Reviewer #1 (Remarks to the Author):

Comments:

Article Number: Nature Communications-119953

Title: Toward the next generation of smart anti-tumor drugs: a nucleolin aptamer-paclitaxel conjugate for tumor-specific targeting in ovarian cancer

Author: Zhang and co-workers

This manuscript reports on synthesis, characterization, and in vitro and in vivo examination of an aptamer drug conjugate (ApDC). Authors used Paclitaxel as their drug, and Nucleolin as the aptamer. They conjugated the two using Cathepsin B sensitive linkage.

Major/Minor issues:

1. Authors claim lack of immunogenicity of the ApDC is advantageous, yet, they don’t demonstrate any results in this regard.
2. HPLC chromatograms in support of Supplement Figure 17 will be beneficial.
3. HPLC chromatograms and retention times of PTX will be useful
4. Authors should provide a table with IC50 values for their in vitro cytotoxicity experiments
5. Authors should indicate amount of Cathepsin B used for their drug release studies? Also, is the concentration comparable to in vivo/in vitro levels. Their studies are conducted at pH 5, which is bit more acidic than typical cellular pH.
6. In the in vivo biodistribution study, how does authors know that the Drug is still intact when it reached the specific tissues? Also, percent injected dose numbers in different tissues will be needed for appropriate comparison.
7. From the bioD data, ApDC is cleared from the system within 4 h. Do you know what is the t1/2 value of this material.
8. Few typo errors in the manuscript were noted. Formatting of references is needed.
9. Authors should define protons in the spectra or at least point out the protons which are new/disappeared in their NMR spectra for better understanding of the characterizations. Also, I encourage to conduct 2D NMR studies.
10. Even though the concept of ApDC is old, overall the authors demonstrate interesting and positive in vitro and in vivo results with this ApDC.

Reviewer #2 (Remarks to the Author):

We thank the authors Li et al. for contributing their manuscript “Toward the next generation of smart anti-tumor drugs: a nucleolin aptamer-paclitaxel conjugate for tumor specific targeting in ovarian cancer.” The novelty is their claim to being the first to create a nucleolin-aptamer direct paclitaxel conjugate whereas other investigators have utilized the nucleolin-aptamer as a targeting ligand on nanoparticles (Subramanian et al 2016. Molecular Therapeutic Acids Nucleolin-aptamer therapy in retinoblastoma molecular changes and mass spectrometry–based imaging); this strength is important advantage in the translatability of aptamers. Audience is geared predominantly towards academics in the field of targeted drug delivery for oncology applications. Recent paper has been described with same nucleolin sequence conjugated to doxorubicin as a proof of principle (Trinh et al. Plos One 2015. A Synthetic Aptamer-Drug Adduct for Targeted Liver Cancer Therapy.) Following review should be cited in your introduction: Keefe et al. Aptamers as therapeutics. Nature Reviews and Drug Discovery 2011.

The authors have demonstrated successful synthesis of an aptamer-paclitaxel bioconjugate cleavable by cathepsin B and that does not affect the active binding site of nucleolin. In Figure 2d, the authors described using a buffer for the cathepsin containing DTT and EDTA and unclear whether the same amount of buffer was added to the control which demonstrates remarkable stability. As DTT would cause disulfide bond reduction, it is important to repeat the control of no cathepsin with the same quantity of buffer added. Gating strategy of flow cytometry characterization should be added to supplemental. Interestingly in figure 3 c,d there is notable non-specific binding of the CRO-PTX with increasing time and concentration, would benefit from clarification in the methods of whether the cells were washed after incubation. Consider placing 3a should be placed in supplemental, and bringing supplemental figure 19 should be moved to main. In Figure 4, colocalization would be better assessed via quantification of several cells to compare the different pathways.

The most significant portion of the paper is the findings of increased anti-tumor efficacy with reduction in side effect profile of these drug conjugates. The authors demonstrates tumor reduction efficacy of NucA-PTX conjugate in comparison to PTX and CRO-PTX alone and in bypassing toxic side effects such as neuropathy, leukopenia and off target necrosis. Please add drug dosing time points to Figure 7b and 8a.
In your discussion, please contemplate the reasoning why AS1411 (nucleolin optima alone), which according to your methods is the same sequence that you used in your studies, has been described in several instances in literature and additionally is being utilized in phase II clinical trials, to demonstrate growth inhibition and apoptosis, when this was not seen in your studies in mice in your control of nucleolin aptamer alone.

