G., et al. *ACS Nano*. 2025, 19(48):40828-40849; Nicolle R., et al. *Cell Reports*, 2017, 21(9): 2458-2470; You X., et al. *Cancer Lett*. 2025, 612:217493). We first validated NPC1L1 expression in human hepatic stellate cells (quiescent LX-2 fibroblasts vs TGF-β1-activated LX-2), and in human triple-negative breast cancer cells MDA-MB-231 cells. Western blot showed that NPC1L1 is upregulated in activated CAFs (LX-2 + TGF-β1) and in MDA-MB-231 cells compared to quiescent LX-2 fibroblasts (Fig. 8a-c). The elevated NPC1L1 expression observed in mouse tumor cells and CAFs was also recapitulated in human cell lines. Cellular uptake of PtD-Chol via NPC1L1 was confirmed in these human cell lines (Fig. 8d-e), and cross-multicellular transport was also occurred (Fig. 8f). Subsequently, we established an orthotopic TNBC CDX model in nude mice. PtD-Chol treatment significantly inhibited tumor growth, reduced liver metastasis, downregulated NPC1L1 and α-SMA expression, and induced both clearance and phenotypic reversion of CAFs (Fig. 8g-q and Extended Data Fig. 9h-i). These data indicate that the proposed mechanism and therapeutic effects are not limited to mouse cells but are also feasible in human-derived systems.

About issues: pharmacokinetics and the core design rationale.

We acknowledge that our nanodrugs were not optimized for prolonged circulation, which is also important for enhancing drug therapeutic efficacy. At the present stage, the strategy was developed to overcome the poor tumor penetration of nanodrugs. Although PtD-Chol showed no advantage over the control nanodrug PtD in terms of blood circulation, it successfully targeted tumors and achieved sustained intratumoral retention via active transcellular transport, leading to high penetration and robust therapeutic efficacy. We acknowledge that further improvement in pharmacokinetics could enhance tumor accumulation and therapeutic outcomes, and we consider this a promising direction for future studies.

About issues: off-target uptake in NPC1L1-rich organs (intestine and liver).

As previously addressed in response to the comment 6, we have evaluated the issues of biodistribution and safety. PtD-Chol shows transient intestine and liver accumulation at 2 h, which resolves by 12 h and 24 h. Repeated dosing caused no histological damage to the intestine or liver. More importantly, we identified a clinically feasible mitigation strategy: oral pre-administration of ezetimibe (EZE), an approved NPC1L1 inhibitor, significantly reduced the transient intestinal retention without compromising tumor targeting or antitumor efficacy (Fig. 6, Extended Data Fig. 6f-i, and Extended Data Fig. 10j). This combination strategy, which utilizes clinically approved drugs, has the potential to solve the off-target problem and supports the potential for clinical translation.

Reviewer #2 (Remarks to the Author):

The manuscript leverages NPC1L1 as a “shared entry portal” for tumor cells and CAFs, and constructs a cholesterol surface–displayed dendrimeric cisplatin nanodrug (PtD-Chol) with the goal of traversing the CAF barrier via NPC1L1-mediated transcytosis to restore antitumor efficacy in TNBC and PDAC models. The Abstract and Introduction clearly articulate the challenge (the CAF barrier and the transcytotic route) and the central premise, maintaining a coherent narrative. In vitro, uptake/inhibitor/co-localization assays together with time-resolved tracking establish an evidence chain of “NPC1L1–ERC–ER/Golgi–exocytosis–re-uptake.” In vivo, conventional-volume, large-volume, and orthotopic tumor models are included, with dosing regimens and predefined endpoints, indicating translational potential. Overall, the innovation is clear and the evidentiary chain is largely complete, but several technical details still require improvement, as detailed below.

We sincerely appreciate the time you have dedicated to reviewing our manuscript. Thank you very much for your thorough and constructive evaluation of this work. In particular, we are grateful that you highlighted the concept of NPC1L1 as a “shared entry portal” for tumor cells and CAFs. This is an insightful and inspiring description. According to your instructions, we have now incorporated the phrase “shared entry portal” into the revised manuscript (Abstract and Introduction sections) to more accurately and vividly convey the core concept of our strategy. In addition, we have carefully addressed all the specific technical points you raised in the detailed comments, and the corresponding improvements have been made throughout the revised manuscript.

1.“Transcytosis” must be demonstrated more directly rather than inferred. The current evidence shows that the nanodrug first enters donor cells, then undergoes ERC/ER/Golgi-mediated exocytosis, followed by re-uptake by neighboring cells; BFA inhibits exocytosis and EZE inhibits re-uptake. While these are strongly correlative data, they still fall short of providing direct experimental proof of the authentic transcytotic route across the CAF barrier. It is recommended to add, in the main text and Methods, relevant experiments such as CAF–tumor cell co-culture systems and dynamic co-localization with vesicular markers.

We appreciate your thoughtful suggestion regarding the need for more direct evidence of cross-multicellular transport across the CAF barrier. We fully agree that further visualization of this process would be needed. To address this, we have performed additional electron microscopy experiments using a CAF-tumor cell co-culture system. Transmission electron microscopy (TEM) images clearly show the presence of dispersed nanodrug particles between CAFs and tumor cells, as well as endocytic events on the surface of recipient tumor cells (Fig. 4j). These ultrastructural observations provide direct morphological evidence for a cross-multicellular transport route, in which nanodrug particles exit donor CAFs and are subsequently taken up by neighboring tumor cells. Although dynamic tracking remains technically challenging, due to the Brownian motion of exocytosed small-sized nanoparticles (PtD-Chol, 8 nm) that prevents confocal microscopy from capturing their extracellular location distribution information under our current experimental conditions, the combination of our

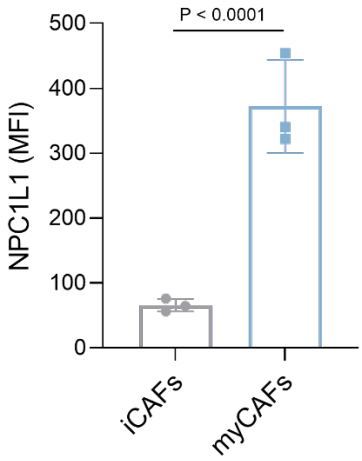
co-culture TEM data, the inhibitory effects of BFA and EZE, along with the ERC–ER–Golgi colocalization, strongly supports the proposed transcytosis mechanism.

