nature portfolio

Peer Review File

Surface-engineered dual drug-loaded tumor-targeted liposomal nanoparticles to overcome the therapeutic resistance in glioblastoma multiforme.

Corresponding Author: Professor Debabrata Mukhopadhyay

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)
Overall, the main claims of the paper seem well justified by the data provided though there are some areas of clarification that could solidify the work presented here, as well as some other minor comments. Though the most important aspect, the novel tumor targeting peptide, is proprietary, it does appear to provide a tumor localization benefit. The drugs studied here have been used in limited ways before for GBM treatment though have not previously demonstrated benefit over standard of care. It is interesting to observe the combination of such with an appropriate carrier can provide a benefit, though the feasibility of translating into larger animal models/humans might be challenging. The use of combination therapies to sensitive GBM to TMZ or radiation is not inherently novel though the combinations presented here have not been employed in this way to my knowledge. This work builds upon the ongoing conversation in the field to achieve BBB penetrating carriers for molecules that have had success in other cancers but have been unable to achieve efficacy through systemic administration. In terms of the transcriptome analysis, the results are somewhat interesting though many of the top identified genes have been well described previously. Of note, though only mentioned briefly was the particular interaction of the drug combinations presented with the DNA repair pathways (ATR/ATM/CHK1 etc) that have been targeted in other similar research initiatives. It would be helpful to have some additional comment on the mTOR/DNA Repair relationship in the introduction or discussion with reference to the many studies on these other combinations. Further comments for consideration:

1. I noticed conflation of LNP and liposome in some instances in the results section. Please maintain one terminology as they are slightly different structures.
2. Missing loading and encapsulation efficiency data for TTL, as well as release study – I see previous work was cited to account for this though I would expect this information to be included for the batches used in this study specifically as this takes away from the novelty as presented.
3. Is there any flow cytometry to quantify uptake differences between TTL and CL as opposed to just imaging? It would be advisable as more quantitative approach over microscopy.
4. The TEM images provided in Figure 1 appear somewhat low contrast and sample preparation method is not described in the provided methods or the methods of the work cited as a justification for not including a full methods section on TTL preparation. Furthermore, the
5. Did IVIS imaging detect any TTL uptake in other organs outside of the brain/tumor? For systemically delivered nanocarriers it is often typical to show extracted organs placed together post-experiment for direct comparison of flux
6. Was any synergy model or analysis performed on the cell line data? There was discussion of an additive effect but it was not confirmed whether there was based on a synergy analysis or just estimated for the EV-TMZ or EV-Radiation combinations
7. In cell lines, there are no E/V/EV only controls without the TTL. Could the authors address if any of the efficacy observed is attributable to the enhanced uptake due to TTL or would naked drug in cell culture behave the same way as loaded TTL?
8. For GBM 22 and GBM 1Z, was the MGMT promoter methylation status confirmed or is there another feature that causes TMZ resistance in these particular PD cell lines? I attempted to find this information in the authors' cited previous work however I did not have access, so it would be helpful to include a statement on this.
9. IDH wild and mutant status not mentioned in the results section when discussing QNS120 and QNS108 which seems relevant from the included Figure S3

10. Why did the authors not perform western blot to measure p-CHK2 alongside the other DNA repair markers? I would expect it to also have been affected due to the lower levels of p-ATR and p-ATM after EV administration
11. No TMZ + Radiation controls were used in the cell-based or in vivo experiments which is important context as it is the SOC typically (as the authors acknowledge multiple times). How would a reader know from the data presented that the EV+radiation combination is better than the concomitant TMZ+radiation SOC?
12. What happened to 5th mouse in TTL-EV+R group between D0 and D15?
13. In Figure 5, plotting the average total photon flux of each group on the same graph would be a more useful way to compare the tumor growth than graphs of the individual groups with each animal
a. The Y axis of these graphs goes from 0 to 5x10^8 immediately and makes it look like the flux is 0 at certain time points which does not appear to be true from the images. Please reformat the Y-axes to be more representative
14. There was no included transcriptomic data on the E or V molecule treatments or, in particular, radiation alone as compared to the combination to understand the contribution of each component to the observed genetic regulation changes. The former is perhaps not necessary for inclusion, though with regard to the latter, it is possible that many of the induced changes were just from radiation based on the data presented.
15. In the discussion second paragraph 'Vinorelbine also has shown relapsing GBM' – a word is probably missing here?
16. The discussion mentions possibility of EV combination with TMZ could help reduce chemo-resistance, which was somewhat supported by the in vitro data, though the in vivo data did not show any benefit
17. Citation numbers in the methods section are not superscript (I think this happened to all superscript numbers in the methods section and may be a compiling issue)