Please note the number of technical and experimental repeats along with the statistics used in the captions. The manuscript would benefit from focused grammatical review as there are several misspellings and word inaccuracies and would encourage resubmission with these edits.

Reviewer #3 (Remarks to the Author):

The manuscript presented by Zhang and colleagues is of high interest. In the field of cancer research where there is a constant search for more specific delivery of chemotherapeutics, whether novel or conventional, a paper describing a ‘simple’ linker to attach a drug to a targeting moiety, such as an aptamer, is certainly an advancement in the field and could lead to other drugs being attached in this manner. However, I do have some concerns regarding the results presented:

In figure 3C and 3D, the standard deviations appear to be very small. Given the differences presented for 500nM aptamer incubated for 2 hours in both 3C and 3D, I find these SDs to be too low. How many replicates were performed and how many times were each experiment repeated? I also appreciate that the authors have shown results from a liver cell line in figure 3E, but did they perform the experiment using the C-rich oligonucleotide as well? How specific is the uptake?

In figure 4 the authors suggest that the NucA aptamer is taken into the cell via macropinocytosis. However, Supp Figure 18 appears to show some macropinocytotic uptake of CRO-PTX-Rh. Has a Pearson’s co-efficient been performed on 50-100 cells to accurately characterise the amount of co-localisation that has occurred? This might strengthen the argument that the aptamer can discriminate between cancer cells and normal cells, an important point raised in the introduction. I do note that Supp figure 19 does suggest a lack of uptake of CRO, but doesn’t really suggest that NucA would be only taken up by cancer cells. Also, have the authors performed these experiments with a normal cell line? Maybe the LO2 line?

In figure 5 the authors show that the NucA-PTX is cytotoxic to a normal liver cell line. I find it worrying that the authors offer no commentary on this in the results or the discussion. While there
was some information presented in the commentary of figure 6b, a Pearson’s co-efficient is required to confirm a lack of co-localisation in the liver as some can be faintly detected by macro-observation. I agree in figure 6b that there is less NucA-PTX in the kidney, but a similar amount of CRO and NucA appears in the rhodamine images presented of the liver – again, can software analysis be performed to quantitate the amounts present. The authors appear to depend on observational skills which are qualitative, rather than quantitative analysis for their commentary on figure 6b. Can the authors also acknowledge that a lack of colocalisation does not mean a lack of uptake or toxicity? Also, the authors present biophotonic imaging at 2 and 4 hours. However, the methods only state 2 hours in the mice modelling section. Should the biophotonic imaging methods be merged with this section?

In figure 8D, the authors state that the level of damage to the heart, liver, spleen and lung is greatly reduced in animals treated with NucA-PTX. How was this evaluated? Were the sections blinded and evaluated by a qualified pathologist? What grading system was used? Biochemical assays for cardiac enzymes and liver function enzymes would be preferred as an indication of damage.

Finally, I find the lack of discussion, given that there are results presented from generation of a drug delivery complex, to characterisation of the aptamer-drug complex, to toxicity in vitro, biodistribution, AND the in vivo treatment of a xenograft, to be concerning. There are a number of papers published in this specific area which demonstrate similar results. Surely the authors could provide a comparison of their results with previous published work?
Dear Prof Zhang,

Your manuscript entitled “Toward the next generation of smart anti-tumor drugs: a nucleolin aptamer-paclitaxel conjugate for tumor-specific targeting in ovarian cancer” has now been seen by 3 referees, whose comments are appended below. Although the referees find some potential interest in your work, in the light of their overall advice I regret to say that we are unable to offer to publish it in Nature Communications, at least in its present form.

I should explain that we do not ask referees to say whether they think that a paper belongs in Nature Communications; instead, we ask them to tell us specifically what they feel to be the paper's contribution, and what its significance might be. It is then on these comments and our own editorial criteria that we decide whether publication in Nature Communications is appropriate. In this regard, the reviewers raise important technical concerns that call into question the findings of the study. In particular, Reviewer #1 was concerned about the lack of sufficient data to justify the claims made. There were also concerns related to the lack of assessment of stability and delivery efficiency at acidic pH. Reviewer #2 was concerned about non-specific binding and the lack of sufficient control groups. Reviewer #3 raised concerns regarding the technical replicates, the need for assessment with non-cancerous cells, the lack of sufficient quantitative data and the need for more elaborate methodology. Thus, we feel that at this stage, the present work is at too preliminary a stage to warrant publication in Nature Communications.