2.In the Results and Methods sections, please add details of Western blot and immunostaining quantification (normalized to Tubulin or GAPDH), and report the number of replicates and statistical tests in the figure legends to avoid relying solely on workflows and representative bands.

Thank you for your constructive suggestions regarding the quantification details of Western blot and immunostaining. In the revised manuscript, we have added the following information in the Methods section: quantification of protein bands was normalized to the internal control (Tubulin or GAPDH), as presented in detail in the "Methods" section of the revised manuscript (Lines 1197-1206). Additionally, we have carefully modified the y-axis labels of all Western blot quantifications to clearly indicate “Target protein/Internal control (GAPDH or Tubulin)” to improve clarity and reproducibility. In the figure legends, we have also included the exact number of replicates and the statistical methods applied, thereby avoiding reliance on representative bands or workflows alone.

3.Based on the data presented, briefly discuss potential differences in NPC1L1 expression between myCAFs and iCAFs.

Thank you for your insightful question regarding potential differences in NPC1L1 expression between myCAFs and iCAFs. We measured NPC1L1 expression in myCAFs and iCAFs using flow cytometry. The results suggested that NPC1L1 expression was relatively higher in myCAFs compared to iCAFs.



Regarding the possible reasons for this differential expression, we note that myCAFs are primarily responsible for depositing and remodeling the extracellular matrix (ECM) within the tumor microenvironment, a process known to be metabolically demanding (Mucciolo G., et al. *Cancer Cell*. 2024, 42(1):101-118.e1.). In contrast, metabolic profiling studies have revealed that iCAFs tend to rely more on glycolysis, whereas myCAFs depend on the tricarboxylic acid (TCA) cycle and its derivatives (Hu B., et al. *Ann Transl Med*. 2022, 10(5): 262). Considering that

NPC1L1 is a key transporter responsible for cellular cholesterol uptake, the higher expression of NPC1L1 in myCAFs may reflect their increased demand for cholesterol to support their ECM-secreting and remodeling functions. It should be noted that our study primarily focuses on matrix-producing myCAFs, which constitute the primary structure of the matrix barrier.

4.For the in vivo studies, please provide sample-size calculations based on statistical power and expected effect sizes; regarding safety, beyond organ H&E and body-weight monitoring, include baseline hematology/serum biochemistry (e.g., ALT/AST, Cr/BUN, etc.), or acknowledge this limitation in the Discussion, given that NPC1L1 has baseline expression in the liver/intestine and NPC1L1 pharmacological intervention (EZE) is used in this study.

Thank you for your suggestions; we have provided sample-size justification based on literature, and have included baseline hematology and serum biochemistry (ALT/AST, Cr/BUN) in the revised manuscript to address the safety concern raised by NPC1L1 expression in the liver/intestine and the use of EZE.

In the current experiment, no formal a priori power analysis was performed for sample size determination. Instead, sample sizes for in vivo studies were justified based on previously published studies employing similar experimental models and comparable endpoints.

Specifically, we reviewed several high-quality papers that employed orthotopic pancreatic and breast cancer models with analogous treatment regimens and detection methods. Based on the animal numbers and observed inter-group differences reported in those studies, which demonstrated sufficient statistical power, we adopted the following sample sizes: For orthotopic pancreatic and orthotopic breast cancer models, we used n = 5 per group, following the references (Zhou Q., et al. *Nat Nanotechnol.* 2019, 14(8): 799-809 and Encarnación-Rosado J, et al. *Nat Cancer.* 2024, 5(1): 85-99), both of which evaluated antitumor and antimetastatic efficacy by dissecting all mice at the end of treatment. For the subcutaneous regular-tumor and large-tumor breast cancer models, we used n = 6 per group, drawing on the references (Jin H, et al. *Nature* 2021;595:730-734 and Saha T., et al. *Nat. Nanotechnol.* 2022, 17: 98-106). In these studies, one mouse from each group was selected for dissection, tumor photography, and histological analyses (e.g., TUNEL and H&E), while the remaining five mice per group were used for survival curve analysis.

For biosafety, we have performed a series of additional assessments. We conducted hematology, serum biochemistry (including ALT, AST, Cr, and BUN), as well as blood coagulation assays (PT and APTT). These results demonstrated that nanodrugs do not induce significant systemic toxicity or coagulation abnormalities (Extended Data Fig. 10). We obtained biodistribution data for major organs, including the liver and intestine. Cholesterol modification induced transient intestinal accumulation of PtD-Chol at 2 h post-injection, which was significantly reduced by 12 h, with no liver enrichment or histopathological damage observed after repeated dosing. Oral administration of EZE (an NPC1L1 inhibitor) could partially eliminate this intestinal retention, without compromising tumor targeting or antitumor efficacy (Extended Data Fig. 6f-i). These additional experiments confirm the

favorable safety profile of our nanomedicine in the context of liver and intestinal exposure. We have now included these data in the revised manuscript.

5.Multiple places use 0.02–0.04 g/L to report concentrations; please standardize to μg/mL or μM and indicate this in the figure legends.

Thank you for your kind suggestion. In the revised manuscript, we have converted all concentration values from g/L to μg/mL as recommended, and have indicated the unit (μg/mL) in both the figure legends and the Methods section.

6.The Methods already detail raw materials, polymerization, and probes (RAFT, FRET, antibodies, etc.). In the characterization section, please add a statement on between-batch consistency of the materials, and provide curves or tables demonstrating batch-to-batch reproducibility either in the main text or the Supplementary Information.