Reviewer #2

(Remarks to the Author)
Summary
Angom, Rachamala et al. present a study investigating tumor-targeted liposomal nanoformulations (TTL) loaded with various chemotherapeutic agents (everolimus, vinorelbine, rapamycin) alone or in combination (TTL-EV, TTL-RV) for the treatment of glioblastoma (GBM). The formulations were evaluated in orthotopic GBM mouse models in combination with standard-of-care radiation and temozolomide. The authors report enhanced tumour uptake, improved survival, and transcriptomic changes implicating pathways related to immune response, DNA damage repair, and metabolism. The data suggest TTL-based combinatorial therapy may sensitize GBM tumours to radiation and offer a novel strategy to overcome resistance.
The manuscript presents a well-rationalized approach combining nanomedicine and synergistic therapy to enhance GBM treatment. The authors provide promising preclinical data on drug delivery, tumour suppression, and molecular pathway modulation. However, key methodological details, statistical analyses, and appropriate controls are missing or insufficient in several figures, limiting the robustness of the conclusions. Clarification and further validation are required to both support the authors conclusions and provide clarity on the relevance of these results.

Specific comments
Major Comments
1. Lack of Comparison to Free Drugs: A fundamental issue is the absence of data directly comparing liposomal versus free drug administration. Demonstrating improved efficacy or reduced toxicity through liposomal delivery is critical to support the central claim. The authors should explicitly include or discuss such comparisons in vivo.
2. Figure 2A – Although the authors show enhanced tumour-specific uptake of TTL-liposomes, only representative images are shown. Quantitative analysis of uptake (eg. % positive cells or mean fluorescence intensity) across multiple biological replicates is necessary, with appropriate statistical analysis to support reproducibility.
3. Figure 2J–K –The Raman data are unconvincing. The signal appears to co-localize with brain regions such as white matter rather than clearly demarcated tumours. Key information is missing:
a. Brain orientation and tumour injection site must be indicated.
b. A negative control (Raman scan from animals without liposome treatment) is required to confirm specificity.
c. Scanning of brain regions outside the tumour, or non-tumour-bearing mice, would help validate that the mCherry signal corresponds specifically to the nanoparticles.
4. Figure 3 – The number of replicates or independent repeats is not stated. While the results are promising, reproducibility must be demonstrated with statistical analysis.
5. Figure 4G–H –The legend states that results are based on three independent experiments, but supporting quantification (eg. densitometry relative to Actin) is not provided. Bar graphs showing mean ± SD across replicates would enhance data interpretation.
6. Figure S4 – Quantification across biological replicates should be provided. The figure currently appears to show only one experiment.
7. Figures 5B–E, S6, S8 – Individual mouse data are well-presented; however, group-level statistical analysis (eg. ANOVA for BLI signal/tumour size) is essential to confirm therapeutic benefit is not just by chance.
8. Figure 6 & Supplementary (S9–S12): The number of animals used and the time points post-treatment in this transcriptomic analysis are unclear. Although the text states the experiments were "repeated 3 times," it is essential to report the actual sample size (n) and clarify if these represent biological replicates.
9. GEPIA Analysis – Confusion Between Mouse and Human Data: The transition from murine in vivo data to human TCGA datasets via GEPIA is insufficiently explained. The manuscript does not clarify that GEPIA data are from human patients, which may confuse readers. The rationale for integrating these datasets and how they validate or complement the in vivo findings should be explicitly discussed.
10. Figures S13–S16 – The link between gene expression data and therapeutic efficacy is not well explained. The

suggestion that these minor transcriptomic changes override the known cytotoxic effects of the drugs is speculative and may overstate the significance of these data.
11. Several differentially expressed genes described in lines 313-319 (eg. CD3e, CD7, IL2R) are T-cell specific, but the authors then compare this to their mouse data which used immunodeficient (nude) mice that lack T cells. Interpretation of immune modulation in this context is inappropriate and should be revised.