Indeed, it seems that a significant amount of additional experimental work and clarification will be necessary in order to support your key claims, and it is far from clear whether the results will continue to appear impressive in the light of this additional work. And while we would not rule out consideration of a revised manuscript that makes a much stronger case in support of the claims due to lack of novelty, as has been highlighted by Reviewers #2, we feel that at this stage, the present work is at too preliminary a stage to warrant publication in Nature Communications.

Whether or not it will be possible for you to address all of these concerns is not something we can assess at this stage. If you do decide to submit a revised version to Nature Communications, it should include a separate response to all the referees' comments, describing the specific changes and additions that you have made. Once you feel that you have addressed the referees' concerns in full, please submit the revised version of your manuscript along with a point-by-point response to these concerns.

It is only fair to say, however, that we would be reluctant to trouble our referees again unless we thought that the entirety of their comments had been fully addressed. In a case such as this, where the revisions are likely to be extensive, we would naturally understand if you preferred to submit your manuscript elsewhere. If the revision process takes significantly longer than three months, we will be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Nature Communications or published elsewhere in the meantime.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if you wish to discuss the revision or if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.
When resubmitting your paper, we also ask that you ensure that your manuscript complies with our editorial policies. Specifically, please ensure that the following requirements are met, and any relevant checklists are completed and uploaded as a Related Manuscript file type with the revised article:

Reporting requirements for life sciences research: http://www.nature.com/article-assets/npg/ncomms/authors/ncomms_lifesciences_checklist.pdf

Characterization of chemical and biomolecular materials: http://www.nature.com/ncomms/journal-policies/editorial-publishing-policies#Characterization-materials

Please use the following link to submit your revised manuscript, point-by-point response to the referees’ comments (which should be in a separate document to any cover letter) and any completed checklist:
http://mts-ncomms.nature.com/cgi-bin/main.plex?el=A6S7sSp7A5DyAl3I1A9ftdZGXpmPib0Cq8b3oN9POAZ

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Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

Dr Amos Matsiko
Associate Editor
Nature Communications
## Point-by-point response to the comments from reviewer #1 of Nature Communications

| Items                      | Comments                                                                                                                                                                                                 | Response                                                                                                                                                                                                 | Line number in updated manuscript |
|----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|
| **Overall remarks**        | This manuscript reports on synthesis, characterization, and *in vitro* and *in vivo* examination of an aptamer drug conjugate (ApDC). Authors used Paclitaxel as their drug, and Nucleolin as the aptamer. They conjugated the two using Cathepsin B sensitive linkage. | Thanks for the comments. The revised contents in our manuscript in response to the reviewer’s suggestions have been highlighted in yellow.                                                                 | N/A                             |
| **Concerns**               | Authors claim lack of immunogenicity of the ApDC is advantageous, yet, they don’t demonstrate any results in this regard.                                                                                     | Understand the concern of the reviewer. In our re-submission, to investigate whether our ApDC led to an immune response, we examined serum cytokine levels after 4-week treatment of NucA-PTX in both nude mice (immune-deficient, the mice we used for our xenograft model), and Balb/c mice (immune-competent). As shown in Supplementary Fig. 11, no significant changes of cytokine levels (TNF-α, INF-γ, IL-1β, IL-6, IL-10) were observed in both mice strains after NucA-PTX treatment, which implies the lack of immunogenicity of NucA-PTX. | Supplementary Fig. 11<br>Line 326-329, 402-403, 758-763 |
|Authors should provide a table with IC$_{50}$ values for their *in vitro* cytotoxicity experiments. | Thanks for the suggestion. According to the previously reported IC$_{50}$ studies for PTX, IC$_{50}$ values are most commonly presented at Supplementary Fig. 8. | Thanks for the suggestion. In our re-submission, HPLC chromatograms in support of Supplement Fig. 17 (now supplementary Fig. 3 in the re-submission) were provided. Please refer to Supplementary Fig. 3d and Supplementary Spectra 16. | Supplementary Spectra 16 |
| HPLC chromatograms and retention times of PTX will be useful. | Thanks for the suggestion. In our re-submission, HPLC chromatograms for our cathepsin B catalyzed PTX release assay including PTX (together with NucA-PTX and NucA) were supplemented and the retention times were provided. Please refer to Supplementary Spectra 16. | Supplementary Spectra 16 |
| HPLC chromatograms in support of Supplement Figure 17 will be beneficial. | Thanks for the suggestion. In our re-submission, HPLC chromatograms in support of Supplement Fig. 17 (now supplementary Fig. 3 in the re-submission) were provided. Please refer to Supplementary Fig. 3d and Supplementary Spectra 16. | Supplementary Spectra 16 |
| Authors claim lack of immunogenicity of the ApDC is advantageous, yet, they don’t demonstrate any results in this regard. | Understand the concern of the reviewer. In our re-submission, to investigate whether our ApDC led to an immune response, we examined serum cytokine levels after 4-week treatment of NucA-PTX in both nude mice (immune-deficient, the mice we used for our xenograft model), and Balb/c mice (immune-competent). As shown in Supplementary Fig. 11, no significant changes of cytokine levels (TNF-α, INF-γ, IL-1β, IL-6, IL-10) were observed in both mice strains after NucA-PTX treatment, which implies the lack of immunogenicity of NucA-PTX. | Supplementary Fig. 11<br>Line 326-329, 402-403, 758-763 |
To be comparable to those values, we conducted our cytotoxicity assays at 72 h, and the data and IC$_{50}$ values were presented in Supplementary Fig. 8 and Supplementary Table 1 respectively. The IC$_{50}$ values of PTX against ovarian cell lines in our studies are comparable to the previously reported values$^{1,2,3}$.