Thank you for your kind suggestion. In the revised manuscript, we have added a statement regarding between-batch consistency in the Characterization section (lines 155-157). Tables demonstrating batch-to-batch reproducibility are now provided in Supplementary Information Table 2.

7.Because NPC1L1 is a clinical drug target (EZE is an approved medicine), please highlight in the Discussion potential drug–drug interactions, the impact of dosing sequence or co-administration on efficacy, and briefly outline tissue-specific risks arising from intestinal/hepatic expression together with mitigation strategies.

Thank you for this insightful suggestion. Following your recommendation, we have now performed additional in vivo experiments in which oral EZE was pre-administered before PtD-Chol injection. The results shown that pre-oral administration of EZE effectively abolished the transient intestinal accumulation of PtD-Chol caused by cholesterol modification, without compromising PtD-Chol tumor accumulation or antitumor efficacy. These findings not only address the safety concern but also reveal a potential strategy to mitigate off-target intestinal retention. In the revised Discussion, we have added a paragraph (lines 667-674) to illustrate the clinical translation potential of this nanodrug, specifically the NPC1L1-targeting strategy and its combination with EZE.

8.Many figure legends state “representative images, repeated three times, mean ± SD, t-test.” For key conclusions, please add bar plots overlaid with individual data points and report exact P values.

Thank you for your kind suggestion. We would like to clarify that in our figure legends, “representative images” refer to the imaging data, while the “mean ± SD, t-test” and replicate information correspond to the quantitative bar plots. For key conclusions, we have now added additional quantitative analyses, including co-localization coefficient analysis (Extended Data Fig. 2g-j), quantification of fluorescence intensity in major organs (Extended Data Fig. 6g-i), and positive cell counting in tissue sections (Fig. 6l, Fig. 7m and Fig. 8q). These data are

presented as bar plots overlaid with individual data points, with exact P values reported in the revised figures and legends.

9.Although the figure legends define abbreviations (EZE, BFA, MON, REC/PM, etc.), please also spell out the full terms at their first occurrence in the main text to improve readability.

Thank you for your suggestion. In the revised manuscript, we have now spelled out the full terms for all abbreviations (EZE, BFA, MON, REC/PM, etc.) at their first occurrence in the main text (Lines 271, 220-222, 248).

10.For the animal experiments, please add standard elements such as the ethics approval ID and housing/environmental conditions.

Thank you for your comment. In the revised manuscript, we have added the standard elements for animal experiments, including the ethics approval ID and housing/environmental condition (Lines 1207-1210).

11.A hemolysis assay has been performed; in the Supplementary Information, please provide positive/negative controls and thresholds, and include coagulation/hemolysis parameters—or acknowledge this limitation in the Discussion.

Thank you for your suggestion regarding the hemolysis and coagulation assays. In the revised manuscript, we have now provided comprehensive data in the Method.

For the hemolysis assay, positive control (2% Triton X-100), negative control (PBS), and a hemolysis threshold of 2% have been included. The hemolysis rate was calculated as (Asample − Anegative)/(Apositive−Anegative) × 100%, with the negative control defined as 0% hemolysis and the positive control as 100% hemolysis (Lines 925-935).

For the coagulation assay, platelet-poor plasma (PPP) from healthy female BALB/c mice was used. Positive control was heparin (1 IU/mL), negative control was PBS (Lines 940-946).

Additionally, we have updated the figure legends to explicitly indicate the positive and negative controls. Specifically, the positive control (2% Triton X-100) and negative control (PBS) for the hemolysis assay, as well as the positive control (heparin) and negative control (PBS) for the coagulation assay, are now clearly stated in the respective legends (Extended Data Fig. 10f-h). We are deeply grateful for your guidance, which has helped improve the clarity and completeness of our work.

Reviewer #3 (Remarks to the Author):

This study addresses the issue of poor nanodrug penetration in stroma-rich tumor tissues by developing a cholesterol surface-displayed dendrimeric cisplatin nanodrug, PtD-Chol. This nanodrug simultaneously targets NPC1L1 on both tumor cells and cancer-associated fibroblasts. A series of experiments validated the transcytosis pathway and its deep penetration into tumors. Furthermore, the therapeutic efficacy and its ability to inhibit metastasis were demonstrated using in vivo breast cancer and pancreatic cancer models. Although the studies are well-designed and the results are impressive, there are still several major issues that need to be addressed.

We are sincerely grateful for the time you have dedicated to reviewing our manuscript. We deeply value your recognition and guidance regarding this work. All comments and suggestions have been thoroughly considered and incorporated into this revised manuscript.

1. NPC1L1 is highly expressed in the intestine (particularly the small intestine) for dietary cholesterol absorption. When analyzing the in vivo distribution of PtD-Chol, it is recommended to supplement data on its accumulation in intestinal tissues (ex vivo fluorescence imaging or histological staining) and discuss the potential intestinal toxicity or other side effects. This is crucial for evaluating the clinical safety profile of this targeting strategy.

We appreciate your concern regarding the potential safety issues related to intestinal and hepatic accumulation of our nanomedicine. To address this, we have performed a series of additional studies. Biodistribution analysis at various time points revealed that, due to the cholesterol-displayed, PtD-Chol transiently accumulated in the intestine at 2 hours post-injection (but not in the liver), and this signal was significant reduced after 12 hours (Extended Data Fig. 6f-i). Importantly, after six repeated injections, H&E staining of the liver and intestine showed no signs of tissue damage (Extended Data Fig. 10j). We also investigated a practical intervention to reduce this transient intestinal retention. Pre-treatment with oral ezetimibe (EZE), a clinically approved NPC1L1 inhibitor that blocks intestinal cholesterol uptake, completely prevented the intestinal enrichment of PtD-Chol without affecting its tumor-targeting ability or therapeutic outcome (Fig. 6 and Extended Data Fig. 6f-i). Together, these results support the favorable safety profile of this nanodrugs and highlight the potential of combining it with EZE to further minimize any intestinal exposure while preserving antitumor efficacy.