Minor comments
1. Line 119 –The authors should define “LNP” upon first use. I would encourage the authors to consider avoiding this abbreviation entirely to prevent confusion with lipid nanoparticles (as used in mRNA vaccines), which differ significantly from liposomes.
2. The rationale for selecting these specific drug combinations (eg. everolimus + vinorelbine) should be expanded. Prior studies demonstrating potential synergy would strengthen the justification.

3. Figure S1 – I would encourage the authors to consider including the quantification from Figure S1 in the main manuscript figures, as these data are critical for evaluating tumour uptake and strongly support the authors conclusions.
4. The claim that TTL-EV impacts immune evasion should be discussed in the context of the immunodeficient model. Molecules such as PD-L1 and CXCL10 rely on T-cell interactions, which are absent in this model. The authors should acknowledge this limitation.
5. Figure 4A/ The lack of synergy of the dual-agent liposomes in the GBM1A cell line requires further discussion.
6. Figures 4C–D –I advise the authors to adjust the y-axis scaling to better visualize distribution and differences in treatment response which are currently uninterpretable.
7. Figure 5A–E –Please clarify why the treatment group was reduced to n = 4. Was this due to animal loss, exclusion, or study design?
8. Lines 315–319 –text refers to TTL-EV treated mice in relation to Figures S13 and S14, but these figures actually show GEPIA (human) data. Please clarify the correct data sources and ensure alignment between figure references and described results.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors provided sufficient additional context and experimental data to clarify most of the comments and questions. I would recommend for publication at this stage.

Reviewer #2

(Remarks to the Author)
I am satisfied that the authors have adequately and comprehensively addressed the prior comments and concerns in this revised manuscript.

Response to Reviewer #1

We thank the reviewers for their thoughtful and constructive feedback. We have addressed each concern point-by-point and revised the manuscript accordingly. Our detailed responses are provided below. Please see the response highlighted in the main text.

Overall Comment:

Though the most important aspect, the novel tumor targeting peptide, is proprietary, it does appear to provide a tumor localization benefit… helpful to have some additional comment on the mTOR/DNA Repair relationship in the introduction or discussion…

Response: *We appreciate the reviewer's overall positive evaluation. In the revised manuscript, we have expanded the discussion to address the interplay between mTOR signaling and DNA damage repair mechanisms, including references to recent studies highlighting how mTOR inhibition modulates the ATR/ATM/CHK1 pathways (see Discussion, page 20). We acknowledge the proprietary nature of the tumor-targeting ligand (TTL) and have included a clarification in the text regarding its function and prior validation.*

1. I noticed conflation of LNP and liposome in some instances in the results section. Please maintain one terminology as they are slightly different structures.

   ***Response**: We thank the reviewer for pointing this out. All references have been revised to consistently refer to our carrier system as "liposomes," which better reflects the structure used in this study. We have corrected the entire paper accordingly in the results section and figure legends.*

2. Missing loading and encapsulation efficiency data for TTL, as well as release study – I see previous work was cited to account for this though I would expect this information to be included for the batches used in this study specifically as this takes away from the novelty as presented.

   ***Response**: We thank the reviewer for this valuable suggestion. We now include encapsulation efficiency and drug loading data for each TTL–drug formulation used in this study (please see Table S3). While our formulation approach is based on prior work, we agree that batch-specific data are essential and have accordingly incorporated these results.*

3. Is there any flow cytometry to quantify uptake differences between TTL and CL as opposed to just imaging? It would be advisable as more quantitative approach over microscopy.