| Authors should indicate amount of Cathepsin B used for their drug release studies? Also, is the concentration comparable to in vivo/in vitro levels. Their studies are conducted at pH 5, which is bit more acidic than typical cellular pH. | Thanks for the suggestions and concerns. The concentration of cathepsin B used in the drug release studies was 0.5 unit/mL (approximately 0.05 mg/mL), and this has been indicated in legend of Fig. 2 and Methods in our re-submission. Cathepsin B is a lysosomal cysteine protease. It takes up 10% of all soluble lysosomal proteases and the concentration of cathepsin B in lysosomes was estimated to be approximately 1 mM (31 mg/mL)$^4$. However, this concentration is too high and unrealistic to be used for in vitro studies. In addition, the ratio between enzyme and the substrate matters more than their absolute concentrations, but the concentration of the substrate (NucA-PTX) that could achieve in lysosomes was not clear. According to the instruction from Sigma (where our cathepsin B was purchased) and the concentration of cathepsin B used in previously reported catalyzed studies$^5$, we used 0.5 unit/mL (0.05 mg/mL) of cathepsin B in this study, with a substrate (FAM-NucA-PTX-Rh) concentration of 2 μM. This concentration ratio between cathepsin B and a substrate could demonstrate the lability of the linker and the effective catalysis by cathepsin B. Consistent result was also shown in the intracellular study data in Fig. 2e and in vivo study data in Supplementary Fig. 10. Lysosomes are the main localization and functional place for cathepsin B. The pH range of lysosomes is normally from 4.5 to 5.0$^6$, which is more acidic than typical cellular pH. Therefore, we |
|---|---|
| 5 | Fig 2, Line 71-76, 544 |
| Supplementary Table 1, Line 223, 225-227, 628, 630-631 |
conducted the cathepsin B catalyzed experiments at pH 5.0 to ensure cathepsin B was active and functional.

Understand the concern of the reviewer. In our re-submission, to illustrate this question, we utilized both mono fluorophore-labeled conjugate (NucA-PTX-Rh) and dual fluorophore-labeled conjugate (FAM-NucA-PTX-Rh) to quantitatively monitor the accumulation and intactness of the conjugate at tumor site upon time. Briefly, NucA-PTX-Rh and FAM-NucA-PTX-Rh at a single dose of 5 mg/kg were given to the tumor-bearing nude mice respectively by intravenous route (i.v.) via the tail vein. Mice were sacrificed at 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h. Tumors were then collected, chopped and homogenized. The fluorescence of Rhodamine in NucA-PTX-Rh treated mouse tumor homogenates (representing the concentration of PTX-Rh in the conjugate) and the fluorescence of rhodamine and FAM in FAM-NucA-PTX-Rh treated mouse tumor homogenates (FAM/Rh representing the intact conjugate illustrated in Fig. 2) were analyzed by a fluorescence spectrophotometer. Please refer to Supplementary Fig. 10 for the results. The PTX-Rh gradually accumulated in tumor tissue in the first 2 hours after injection, and started being cleared from the tissue after 2 hours (Supplementary Fig. 10a). Interestingly, the relative fluorescence intensity (RFI) Rh/FAM exhibited a plateau at the beginning, and then gradually decreased after 2 hours (Supplementary Fig. 10b). The initial plateau of RFI Rh/FAM could be explained by the continuous accumulation of intact conjugate at the tumor site. The subsequent reduction of RFI Rh/FAM could possibly because when there was no accumulation of new intact conjugate after 2h, the release of PTX-Rh from the conjugate in the tumor tissue could be observed.