2. Regarding the NPC1L1-mediated transcytosis pathway (via the ERC-ER-Golgi pathway) that shuttles PtD-Chol across the barrier in both tumor cells and fibroblasts: the schematic diagram (Fig. 1c) depicts all internalized PtD-Chol being transcytosed. However, in reality, a portion of the internalized PtD-Chol is likely retained within the cell. It is necessary to determine the proportion of cell-internalized PtD-Chol that is retained intracellularly versus the fraction that is released via transcytosis.

We appreciate your raising this important point. The original schematic diagram shown in Fig. 1c inadvertently implied that all internalized PtD-Chol undergoes cross-multicellular redistribution, which is indeed not the case. To address this, we have revised the schematic to more accurately reflect that a portion of the internalized nanodrug is retained within cells while the remaining fraction is released via cross-multicellular transport. Furthermore, we performed additional flow cytometry experiments to quantify the ratio of PtD-Chol that is transported versus that retained intracellularly. The results shown that the ratio of the transport fraction (delivered to recipient cells) to the intracellularly retained fraction (remaining in donor cells) is approximately 3:5. These data have now been incorporated into the revised manuscript (Extended Data Fig. 3d).

3. In Figures 3d-f, after the removal of the PtD-Chol-containing medium, transcytosis requires secretion into the medium before uptake by other cells. This implies that a portion of PtD-Chol should be present in the medium. However, the intracellular fluorescence intensity shown in the figure (0-4h) does not change. Please explain this observation.

Thank you for raising this important question. In this experiment, after the removal of the PtD-Chol-containing medium, the processes of cellular uptake and transport continue simultaneously. This means that each cell acts as both a donor and a recipient. Specifically, cells that have internalized PtD-Chol can exocytose the nanodrug into the extracellular medium, while at the same time, they can take up PtD-Chol that has been exocytosed by other cells or even by themselves. This establishes a dynamic equilibrium of continuous exocytosis and re-uptake.

However, it is important to note that the overall uniformity of fluorescence distribution among cells does change over time. Quantitative analysis of 30 individual cells revealed that while the average intensity remained steady, the cell-to-cell homogeneity gradually decreased. Moreover, we performed flow cytometry to measure the mean fluorescence intensity (MFI) of the cell population during this period. The results confirmed a gradual loss of total fluorescence over time, indicating that not all exocytosed PtD-Chol is recaptured; some fraction is diluted or lost in the medium. These additional data have now been incorporated into the revised manuscript (Extended Data Fig. 3c).

4. In Fig.4h-i, the labels (i, ii, iii) should be clearly marked to correspond with the numbering used in the manuscript.

Thank you for pointing this out. In the revised manuscript, we have clearly marked the labels (i, ii, iii) in Fig. 4h-i to correspond with the numbering used in the text.

5. Lines 454-456 describing the inhibition of spheroid growth by PtD-Chol, the phrasing "resulting in a final volume increase of only 55%" could be ambiguous. For greater accuracy and clarity, this should be more precisely described as "decreasing to 55% of its initial volume" or "resulting in a final volume that was only 55% of the initial volume." The current phrasing

is ambiguous.

Thank you for your kind reminder. We have revised the ambiguous phrasing (Lines 533-535 in the revised manuscript) as you suggested. The sentence now clearly states that the spheroid volume decreased to 55% of its initial volume.

Reviewer #4 (Remarks to the Author):

The manuscript by Peiyi He and colleagues entitled “NPC1L1-mediated transcytosis enables nanodrug delivery across CAF barrier to restore antitumor efficacy in triple-negative breast cancer and pancreatic cancer” reports on the development of PtD-Chol, a cholesterol-surface displayed dendrimeric cisplatin nanodrug engineered to exploit transcytosis for deep tumor penetration. Authors show that the strategy facilitates efficient tissue penetration through NPC1L1-mediated cellular uptake coupled with multicellular transcytosis. This improved tumor-penetration capacity translates into potent anti-cancer effects in both in vitro and in vivo models of breast and pancreatic cancer.

This manuscript describes very relevant and robust findings that can set the stage for original treatment improving cancer treatment. However, authors should provide few additional clarifications to this study.

We sincerely appreciate the time you have dedicated to reviewing our manuscript. We are deeply grateful for your recognition and guidance regarding this work. All comments and suggestions have been thoroughly considered and incorporated into this revised manuscript.

In the Introduction section, please avoid referencing data/results that are described later in the Results. Instead, expand on the problem and the current state of the art prior to this study, including existing knowledge about NPC1L1 in the clinical and oncological settings

Thank you for your kind suggestions regarding the Introduction section. In the revised manuscript, we have expanded the background on NPC1L1 (Lines 65-71). At the same time, we have removed references to the results from the Introduction, as you recommended. We now present only a brief overview of the main work and the schematic diagram (Fig. 1a) at the end of the Introduction.

Line 104/Line 160: The current structure of the two first sections of Results is confusing, as the first section describes the development of the drug targeting NPC1L1, but the relevance of targeting NPC1L1 appears only in the following section. A reorganization would improve clarity.

We appreciate for you pointing out the confusing structure in the first two sections of the Results. In the revised manuscript, we have reorganized these sections to improve clarity. Specifically, the first subsection now begins with an analysis of NPC1L1 expression in various cell lines, followed by the characterization of the NPC1L1-targeted nanodrug and the demonstration of its NPC1L1-dependent efficacy. The content originally appearing in the second section has been integrated accordingly. As a result, the relevance of targeting NPC1L1 is now presented upfront, making the logical flow more coherent (Lines 108-196).

Line 105: “Given the highly hydrophobic nature of cholesterol, it tends to localize within the hydrophobic core of conventional nanoparticles, thereby restricting surface display and impairing molecular recognition functions.” Please provide references supporting this statement.

Thank you for pointing this out. We have now added appropriate references to support the statement regarding the hydrophobic nature of cholesterol and its impact on surface display and molecular recognition (see Lines 114-116 in the revised manuscript).