   ***Response:** We thank the reviewer for this insightful suggestion. As recommended, we performed flow cytometry to quantify cellular uptake of TTL compared with conventional liposomes (CL) in GBM cell lines (Fig. 2G–2R). The results demonstrated significantly higher*

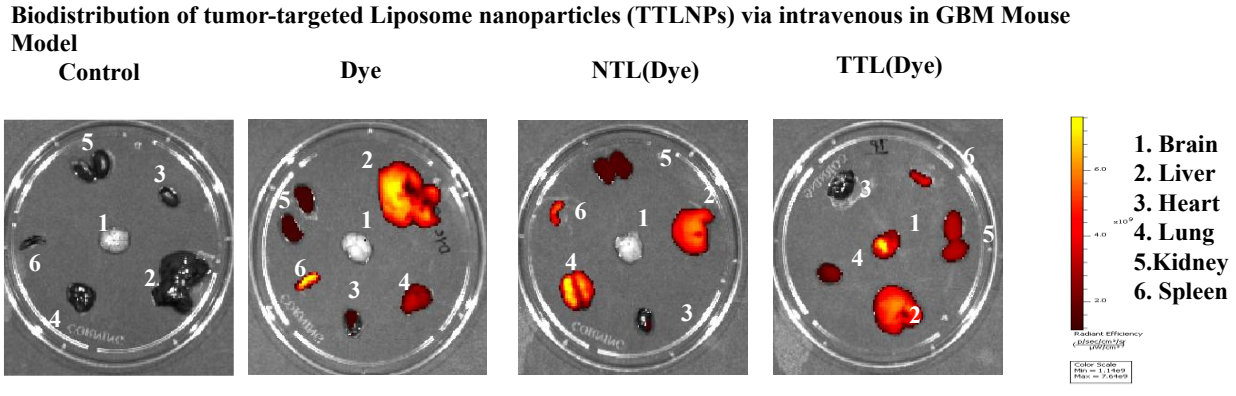
TTL uptake in both GBM22 and GBM1Z cells, consistent with and supporting our imaging findings.

4. The TEM images provided in Figure 1 appear somewhat low contrast and sample preparation method is not described in the provided methods or the methods of the work cited as a justification for not including a full methods section on TTL preparation.

Response: *We thank the reviewer for highlighting this point. We have replaced the TEM images in Figure 1 with higher-contrast versions and added detailed sample preparation methods under 'Supplementary Materials and Methods' (Supplementary Data, page 4). We apologize for the initial omission and have corrected this in both the main text and the supplementary materials.*

5. Did IVIS imaging detect any TTL uptake in other organs outside of the brain/tumor? For systemically delivered nanocarriers it is often typical to show extracted organs placed together post-experiment for direct comparison of flux.

Response: *We thank the reviewer for raising this point. For the reviewer's reference, we have provided representative ex vivo IVIS images of major organs (brain, liver, lung, spleen, and kidney) collected post-euthanasia (Figure below). Comparing fluorescence intensity across organs confirmed that, trace signals were observed in the liver; however, the accumulation in the brain tumor was significantly higher. In control animals, no signal was detected in any organ. When only free dye was injected, higher signals were observed in the liver, with trace levels in the lungs, kidney, and spleen. In contrast, animals treated with the non-targeted liposome formulation showed signal in liver, lung, kidney, and spleen and negligible in heart and brain, whereas the TTL-targeted liposome formulation accumulated predominantly in the tumor-bearing brain, confirming the specificity of TTL delivery. Please kindly see the image below.*



6. Was any synergy model or analysis performed on the cell line data? There was discussion of an additive effect but it was not confirmed whether there was based on a synergy analysis or just estimated for the EV-TMZ or EV-Radiation combinations.

Response: *We thank the reviewer for this insightful comment. Based on the Chou–Talalay equation, we observed the following: in GBM22 cells, TTLEV + TMZ yielded a combination index (CI) of 0.761, and in GBM1A cells, TTLEV + TMZ yielded a CI of 0.452. Since CI < 1 indicates synergy, these results demonstrate a synergistic effect (drugs work better together) in vitro. In GBM22 cells, TTLEV treatment without radiation also showed synergy, while in GBM1A cells, TTLEV combined with radiation demonstrated synergy as well. Please see highlighted text in the revised manuscript on page 11, Line 285-293.*

7. In cell lines, there are no E/V/EV only controls without the TTL. Could the authors address if any of the efficacy observed is attributable to the enhanced uptake due to TTL or would naked drug in cell culture behave the same way as loaded TTL?