Supplementary Fig. 10, Line 262-274, 342-345, 690-696

Thanks for the constructive suggestion. Percentage of injected dose (%ID) in tumor and major organs at 2 and 4 h after NucA-PTX-Rh or CRO-PTX-Rh administration were evaluated by Fig. 6b Line 247-251, 680-688
detecting the fluorescence of rhodamine in the tissue homogenates according to the method previously reported\(^7\). Please refer to Fig. 6b for the data. Related experimental information and comments were added to methods and results. The percentage of injected dose of NucA-PTX-Rh in tumor tissue was significant higher than that of CRO-PTX-Rh in both time points, which was consistent with the bio-distribution data.

From the bioD data, ApDC is cleared from the system within 4 h. Do you know what is the \(t_{1/2}\) value of this material.

Thanks for the constructive suggestion. In our re-submission, serum half-life (\(t_{1/2}\)) of NucA-PTX was evaluated after a single injection and the \(t_{1/2}\) value was 2.17 h. Please refer to Supplementary Fig. 9 for the drug clearance curve.

Few typo errors in the manuscript were noted. Formatting of references is needed.

Thanks for the suggestions. We have carefully examined and corrected typo and grammatical errors throughout the updated manuscript. The formatting of references was also conducted.

Authors should define protons in the spectra or at least point out the protons which are new/disappeared in their NMR spectra for better understanding of the characterizations. Also, I encourage to conduct 2D NMR studies.

Thanks for the comments. In our re-submission, the protons in the \(^1\)H NMR spectra of important intermediates were defined. In addition, 2D NMR studies on bigger molecules 4, 6 and NucA-PTX were conducted for proton definition and better understanding of the characterizations. The new appeared cross-peaks in 2D NMR spectra of NucA-PTX were defined in comparison of the spectra of NucA and compound 6 (the PTX derivative). Please refer to Supplementary Spectra 1-6.

Even though the concept of ApDC is old, overall the authors demonstrate interesting and positive \textit{in vitro} and \textit{in vivo} results with this ApDC.