Line 151: “The cisplatin drug release profiles from the nanodrugs were evaluated under a reducing condition that mimics the tumor microenvironment (5 mM ascorbic acid, pH 7.4).” Please provide data supporting that this condition mimics the tumor microenvironment and to what extent.

Thank you for your comment. The release condition (5 mM ascorbic acid, pH 7.4) was chosen based on some published works (e.g. Li H., et al. *Proc Natl Acad Sci U S A*. 2016, 113(15): 4164-4169). To further validate the suitability of this condition, we also performed cisplatin release experiments under glutathione (GSH)-responsive conditions, which are widely recognized to reflect the intracellular reducing milieu. The release profile obtained under GSH was highly consistent with that observed under ascorbic acid, confirming that ascorbic acid effectively recapitulates the tumor-relevant reducing environment. These data have been added to the revised manuscript (Extended Data Fig. 1r).

Figures 2 and 3 demonstrate extensive accumulation of PtD-Chol in the cytoplasm of target cells. However, given that platinum-based drugs act on DNA, it is important to clarify whether PtD-Chol is able to penetrate the nucleus. Additionally, the authors should provide quantitative data on how much platinum is retained within the cells relative to the fraction of the compound undergoing exocytosis.

We appreciate your insightful questions regarding the nuclear penetration of PtD-Chol and the quantitative analysis of platinum retention versus exocytosis.

First, to determine whether PtD-Chol can enter the nucleus, we isolated cell nuclei and measured the associated PtD-Chol signal. A measurable amount of PtD-Chol was detected within the nucleus, which may be attributed to its small hydrodynamic size (~8 nm). Furthermore, time-course analysis revealed that while the fluorescence signal of PtD-Chol gradually decreased, the total platinum content in the nuclei increased over time. This suggests that a fraction of cisplatin was released from the nanodrugs and subsequently localized in the nucleus (Extended Data Fig. 5j,k).

Second, to quantify the balance between intracellular retention and exocytosis during cross-multicellular transport, we measured the platinum content retained inside donor cells versus that released into the extracellular medium. The results shown that a fraction of platinum remains inside the cells even when most of the PtD-Chol has been transported (Extended Data Fig. 3b). Importantly, we also extracted DNA from PtD-Chol-treated cells and confirmed that platinum binds to cellular DNA, demonstrating that the released cisplatin exerts its pharmacological effect (Extended Data Fig. 5l). Together, these results clarify that during cross-multicellular transport, PtD-Chol releases cisplatin intracellularly, leading to sustained platinum accumulation in the nucleus, while a portion of the nanodrugs is exocytosed for intercellular transfer. These quantitative data have now been included in the revised manuscript.

Line 271: “Transcytosis has held considerable promise for enhancing nanodrug penetration in solid tumors. However, its efficacy is predominantly confined to cell-to-cell interactions between tumor cells.” Please provide references supporting this statement.

We appreciate your comment. We have now added appropriate references to support the statement “Transcytosis has held considerable promise for enhancing nanodrug penetration in solid tumors. However, its efficacy is predominantly confined to cell-to-cell interactions between tumor cells.” (see Lines 285-286 in the revised manuscript).

Line 346: “To evaluate penetration through CAF-rich barriers….”. In Figure 5d, α-SMA staining indicates only minor CAF infiltration within the tumor, making this specific breast cancer model unsuitable for the authors’ claim. Could the authors provide data from a breast cancer model with more substantial CAF accumulation in the tumor (similar to the pancreatic cancer model presented later (ie. extended Figure 8)?

We appreciate your insightful comment. Indeed, using a tumor model with more abundant CAFs would provide stronger evidence for the ability of the nanodrug to penetrate the CAF barrier, which was insufficiently considered in our previous manuscript. To address this, we have now performed additional penetration experiments using a breast cancer model with enriched CAFs, specifically by co-inoculating 4T1 cells with NIH-3T3 fibroblasts to generate a mixed tumor model. The results demonstrated that PtD-Chol effectively penetrated the CAF-rich barrier, consistent with our previous findings. These new data have been included in the revised manuscript (Fig. 5k).

On another note, Figure 5c suggests colocalization of CD31 and PtD-Chol (overlap of red and green staining produces yellow). In contrast, α-SMA and PtD-Chol colocalization is not evident in Figure 5d. Does PtD-Chol penetrate tumor endothelial cells in vivo more effectively? Could this enhance treatment penetration? Please provide data comparing PtD-Chol uptake in endothelial cells, cancer cells, and CAFs in the tumor in vivo.

We appreciate your careful observation and insightful questions. Regarding Figure 5d (Fig. 5k in revised manuscript), we acknowledge that the colocalization between α-SMA and PtD-Chol (along with NPC1L1) is not as visually apparent as the dual-color colocalization shown in Figure 5c (Fig. 5h in revised manuscript). This is primarily because Fig. 5k is a four-color image; triple-color colocalization is intrinsically less conspicuous than the yellow merge from two channels. To address this, we have now included line-scan intensity profiles for the indicated arrow positions in the revised figure. These profiles clearly demonstrate that α-SMA, PtD-Chol, and NPC1L1 are indeed colocalized (Fig. 5l,m), even though the pseudocolor makes the overlap less striking to the eye.

Regarding the comparative uptake of PtD-Chol among different cells in vivo, we performed flow cytometry to quantify nanomaterial accumulation in endothelial cells, CAF, and tumor cells within the tumor microenvironment at 2 hours and 12 hours. The results show that uptake by tumor endothelial cells did not change significantly over the two time points examined. In contrast, PtD-Chol accumulation in CAFs and tumor cells increased over time, with the majority of PtD-Chol eventually localizing in these two populations (Extended Data

Fig. 6k,l). This indicates that while endothelial cells serve as a vascular entry point and transiently deliver the nanodrug into the tumor, the subsequent cross-multicellular transport and retention occur predominantly within CAFs and tumor cells. Collectively, these data suggest that PtD-Chol can traverse the endothelial barrier and then be efficiently taken up by perivascular CAFs and tumor cells, thereby facilitating deep tumor penetration.