Response*: We appreciate the reviewer's important point. To address the reviewer's concern, we have performed an in-vitro cytotoxicity assay in GBM22 cells. The unformulated everolimus (E) and vinorelbine (V), as well as their combination at different concentrations, were tested in GBM22 cell lines. We observed that the cytotoxicity of the formulated drugs was greater than that of the unformulated drugs. These new results have been updated in Fig. S5. Please see the highlighted text in page 12.*

8. For GBM 22 and GBM 1Z, was the MGMT promoter methylation status confirmed or is there another feature that causes TMZ resistance in these particular PD cell lines? I attempted to find this information in the authors' cited previous work however I did not have access, so it would be helpful to include a statement on this.

Response*: We confirm that GBM22 and GBM1A displayed TMZ resistance/sensitivity profiles. This information, with citations to relevant sources, is now included in the Results and Methods sections.*

9. IDH wild and mutant status not mentioned in the results section when discussing QNS120 and QNS108 which seems relevant from the included Figure S3

Response: *Thank you for this observation. We have added statements indicating that GBM22 is both MGMT non-methylated (Vaubel RA, et al. Genomic and Phenotypic Characterization of a Broad Panel of Patient-Derived Xenografts Reflects the Diversity of Glioblastoma. Clin Cancer Res. 2020 . PMID: 3185283. and IDH wildtype (Nguyen, T.T.T., et al. Aurora kinase A inhibition reverses the Warburg effect and elicits unique metabolic vulnerabilities in glioblastoma. Nat Commun 12, 5203 (2021), Both QNS120 and QNS 108*

are IDH-wildtype and QNS108 is MGMT unmethylated while QNS120 is methylated(Garcia CA. Functional Characterization of Brain Tumor-Initiating Cells and Establishment of GBM Preclinical Models that Incorporate Heterogeneity, Therapy, and Sex Differences. Mol Cancer Ther. 2021). Please see the highlighted text on page 7.

10. Why did the authors not perform western blot to measure p-CHK2 alongside the other DNA repair markers? I would expect it to also have been affected due to the lower levels of p-ATR and p-ATM after EV administration

Response: *We now include western blot analysis for p-CHK2 in response to EV treatment (Figure 4I and 4Q). As anticipated, levels of p-CHK2 decreased in parallel with p-ATM and p-ATR, further supporting our conclusion of DNA damage pathway suppression. Please see updated Figure 4.*

11. No TMZ + Radiation controls were used in the cell-based or in vivo experiments which is important context as it is the SOC typically (as the authors acknowledge multiple times). How would a reader know from the data presented that the EV+radiation combination is better than the concomitant TMZ+radiation SOC?

Response: *We thank the reviewer for highlighting this important point, and we do agree with the reviewer's suggestion. In this study, while we did not include TMZ + radiation controls, which represent the current standard of care (SOC), our focus was to investigate the additive or synergistic effects of radiation when combined with our targeted EV platform. Prior studies have demonstrated the limited efficacy of TMZ in resistant GBM subtypes (Hegi ME, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005), thereby underscoring the need for alternative therapeutic strategies. Future work will extend these findings by directly comparing EV + radiation against TMZ + radiation in both sensitive and resistant GBM models to more comprehensively position our approach within the context of SOC therapies.*

12. What happened to 5th mouse in TTL-EV+R group between D0 and D15?

Response: *We thank the reviewer for drawing attention to this point. The 5th mouse in the TTL-EV + R group was euthanized on Day 7 due to ulcerative dermatitis unrelated to treatment. This information has now been clearly noted in the revised figure legend on page 29.*

13. In Figure 5, plotting the average total photon flux of each group on the same graph would be a more useful way to compare the tumor growth than graphs of the individual groups with each animal

a. The Y axis of these graphs goes from 0 to 5x10^8 immediately and makes it look like the flux is 0 at certain time points which does not appear to be true from the images. Please reformat the Y-axes to be more representative

Response: *Thank You. We revised Figure 5 to include a single graph comparing the average photon flux of each group over time. Y-axis scaling has also been corrected to accurately reflect flux values, eliminating the appearance of a zero signal. Please see updated Figure 5.*

14. There was no included transcriptomic data on the E or V molecule treatments or, in particular, radiation alone as compared to the combination to understand the contribution of each component to the observed genetic regulation changes. The former is perhaps not necessary for inclusion, though with regard to the latter, it is possible that many of the induced changes were just from radiation based on the data presented.