Thanks for the encouraging comments. Although the concept of ApDC was previously proposed, to our knowledge, this was the first time to create an ApDC as a single macromolecule with direct connection of a nucleolin aptamer with paclitaxel. The advantages of this direct conjugation in terms of stability, homogeneity, low immunogenicity and limited toxicity represent a step further towards the clinical translation for aptamers.
| Items          | Comments                                                                                                                                                                                                 | Response                                                                                                                                                                                                 | Line number in updated manuscript |
|---------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| Overall       | We thank the authors Li et al. for contributing their manuscript “Toward the next generation of smart anti-tumor drugs: a nucleolin aptamer-paclitaxel conjugate for tumor specific targeting in ovarian cancer.” The novelty is their claim to being the first to create a nucleolin-aptamer direct paclitaxel conjugate whereas other investigators have utilized the nucleolin-aptamer as a targeting ligand on nanoparticles (Subramanian et al 2016. Molecular Therapeutic Acids Nucleolin-aptamer therapy in retinoblastoma molecular changes and mass spectrometry–based imaging); this strength is important advantage in the translatability of aptamers. Audience is geared predominantly towards academics in the field of targeted drug delivery for oncology applications. Recent paper has been described with same nucleolin sequence conjugated to doxorubicin as a proof of principle (Trinh et al. Plos One 2015. A Synthetic Aptamer-Drug Adduct for Targeted Liver Cancer Therapy.) Following review should be cited in your introduction: Keefe et al. Aptamers as therapeutics. Nature Reviews and Drug Discovery 2011. | Thanks for the encouraging comments. In our re-submission, the review (Keefe et al. Aptamers as therapeutics. Nature Reviews and Drug Discovery 2011.) has been cited in the introduction. The revised contents in our manuscript in response to the reviewer’s suggestions have been highlighted in yellow. | Line 58, 806-807                  |
| Overall       | The authors have demonstrated successful synthesis of an aptamer-paclitaxel bioconjugate cleavable by cathepsin B and that does not affect the active binding site of nucleolin. In Figure 2d, the authors described using a buffer for the cathepsin containing DTT and EDTA and unclear whether the same amount of buffer was added to the control which demonstrates remarkable stability. As DTT would cause | Understand the concerns. In our re-submission, we performed the control experiment by adding NucA-PTX to the cathepsin B buffer only (containing DTT and EDTA) and monitored the fluorescence change for 48 hr. The results showed that NucA-PTX remained stable in the buffer within 48 hr. Please refer to Fig. 2d for the data. The related information and comments have also been added to the methods and results. | Fig. 2d, Line 142-144, 541-546    |
| Comment | Response | Supplementary Information |
|---------|----------|---------------------------|
| disulfide bond reduction, it is important to repeat the control of no cathepsin with the same quantity of buffer added. | Thanks for the suggestion. Gating strategy of flow cytometry characterization has now been supplemented as Supplementary Fig. 5 in our re-submission. | Supplementary Fig. 5 |
| Gating strategy of flow cytometry characterization should be added to supplemental. | Thanks for the suggestion. In time and concentration-dependent experiments, after 2 h incubation with NucA-PTX or CRO-PTX, the cells were washed three times before analyzed by flow cytometry. The description of the washing step has now been added to the methods. The comments on non-specific binding of CRO-PTX were also added to results and discussion. | Line 585-586, 181-182, 187-190, 347-349, 361-367 |
| Interestingly in figure 3 c,d there is notable non-specific binding of the CRO-PTX with increasing time and concentration, would benefit from clarification in the methods of whether the cells were washed after incubation. | Thanks for the suggestion. Fig. 3a in last submission has now been placed in supplemental as Supplementary Fig. 4, and Supplementary Fig. 19 in last submission has now been moved to Fig. 4 as Fig. 4c-e. | Supplementary Fig. 4, Fig. 4c-e |
| Consider placing 3a should be placed in supplemental, and bringing supplemental figure 19 should be moved to main. | Thanks for the suggestion. All the confocal co-localization studies in our re-submission were quantitatively characterized by performing Pearson's correlation coefficient analysis on 50 cells using Image J Coloc2 software. The data of quantitative analysis were shown in Fig. 4b, Fig. 4g, Fig. 6d, Supplementary Fig. 6b and Supplementary Fig. 7b. | Fig. 4b, Fig. 4g, Fig. 6d, Supplementary Fig. 6b, Supplementary Fig. 7b |
| In Figure 4, colocalization would be better assessed via quantification of several cells to compare the different pathways. | | |
| The most significant portion of the paper is the findings of increased anti-tumor efficacy with reduction in side effect profile of these drug conjugates. The authors demonstrate tumor reduction efficacy of NucA-PTX conjugate in comparison to PTX and CRO-PTX alone and in bypassing toxic side effects such as neuropathy, leukopenia and off target necrosis. Please add drug dosing time points to Figure 7b and 8a. | Thanks for the comments. Drug dosing time points have now been marked in Fig. 7b and Fig. 8a. | Fig. 7b, Fig. 8a |
| In your discussion, please contemplate the reasoning why AS1411 (nucleolin optima alone), which according to your methods is the same sequence that you used in your | Thanks for the constructive suggestion. This discrepancy could possibly attribute to the following reasons. | Line 379-392 |
studies, has been described in several instances in literature and additionally is being utilized in phase II clinical trials, to demonstrate growth inhibition and apoptosis, when this was not seen in your studies in mice in your control of nucleolin aptamer alone.

Firstly, for *in vitro* cytotoxicity study, the concentration range that we examined was set based upon the activity of PTX, which was within nanomolar range. While AS1411 was reported to be cytostasis and only inhibit the growth of cancer cells in micromolar range. In addition, the induction of cell death by AS1411 occurs only after prolonged exposure to AS1411 and varies from different cell lines. Therefore, it is reasonable that we did not observe anti-cancer activities for NucA *in vitro*.

Secondly, for *in vivo* anticancer efficacy studies, normally a continuous infusion of AS1411 for 4 or 7 days was chosen as the route of administration for clinical studies, and the effective doses were reported 5-40 mg/kg/day i.v. for 5 to 7 days. However, the *in vivo* dose used in our study was set based on PTX, which was lower and less frequent – twice a week. Besides, ovarian cancer was probably not among the most sensitive cancer types to AS1411 compared to acute myeloid leukaemia, or metastatic renal cell carcinoma, which were chosen as indications of AS1411 for phase II studies. Therefore, it is also reasonable that we did not observe anti-cancer activities for NucA in our *in vivo* study.

These comments have been added to the discussion in our re-submission.

Please note the number of technical and experimental repeats along with the statistics used in the captions. The manuscript would benefit from focused grammatical review as there are several misspellings and word inaccuracies and would encourage resubmission with these edits.