Line 366: “The survival benefit was equally remarkable…”. Was the survival endpoint based solely on tumor size, as described in the Methods? The authors should clarify whether humane endpoints, such as body weight loss, reduced mobility, or other clinical signs, were also considered. Integrating these measures ensures both ethical treatment of animals and accurate interpretation of survival data.

Thank you for your important remark regarding the survival endpoint. As originally described in the Methods, our survival analysis was based solely on tumor size. In the current study, we did not incorporate humane endpoints (e.g., body weight loss, reduced mobility, or other clinical signs), which represents a limitation of this work. According to your instructions, we have revised the relevant sentence in the manuscript from “The survival benefit was equally remarkable…” to “The prolongation of survival time was equally significant.” We fully agree that future studies should integrate additional humane endpoints to ensure ethical animal treatment and more accurate interpretation of survival data.

Line 369: “the treatment regimens across all groups showed no adverse effects on the functions of the heart, liver, spleen, lungs, or kidneys indicating negligible systemic toxicity.” Of note, NPC1L1 is highly expressed in the intestine, making it potentially susceptible to adverse effects of PtD-Chol. However, this organ was not examined. Could the authors provide data on PtD-Chol uptake and effect (eg. apoptosis) in the intestine?

We appreciate your concern regarding the intestine. In the revised manuscript, we have now provided additional experiments to address PtD Chol uptake and its potential effects in the intestine.

First, biodistribution analysis revealed that PtD-Chol exhibited a transient accumulation in the intestine at 2 h post-injection, which significantly reduced after 12 h. No significant increased accumulation was observed in the liver (Extended Data Fig. 6f-i). Second, to assess whether this transient intestinal exposure causes any pathological damage, we performed H&E staining on intestinal sections from mice that received six repeated injections of PtD-Chol (at the end of the therapeutic regimen). The results showed no signs of epithelial necrosis, villus atrophy, or inflammatory infiltration (Extended Data Fig. 10j). Finally, we explored a clinically feasible mitigation strategy: oral pre-administration of ezetimibe (EZE), an NPC1L1 inhibitor, completely abolished the transient intestinal accumulation of PtD-Chol without affecting its tumor targeting or efficacy (Fig. 6 and Extended Data Fig. 6f-i). Collectively, these data demonstrate that PtD-Chol does not cause appreciable intestinal toxicity under the tested conditions, and transient exposure can be partially reduced by co-administration with EZE.

Line 400: “PtD-Chol potently inhibited both migration and invasion….”. Please discuss the

potential reasons for the anti-migratory/invasion effect observed with this Pt-based compound.

We appreciate your question regarding the potential reasons for the anti-migratory and anti-invasive effects of PtD-Chol. As shown in the revised manuscript (Extended Data Fig. 5p-u), we have demonstrated that PtD-Chol interferes with the TGF-β and β-catenin signaling pathways, thereby inhibiting epithelial-mesenchymal transition (EMT) in tumor cells. This mechanism is responsible for the observed suppression of tumor cell migration and invasion. A detailed discussion of these results has been included in the corresponding Results sections (see Lines 448-451 in the revised manuscript).

Line 411: "The phenotypic reversion of CAFs induced by PtD-Chol ….". Please discuss the potential mechanisms underlying CAF phenotypic reversion driven by PtD-Chol.

We appreciate your question about how PtD-Chol drives CAF phenotypic reversion. It is well recognized that myCAFs are primarily activated by the TGF-β/SMAD signaling pathway. Upon TGF-β stimulation, SMAD2/3 are phosphorylated and translocate into the nucleus, promoting expression of α-SMA and other matrix-remodeling genes. Given this connection, we speculated that PtD-Chol might interfere with the TGF-β/SMAD axis, thereby pushing CAFs back toward a more normal fibroblast (NAF)-like state. To inspect this, we treated CAFs with PtD-Chol and examined key components of the pathway by western blot. The results showed that PtD-Chol markedly downregulates both the p-SMAD3/SMAD3 ratio, along with a decrease in α-SMA level. This indicates that the PtD-Chol effectively inhibits the TGF-β/SMAD signaling cascade in CAFs (Extended Data Fig. 7e-g), which contributes to the observed phenotypic reversion. A detailed discussion of these findings has been included in the corresponding Results sections (see Lines 462-463 in the revised manuscript).

Line 432: "The marked reduction in α-SMA and NPC1L1 levels following PtD-Chol treatment indicates decreased CAF infiltration and phenotypic reversion toward normal fibroblasts (Fig. 6j)." In view of the data provided, it is unclear whether the reduction in α-SMA correlates with CAF reversion to NAF, CAF clearance, or both. Please provide an objective analysis of positive cell counts to support the statement.

We sincerely appreciate the valuable comments. To distinguish whether the reduction in α-SMA correlates with CAF reversion to NAF, CAF clearance, or both. We distinguished NAFs from CAFs in the tumor by performing objective cell counting on CD34 and α-SMA staining in both the PDAC and TNBC models. The results showed a clear reduction in total fibroblasts, and more importantly, a lower proportion of CAFs within the fibroblast population. These quantitative data indicate that the reduced CAF infiltration results from both phenotypic reversion and clearance occurring simultaneously. The updated cell counts and the corresponding analysis have been added to the revised manuscript (Fig. 6l, Fig. 7m, Fig. 8q).

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.

We sincerely appreciate the time you have dedicated to reviewing our manuscript.

RESPONSES TO REFEREES:
(Reviewer comments in black, our response in blue)

Reviewer #1 (Remarks to the Author):

The reviewer appreciates the additional experiments and substantial revisions provided by the authors. However, several major conceptual concerns remain regarding the proposed delivery mechanism.

We sincerely appreciate for recognizing our additional experiments and revisions. In our point-by-point response below, we have addressed each concern in detail with further explanations and supplementary data, and we trust that our responses will resolve these issues satisfactorily. All comments and suggestions have been thoroughly considered and incorporated into this revised manuscript.