Response: *We thank the reviewer for this insightful comment and fully agree that dissecting the contribution of radiation alone is important for interpreting the transcriptomic changes observed in the combination treatment. While we did not include radiation-only groups in our transcriptomic analysis, we performed targeted validation of selected genes using RT-based methods. These data indicate that some gene expression changes are indeed attributable to radiation alone; however, several key pathways including DNA repair and mTOR signaling were specifically altered in the combination group, suggesting a distinct regulatory effect of the EV + radiation treatment. We acknowledge the value of more comprehensive comparisons in future studies.*

15. In the discussion second paragraph 'Vinorelbine also has shown relapsing GBM' – a word is probably missing here?

Response: *Vinorelbine has also shown relapsing GBM in 19-year-old female patients diagnosed with GBM located in the deep temporal region.*

16. The discussion mentions possibility of EV combination with TMZ could help reduce chemo-resistance, which was somewhat supported by the in vitro data, though the in vivo data did not show any benefit.

Response: *We agree and have updated the discussion to clarify that, while EV+TMZ showed in vitro synergy, in vivo benefit was not observed. We suggest this may relate to pharmacokinetic limitations and note that additional optimization is needed for this combination.*

17. Citation numbers in the methods section are not superscript (I think this happened to all superscript numbers in the methods section and may be a compiling issue)

Response: *This issue was due to a reference style conflict during compilation. We have now corrected all citation numbers in the Methods section to be properly superscripted.*

We hope that the revisions and additions fully address your comments and further strengthen the manuscript. We are deeply grateful for your thorough review and valuable suggestions, which have significantly improved the quality and clarity of our work.

Response to Reviewer #2

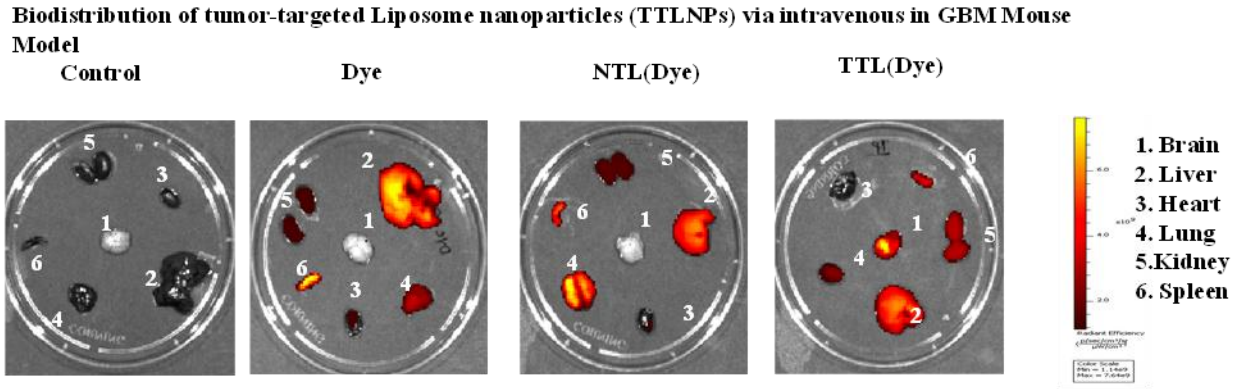
We thank the reviewers for their careful evaluation and valuable feedback. We have revised the manuscript and supplementary materials in response to the comments below. All major and minor concerns have been addressed to improve clarity, rigor, and scientific context. Please see our point-by-point response below.

Major Comments

1. Lack of Comparison to Free Drugs: A fundamental issue is the absence of data directly comparing liposomal versus free drug administration. Demonstrating improved efficacy or reduced toxicity through liposomal delivery is critical to support the central claim. The authors should explicitly include or discuss such comparisons in vivo.