Thanks for the suggestion. The number of technical and experimental repeats along with the statistics have now been noted in the legend of figures where necessary. We also have carefully examined and corrected the misspellings and word inaccuracies throughout the updated manuscript.
### Point-by-point response to the comments from reviewer #3 of Nature Communications

| Items                  | Comments                                                                                                                                                                                                 | Response                                                                                                                                                                                                 | Line number in updated manuscript |
|------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|
| Overall remarks        | The manuscript presented by Zhang and colleagues is of high interest. In the field of cancer research where there is a constant search for more specific delivery of chemotherapeutics, whether novel or conventional, a paper describing a 'simple' linker to attach a drug to a targeting moiety, such as an aptamer, is certainly an advancement in the field and could lead to other drugs being attached in this manner. | Thanks for the encouraging comments. The revised contents in our manuscript in response to the reviewer's suggestions have been highlighted in yellow.                                                                 | N/A                               |
| Concerns               | In figure 3C and 3D, the standard deviations appear to be very small. Given the differences presented for 500nM aptamer incubated for 2 hours in both 3C and 3D, I find these SDs to be too low. How many replicates were performed and how many times were each experiment repeated? | Thanks for the concerns. The experiments in Fig. 3c and 3d in the last submission were re-performed with triplicates and were repeated for three times. The SDs of the updated data were within the reasonable range and the data were shown in Fig. 3b and 3c in our updated manuscript. | Fig. 3b, 3c, Line 592-593          |
|                        | I also appreciate that the authors have shown results from a liver cell line in figure 3E, but did they perform the experiment using the C-rich oligonucleotide as well? How specific is the uptake? | Thanks for the suggestion. In our re-submission, the uptake of the C-rich oligonucleotide – PTX conjugate (CRO-PTX) in a liver cell line (L02) and two ovarian cancer cell lines (SKOV3 and OVCAR2) were evaluated and added to Fig. 3d (Fig. 3e in the last submission). The result showed that there was no significant difference in the uptake of CRO-PTX in the liver cell line and cancer cell lines, which further suggests the specific uptake of NucA-PTX in high nucleolin expression cancer cells. | Fig. 3d, Line 180-181, 595         |
|                        | In figure 4 the authors suggest that the NucA aptamer is taken into the cell via macropinocytosis. However, Supp Figure 18 appears to show some macropinocytotic uptake of CRO-PTX-Rh. Has a Pearson’s co-efficient been performed on 50-100 cells to accurately characterise the uptake? | Thanks for the constructive suggestion. All the confocal co-localization studies were quantitatively characterized by performing Pearson’s correlation coefficient analysis on 50 cells using Image J Coloc2 software. The data of quantitative analysis were shown in Fig. 4b, Fig. 4g, Fig. 6d, Supplementary Fig. 6b, Supplementary Fig. 6d, Supplementary. | Fig. 4b, Fig. 4g, Fig. 6d, Supplementary Fig. 6b, SupplementaryFig. 6d, Supplementary |
amount of co-localisation that has occurred? This might strengthen the argument that the aptamer can discriminate between cancer cells and normal cells, an important point raised in the introduction. I do note that Supp figure 19 does suggest a lack of uptake of CRO, but doesn’t really suggest that NucA would be only taken up by cancer cells. Also, have the authors performed these experiments with a normal cell line? Maybe the L02 line?

In figure 5 the authors show that the NucA-PTX is cytotoxic to a normal liver cell line. I find it worrying that the authors offer no commentary on this in the results or the discussion.

While there was some information presented in the commentary of figure 6b, a Pearson’s co-efficient is required to confirm a lack of co-localisation in the liver as some can be faintly detected by macro-observation. I agree in figure 6b that there is less NucA-PTX in the kidney, but a similar amount of CRO and NucA appears in the rhodamine images presented of the liver – again, can

Understand the concern of the reviewer. Although the viability curves showed that NucA-PTX was cytotoxic to the normal liver cell line, the cytotoxicity was still less compared to PTX and CRO-PTX in vitro (Fig. 5a, Supplementary Fig. 8). This could be caused by non-specific binding by aptamers. More importantly, the in vitro cytotoxicity to normal cells could not represent the in vivo condition, as the in vitro incubation time was quite long (24 h to 72h) and no cancer cells were presented for the selective accumulation of NucA-PTX. Comments on this have now been added in the results and discussion in our re-submission.