1. Concern regarding active nanoparticle transport by dying cells. It remains unclear whether the proposed active transport mechanism can operate efficiently during therapy. As treatment progresses, many donor cells are expected to undergo apoptosis or become severely damaged. It therefore remains unclear whether these dying cells retain the capacity to efficiently transport nanoparticles to neighboring cells.

We thank you for raising this critical question. We acknowledge that the potential impact of apoptosis on cellular transport capacity was not addressed in our initial study, and we are grateful for this valuable suggestion. To investigate this issue, we first induced apoptosis in hetero-spheroids using unlabeled PtD-Chol at a series of graded concentrations, generating spheroids with varying degrees of apoptosis to mimic the apoptotic states encountered during treatment. These spheroids were then incubated with fluorescently labeled PtD-Chol or PtD to assess their penetration efficiency.

Our results revealed that apoptosis indeed attenuated PtD-Chol penetration to some extent. However, prior to the onset of extensive apoptosis, the penetration of PtD-Chol remained significantly higher than that of PtD (Extended Data Fig. 7k,l). Given that this enhanced penetration was observed at early time points and was dependent on NPC1L1-mediated uptake, these findings indicate that even in the presence of apoptotic cells, the NPC1L1-mediated transport pathway remains sufficiently functional to support superior tumor penetration.

2. The authors claimed a novel cell-to-cell transport pathway (Fig. 1c; 4h), yet all of the presented experiments remain consistent with a much simpler explanation: PtD-Chol is exocytosed into the extracellular space, diffuses locally, and is subsequently internalized by neighboring NPC1L1-positive cells. The current data do not establish, in reviewer's opinion, that nanoparticles are "actively" transported from one cell to another as a "continuous" multicellular transport process (i.e., from peri-endothelial tumor cells to deep tumor cells).

We are grateful for your insightful comment. We agree that upon exocytosis, PtD-Chol diffuse in the extracellular space and may contact neighboring NPC1L1-positive cells, leading to their

subsequent internalization. Accordingly, we have revised the relevant descriptions in the manuscript and supplemented the explanations to more accurately reflect this process. Specifically, we have fully depicted the entire intercellular transport process, including the extracellular diffusion step, in the revised figure legend (Fig. 1c, lines 104-105).

We also acknowledge that among the steps involved in intercellular transport—cellular uptake, efflux, and reuptake—are all active processes, whereas extracellular diffusion is not. Therefore, the previous use of the term "active" to describe the overall transport process was not entirely precise. To address this, we have removed the term "active" from the relevant sections where it was inappropriately applied (lines 21-22).

Regarding the directional transport of PtD-Chol from peri-endothelial tumor cells to deeper tumor cells, we propose the following sequence: peri-endothelial tumor cells first take up PtD-Chol, followed by their exocytosis into the extracellular space. Although it remains possible that these same peri-endothelial cells may re-internalize a portion of the released PtD-Chol, a fraction of the released PtD-Chol nonetheless reaches and contacts the deeper tumor cells, thereby achieving transfer to cells at a greater distance from the vasculature. We believe that this description, more accurately represents the transport mechanism without overstating its nature.

3. The authors assumed that NPC1L1 expression alone is sufficient to enable cross-multicellular transport. However, receptor expression does not necessarily confer transport competence. It remains unclear whether CAFs and tumor cells possess comparable NPC1L1-mediated endocytic recycling kinetics, intracellular trafficking, exocytic efficiency, and membrane recycling capacity. Therefore, the conclusion that both cell types contribute equivalently to the proposed transport cascade is insufficiently supported.

Thank you for raising this important point regarding that NPC1L1 expression alone is sufficient to enable cross-multicellular transport. In our previous manuscript, we had already addressed this issue by performing not only NPC1L1 expression analysis but also functional assays in CAFs, including inhibition of cellular uptake with the NPC1L1 inhibitor EZE, and co-localization studies of NPC1L1 with internalized PtD-Chol (Fig. 4e-g). These experiments collectively demonstrated that CAFs, akin to tumor cells, are capable of efficient PtD-Chol uptake via NPC1L1. Subsequently, cross-multicellular transport assays confirmed that PtD-Chol could be shuttled between CAFs and tumor cells in both breast and pancreatic cancer models (Fig. 4h-i and Extended Data Fig. 4d). Furthermore, in hetero-spheroids penetration experiments, PtD-Chol also achieved effective penetration (Fig. 4k-l). These results confirm cross-multicellular transport between CAFs and tumor cells.

In response to your concern, we have performed additional experiments to provide more robust evidence. Specifically, we conducted time-course co-localization analyses of PtD-Chol with NPC1L1, ERC, ER, and Golgi, which revealed that PtD-Chol follow the NPC1L1-ERC-ER-Golgi intracellular trafficking route in CAFs (Extended Data Fig. 3m-o). We also supplemented exocytosis assays showing that CAFs, like tumor cells, possess comparable efflux and reuptake capabilities, enabling efficient PtD-Chol recycling (Extended Data Fig. 3k-l). Furthermore, we performed additional inhibition experiments using NPC1L1 inhibitors EZE

and the ER-Golgi trafficking inhibitor BFA, which confirmed that CAFs share the same cross-multicellular transport pathways as tumor cells. We hope that these supplementary data adequately address your concerns.

4. The TEM images presented in Fig. 4j are difficult to interpret due to their limited resolution. How did the authors confirm that the observed electron-dense black dots indeed represent PtD-Chol nanoparticles?

We thank you for raising this important technical concern. For cell TEM imaging, we used the platinum (Pt) element within PtD-Chol for visualization. The use of noble metals such as gold, silver, and platinum for TEM imaging of cells is well established in the literature (see Fig. 1 below). However, given that the PtD-Chol are approximately 2-4 nm in diameter, the imaging resolution is indeed limited compared to that of larger noble metal nanoparticles. To validate our assignment of these electron-dense dots, we imaged untreated cells as a negative control (Fig. 2 below). In contrast, cells incubated with PtD-Chol displayed numerous electron-dense black dots within the ER and Golgi, consistent with the intracellular trafficking route we observed by confocal microscopy, which were absent in the control group. These observations strongly support that the electron-dense black dots correspond to internalized PtD-Chol.