Response: *We appreciate the reviewer's insightful comment. In response, we have now included in vitro comparison data of free drugs (E, V, and EV) versus their TTL-formulated counterparts (see new data in Figure S5). These results demonstrate that TTL-formulated drugs exhibit enhanced potency compared to free drug treatment in vitro. While we did not perform additional in vivo free drug comparison studies in the current work, our previous in vivo studies have consistently demonstrated that TTL-formulated drugs yield superior therapeutic outcomes and reduced systemic toxicity relative to free drug administration. We believe that the newly added in vitro data, together with our prior in vivo evidence, strengthen our central claim that TTL delivery improves the therapeutic index over free drug administration. Further to address the reviewer's suggestion, we have provided the results below. For the reviewer's reference, we have provided representative ex vivo IVIS images of major organs (brain, liver, lung, spleen, and kidney) collected post-euthanasia (Figure below). Comparing fluorescence intensity across organs confirmed that trace signals were observed in the liver; however, the accumulation in the brain tumor was significantly higher. In control animals, no signal was detected in any organ. When only free dye was injected,*

higher signals were observed in the liver, with trace levels in the lungs, kidneys, and spleen. In contrast, animals treated with the non-targeted liposome formulation showed signal in liver, lung, kidney, and spleen and negligible in heart and brain, whereas the TTL-targeted liposome formulation accumulated predominantly in the tumor-bearing brain, confirming the specificity of TTL delivery. Please kindly see the image below.



2. Figure 2A – Although the authors show enhanced tumour-specific uptake of TTL-liposomes, only representative images are shown. Quantitative analysis of uptake (eg. % positive cells or mean fluorescence intensity) across multiple biological replicates is necessary, with appropriate statistical analysis to support reproducibility.

Response: *We thank the reviewer for this insightful suggestion. As recommended, we performed flow cytometry to quantify cellular uptake of TTL compared with conventional liposomes (CL) in GBM cell lines (Fig. 2G–2R). The results demonstrated significantly higher TTL uptake in both GBM22 and GBM1A cells, consistent with and supporting our imaging findings.*

3. Figure 2J–K –The Raman data are unconvincing. The signal appears to co-localize with brain regions such as white matter rather than clearly demarcated tumours. Key information is missing:

a. Brain orientation and tumour injection site must be indicated.

Response: *Thank you for highlighting these important points. In the revised figure and legend (now Figure 2L-Q and Figure S2), we have indicated brain orientation and injection site. We have now included Raman scans from control (non-treated) and non-tumor-bearing mice to demonstrate specificity and provided comparative spectra from tumor vs. non-tumor regions. We hope that these additions validate the specificity of the Raman signal to TTL accumulation in tumors.*

4. Figure 3 – The number of replicates or independent repeats is not stated. While the results are promising, reproducibility must be demonstrated with statistical analysis.

Response: *We now clearly state the number of biological replicates per group in the figure legend and Methods. Statistical analysis using one-way ANOVA and Tukey's multiple comparison test is included to validate reproducibility. Error bars and p-values are added in the updated version of Figure 3.*

5. Figure 4G–H –The legend states that results are based on three independent experiments, but supporting quantification (eg. densitometry relative to Actin) is not provided. Bar graphs showing mean ± SD across replicates would enhance data interpretation.

Response*: We thank the reviewer for this suggestion. We now provide densitometric quantification for all Western blots relative to β-Actin controls (see new Figure 4J-Q). Results are presented as mean ± SD from three independent experiments and were statistically analyzed using one-way ANOVA followed by appropriate post hoc tests.*

6. Figure S4 – Quantification across biological replicates should be provided. The figure currently appears to show only one experiment.

Response: *We thank the reviewer for this helpful suggestion. We have updated Figure S4, which is now updated Figure S6, in the revised manuscript to include quantification from three independent experiments.*

7. Figures 5B–E, S6, S8 – Individual mouse data are well-presented; however, group-level statistical analysis (eg. ANOVA for BLI signal/tumour size) is essential to confirm therapeutic benefit is not just by chance.

Response: *We agree and now include ANOVA analyses for all tumor growth and survival data across treatment groups in Figures 5B–E and Supplementary Figures S8, S10. Please kindly see the updated Figures.*

8. Figure 6 & Supplementary (S9–S12): The number of animals used and the time points post-treatment in this transcriptomic analysis are unclear. Although the text states the experiments were "repeated 3 times," it is essential to report the actual sample size (n) and clarify if these represent biological replicates.