Agree with the reviewer and thanks for the comments. According to the reviewer’s suggestion, we performed the Pearson’s co-efficient analysis on the fluorescent micrographs of tumor cryosection by ImageJ software to show that there were the more co-localizations of PTX-Rh with nucleolin-expressing tumor cells in NucA-PTX group as compared to CRO-PTX group at 4h after injection. Moreover, to

The NucA-PTX endocytosis pathways in L02 cell line (a normal cell line) were conducted and the results were presented as Supplementary Fig. 7.

The quantitative analysis of co-localization of NucA-PTX (or CRO-PTX) with endocytosis markers in SKOV3 and L02 cells further demonstrated that NucA-PTX was specifically taken up in cancer cells by macropinocytosis, while no specific endocytic pathways was observed in terms of either CRO-PTX in cancer cells or NucA-PTX in normal cells. These results further strengthen the argument that NucA-PTX could discriminate between cancer cells and normal cells.
software analysis be performed to quantitate the amounts present. The authors appear to depend on observational skills which are qualitative, rather than quantitative analysis for their commentary on figure 6b. Can the authors also acknowledge that a lack of colocalisation does not mean a lack of uptake or toxicity?

| Line | Text |
|------|------|
| 655  | Thanks for the suggestion. To be consistent, the drug administration and sampling parts from mice modelling section have now distributed to each assay. In the “Tissue distribution by biophotonic imaging analysis” section, sampling and imaging at 2 and 4 hours were merged in this section. |
| 659  | Line 655-659 |

Also, the authors present biophotonic imaging at 2 and 4 hours. However, the methods only state 2 hours in the mice modelling section. Should the biophotonic imaging methods be merged with this section?

| Line | Text |
|------|------|
| 776  | Thanks for the comments. Indeed, the histology sections with H&E staining were blindly evaluated by a qualified pathologist using the qualitative methods that have been widely applied in several previous studies on PTX. Briefly, the heart damages were indicated by the presence of bleeding, myofibrillar loss, and atrophy of myocardial cells. The liver damages were indicated by the hepatic lesions, hepatic cells atrophy, mild steatosis, hepatic cords loss, and dilatation of blood sinus. The lung damages were indicated by the breakage of lung fibers, formation of a pneumatocele and widening of the red pulp. The spleen damages were indicated by the atrophy of the white pulp. The kidney damages were indicated by the loss of glomerulus and the disorganization of renal tubular. To further release the reviewer’s concerns, we applied the semi-quantitative analysis with the previously reported grading Fig. 8d, 8e Line 319-322, 729-742 |
Biochemical assays for cardiac enzymes and liver function enzymes would be preferred as an indication of damage. In addition, we have provided the data of liver function enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) and cardiac function enzymes (creatine phosphokinase (CPK) and creatine kinase myocardial bound (CK-MB)), respectively. Consistently, the data showed that the NucA-PTX-treated mice had significant lower levels of AST, ALT, CPK and CK-MB comparing to the mice treated with PTX or CRO-PTX. Please refer to the Supplementary Table 2, results and methods highlighted in the updated manuscript.

Finally, I find the lack of discussion, given that there are results presented from generation of a drug delivery complex, to characterisation of the aptamer-drug complex, to toxicity in vitro, biodistribution, AND the in vivo treatment of a xenograft, to be concerning. There are a number of papers published in this specific area which demonstrate similar results. Surely the authors could provide a comparison of their results with previous published work? Thanks for the suggestion. In our re-submission, the discussion has been fully revised, and the comparison of our work with previous published work in this area has been added. Please refer to the discussion section for the revisions.

| Biochemical assays for cardiac enzymes and liver function enzymes would be preferred as an indication of damage. | Systems on the above sections of liver, kidney, lung, heart and spleen, respectively. In line with the qualitative observations, the histological scores of liver, kidney, lung, heart and spleen were significantly lower in the NucA-PTX-treated mice as compared to the mice treated with PTX or CRO-PTX. Please refer to Figure 8d, 8e, result and methods highlighted in the updated manuscript. | Supplementary Table 2, Line 311-313, 322-326, 765-770 |
| Finally, I find the lack of discussion, given that there are results presented from generation of a drug delivery complex, to characterisation of the aptamer-drug complex, to toxicity in vitro, biodistribution, AND the in vivo treatment of a xenograft, to be concerning. There are a number of papers published in this specific area which demonstrate similar results. Surely the authors could provide a comparison of their results with previous published work? | Thanks for the suggestion. In our re-submission, the discussion has been fully revised, and the comparison of our work with previous published work in this area has been added. Please refer to the discussion section for the revisions. | Line 331-409 |
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Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns and I do not have additional comments.

Reviewer #3 (Remarks to the Author):

I am happy that the authors have answered my concerns.