[editorial note: third party material redacted]

Fig. 1

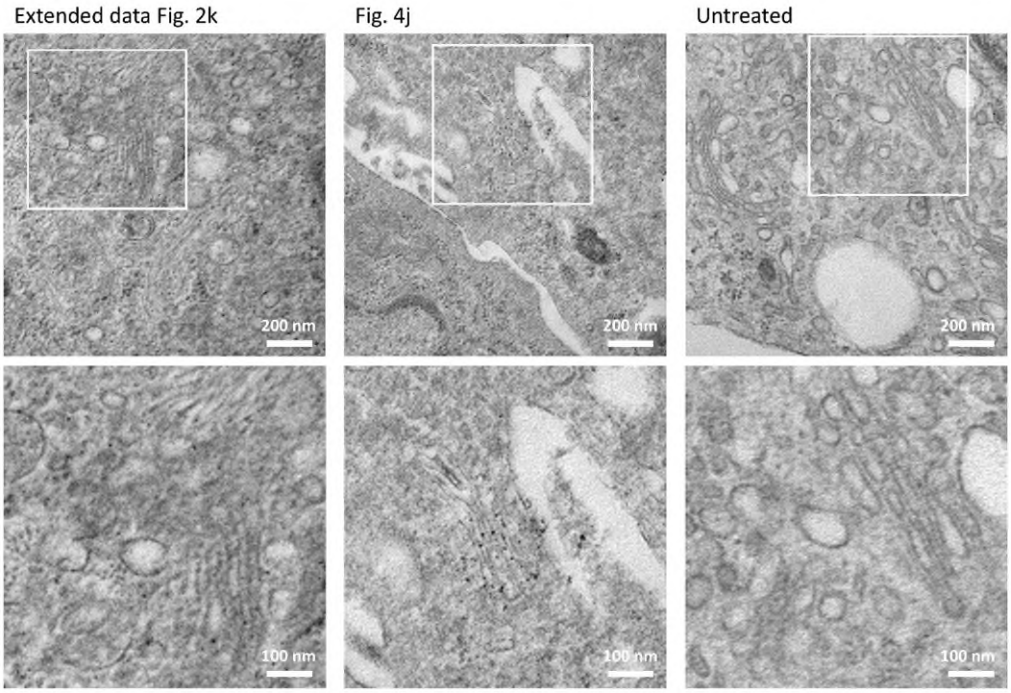


Fig. 2

Reviewer #2 (Remarks to the Author):

I am pleased to have the opportunity to review this manuscript again. The authors have adequately addressed my previous concerns, which has substantially improved the completeness and overall value of the manuscript. I therefore recommend its acceptance.

We sincerely thank you for your encouraging evaluation and for recognizing the improvements we have made. Your constructive comments throughout the review process have been truly invaluable.

Reviewer #4 (Remarks to the Author):

The authors have addressed my previous comments and concerns. I appreciate their responses and the substantial revisions made, which have improved the quality and clarity of the manuscript.

We deeply appreciate your favorable assessment and your recommendation for acceptance. Your insightful comments had significantly strengthened our manuscript, and we are grateful for the time and effort you have devoted to this review.

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.

We sincerely thank you for your encouraging evaluation and for recognizing the improvements we have made. Your constructive comments throughout the review process have been truly invaluable.

RESPONSES TO REFEREES:
(Reviewer comments in black, our response in blue)

Reviewer #1 (Remarks to the Author):

The reviewer appreciates the authors' efforts to address the concerns by incorporating additional experimental data, which have strengthened the manuscript. The new in vitro results provide further support for the occurrence of the observed transcytosis phenomenon among apoptotic cells under cell culture conditions. However, an important question is the robustness and relevance of this effect in a therapeutic setting. Moreover, the proposed mechanism appears to serve as a central premise throughout the manuscript (e.g., Fig. 1) - whether this mechanism is indeed the dominant contributor to the observed effects.

We sincerely appreciate your favorable evaluation of our revised manuscript, as well as your thoughtful comments regarding the key mechanistic and translational issues.

Regarding the robustness and relevance in a therapeutic setting: We agree that the in vitro observation needs to be examined in more complex therapeutic contexts. In this work, we reported multiple in vivo outcomes—including tumor growth inhibition, prolonged survival, and reduced metastasis—that collectively highlight the robust therapeutic efficacy of PtD Chol against CAF-rich solid tumors, as demonstrated in TNBC and PDAC models, even when challenged with large tumor burdens. These findings reinforce the clinical relevance and translational potential of this mechanism.

Concerning the dominant contribution of the proposed mechanism: We acknowledge that multiple transport routes could, in theory, contribute to the overall internalization and trafficking of PtD Chol. However, our mechanistic dissection—encompassing pharmacological inhibition (e.g., ezetimibe) and organelle trafficking blockers—consistently abolished both the deep tumor penetration and the therapeutic efficacy of PtD Chol. These loss-of-function results, together with colocalization data tracing CMCT along the ERC–ER–Golgi axis, collectively indicate that NPC1L1-mediated CMCT represents the predominant—if not exclusive—pathway responsible for the observed antitumor activity. We have now explicitly articulated this conclusion in the Results section and clarified the strength of the evidence in the Discussion, while also acknowledging that minor parallel pathways may exist. Additionally, we have included a limitation statement in the Discussion, recognizing the potential contribution of alternative routes and outlining directions for future investigation. We hope our additional explanation could address your concerns.

Reviewer #2 (Remarks to the Author):

The authors have addressed all the comments and concerns. I recommend publication.

We are most grateful for your favorable assessment and for acknowledging the refinements we have incorporated. The insightful suggestions you offered during the review have been immensely helpful.