Response: *The transcriptomic study included N = 3 biological replicates per treatment for Control and EV+R groups and N=2 for EV group, each from a unique animal at Day 7 post-final treatment. We have clarified this in the Methods on Page 7 in the supplementary material and method. All samples represent biological replicates.*

9. GEPIA Analysis – Confusion Between Mouse and Human Data: The transition from murine in vivo data to human TCGA datasets via GEPIA is insufficiently explained. The manuscript does not clarify that GEPIA data are from human patients, which may confuse readers. The rationale for

integrating these datasets and how they validate or complement the in vivo findings should be explicitly discussed.

Response: *We thank the reviewer for this valuable suggestion. We now explicitly state in the revised text that GEPIA represents human TCGA data and is used to provide comparative translational insight. We have clarified the rationale: to assess whether pathways altered in murine tumors following TTL therapy are relevant in human GBM. We also emphasize that these comparisons are intended for interpretive purposes rather than to establish mechanistic conclusions.*

10. Figures S13–S16 – The link between gene expression data and therapeutic efficacy is not well explained. The suggestion that these minor transcriptomic changes override the known cytotoxic effects of the drugs is speculative and may overstate the significance of these data.

Response: *We thank the reviewer for this thoughtful comment. We acknowledge the speculative tone of the previous interpretation. In the revised Discussion, we have tempered these claims and emphasize that the observed transcriptomic changes are supportive but not solely explanatory of therapeutic efficacy. We also clarify that these findings are intended to be hypothesis-generating.*

11. Several differentially expressed genes described in lines 313-319 (eg. CD3e, CD7, IL2R) are T-cell specific, but the authors then compare this to their mouse data which used immunodeficient (nude) mice that lack T cells. Interpretation of immune modulation in this context is inappropriate and should be revised.

Response: *We thank the reviewer for highlighting this important point. We now acknowledge that genes such as CD3e, CD7, and IL2R are typically T-cell specific, and that the use of immunodeficient (nude) mice limits the interpretation of immune modulation. Accordingly, we have revised the Discussion to avoid overinterpretation and have added caveats regarding this model limitation.*

Minor Comments

1. Line 119 – LNP Definition and Use

Response: *We have removed "LNP" throughout the manuscript to avoid confusion with lipid nanoparticles used in mRNA delivery. The correct term "liposome" is now consistently used.*

2. Rationale for Drug Combinations (E + V)

Response: *We expanded the introduction to include prior studies showing mTOR inhibitors (like Everolimus) sensitize tumors to microtubule inhibitors (like Vinorelbine) through*

modulation of autophagy and DNA repair. References have been added to support the synergy rationale (see Introduction, page XX).

3. Quantification from Figure S1 in Main Figures

Response: *We respectfully thank the reviewer for this suggestion. While we agree that quantification of tumor uptake is important. We believe it is most appropriate to retain it in its original location in Figure S1 rather than moving it to the main Figure 2C. We have now included flow cytometry analysis and quantified the uptake, and its placement in Figure 2. We believe this placement appropriately highlights the data and supports the claim of targeted delivery. However, we would be happy to adjust the figure placement further if the reviewer felt it would improve the manuscript.*

4. Immune Evasion Claims in Immunodeficient Mice

Response: *We now acknowledge that immune modulation by TTL-EV cannot be conclusively assessed in nude mice. The mention of PD-L1 and CXCL10 has been revised to reflect this limitation, and we have clarified that further validation in immunocompetent models is required.*

5. Lack of Synergy in GBM1A Cell Line

Response: *We have added a discussion note that the lack of synergy in GBM1A could be due to intrinsic drug resistance, lower basal mTOR activity, or altered uptake dynamics. This highlights the heterogeneity of GBM responses and potential for biomarker-driven stratification.*

6. Y-axis Scaling in Figures 4C–D

Response*: Figures 4C–D have been reformatted with adjusted y-axis scaling to better visualize differences in cell viability and response distributions across treatment conditions.*

7. Figure 5A–E – Clarification of Reduced n = 4

Response*: We now clarify in the legend and Methods that one mouse was removed from the TTL-EV+R group due to ulcerative dermatitis unrelated to treatment, reducing n = 5 to n = 4.*

8. Lines 315–319 – Incorrect Figure Reference

Response*: We corrected the text and figure citations.*

We are sincerely grateful to the reviewer for their detailed and constructive feedback, which has helped us significantly strengthen the manuscript. All suggested changes have been incorporated in the revised version, and we welcome any additional input or guidance.