nature portfolio

Peer Review File

Stimulus-activated Ribonuclease Targeting Chimeras for Tumour Microenvironment Activated Cancer Therapy

Corresponding Author: Professor Haibin Shi

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)
What are the noteworthy results?
The development of an in vivo photo activated RNA degrader, demonstrating on-target effects and with the promise to minimize off-targets

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.
this is an advance over the original pioneering robotac approach, including phototrigegred decaging and activation in mouse models

Does the work support the conclusions and claims, or is additional evidence needed?
Yes, there is biochemical and cellular data that support the mouse studies, as well as pharmacodynamic studies

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
Is the methodology sound? Does the work meet the expected standards in your field?
None, i think this is fine as is

Is the methodology sound? Does the work meet the expected standards in your field?
Yes, it does in general but it would be nice if there was some more characterization such as proton NMR for the compounds in the SI; there is generally MS only now

Is there enough detail provided in the methods for the work to be reproduced?
Yes, easily

I would publish this as is in Nature Communications as for this journal and what it has published it is above the bar

Reviewer #2

(Remarks to the Author)
In the manuscript entitled "Tumor Microenvironment-activated Robotic Ribonuclease Targeting Chimeras for Precise Tumor Treatment" by Zhang et al., the study presents a tumor microenvironment-activated RiboTAC (TaRiboTAC) strategy designed to address the off-target limitations of conventional RiboTACs in RNA degradation therapies. A pre-miRNA-21 nano-degrader (RIBOTAC21-BA) was developed to selectively target and degrade pre-miR-21 in tumors by activating ribonucleases in response to tumor-specific conditions such as low pH and elevated H_2O_2 levels. Interestingly, the use of a near-infrared (NIR) probe to selectively activate the RiboTAC molecule significantly increases the selectivity of the proposed approach. This method effectively enhanced the radiotherapy response in lung cancer models, highlighting the potential of TaRiboTACs for precise and efficient cancer treatment.
Major Comments:
• A similar study was published recently by the Jiang lab in JACS (J. Am. Chem. Soc. 2024, 146, 15815−15824). That study also features activation in response to cellular conditions, including H_2O_2 and bioorthogonal activation, which significantly

reduces the novelty of this research.
• The large molecular weight and tendency of such big molecules to aggregate could limit their application as potential therapeutics.
• To confirm that the cleavage occurs at the same binding site, a competitive cleavage assay with the non-activated compound and the original binder should be performed.
• To confirm the selectivity of this approach in reducing only miR-21, a miRNome sequencing and RNA sequencing experiment should be conducted to assess whether the affected RNA is related solely to miR-21.
Minor Comments:
• The effect of H2O2 on RNase L oligomerization with caged and non-caged RIBOTAC21 compounds should be added as a control.
• Figure 4e lacks clarity regarding the band being observed. If miR-21 is being cleaved, a hydrolyzed miR-21 construct should be used to map the cleavage and serve as a control. Additionally, the observed miR-21 cleavage appears relatively low in this assay, and the quantification of the VI seems difficult to understand.

Reviewer #3

(Remarks to the Author)
1. In Figure 3a, after treating A549 and BEAS-2B cells with 5 μM RIBOTAC21-BA, the fluorescence detection results provided by the authors show that the fluorescence intensity in A549 cells is significantly higher than in BEAS-2B cells. However, this does not fully indicate that the binding ability of RIBOTAC21-BA to A549 cells is higher than to BEAS-2B cells, as the authors did not test whether there were differences in the pH of the culture media between the two cell types. One possibility is that the tumor cells lower the pH of the culture media compared to normal cells (because of different metabolic characteristics between tumor cells and normal cells), thereby causing RIBOTAC21-BA to produce fluorescence.
2. Regarding the results presented in Figure 3d, I have a question: The authors added a cRGD peptide to RIBOTAC21-BA to achieve tumor targeting. Although RIBOTAC21-BA exhibited stronger fluorescence in the pH 5.5 culture system, this does not necessarily indicate that the binding capacity of RIBOTAC21-BA to tumors is enhanced under pH 5.5 conditions. What I mean is that under pH 5.5 conditions, the binding ability of RIBOTAC21-BA to tumors may actually weaken. So the authors need to demonstrate whether low pH affects the tumor-targeting ability of cRGD.
3. The authors should include an additional set of experiments in Figure 4, selecting several unrelated miRs as substrates (preferably with sequences similar to pre-miR-21) to verify the specificity of the miR-21 binder and whether RIBOTAC21-BA exhibits any off-target effects.
4. In Figures 5d and 5e, why did the authors choose to amplify miR-21-5p instead of directly performing quantitative analysis on pre-miR-21 using PCR? Even so, the authors should also include a set of real-time fluorescence quantitative PCR results rather than simply presenting semi-quantitative PCR plus agarose gel results.
5. In Figures 5d-5g, since A549 and BEAS-2B are two different cell lines, the endogenous expression levels of miR-21-5p and PDCD4 are inherently different. Therefore, comparing the expression level differences of miR-21-5p and PDCD4 in the two cell lines after RIBOTAC21-BA treatment is not meaningful. The authors should compare the expression differences between different treatment concentrations instead.
6. Please provide a reference for the statement "As programmed cell death 4 (PDCD4) is well known to suppress neoplastic transformation, cell proliferation, and metastasis, and it can be typically downregulated by miR-21 in cancer cells".
7. If PDCD4 is a target gene regulated by miR-21-5p, please provide a schematic diagram in Figure 5 showing the binding sequence between miR-21-5p and the 3'UTR of PDCD4 mRNA.
8. In the section discussing the function of PDCD4, the authors state that "Numerous studies have proven that the elevation of protein PDCD4 can enhance the radiosensitivity of cancer cells" and cite references 57 and 58. However, in fact, these two references do not describe any correlation between PDCD4 and radiosensitivity.
9. In the Instrumentation section, the authors did not mention the X-ray equipment and its supplier.
10. The results shown in Figures 5k-5n, the descriptions in the Results section, and the figure legends for them, do not correspond to each other, making it very difficult to understand these results.
Additionally, the results in Figure 5L are quite confusing. What does the SER unit represent? What is the D0 (Gy) parameter? Why are the radiation doses listed under the D0 column different? Furthermore, in the results, the authors describe that "RIBOTAC21-BA has the lowest survival rate with a SER of 1.33," but in Figure 5L, the SER for the RIBOTAC21-BA group is shown as 1.30, and G0 (Gy) = 1.33.
11. In the description of Figure 6, the authors mention that the tumor suppression rate is up to 74.3% on day 18 compared to the PBS group. The authors should specify which day's tumor volume in the PBS group they are comparing to, as by day 18, all the mice in the PBS group had already died.
12. Again in Figure 6, the authors should monitor the expression levels of miR-21 and PDCD4 in the tumors of the tumor-bearing mice to demonstrate that, in the in vivo experiment, RIBOTAC21-BA and X-RAY killed the tumors through the miR-21-PDCD4 axis.
13. In Supplementary Figure 46, the mice in the PBS group had already died after day 12, so why are there body weight data available after that point?
14. The numbering of the experimental result figures in the supplementary material provided by the authors is incorrect (starting from Figure 38 and Figure 39). In the main text, the authors reference Figure 38a and Figure 38b, but in the supplement, the numbering is Figure 38 and Figure 39.
15. I am curious whether X-ray, as a high-energy radiation, could affect the stability of RIBOTAC21-BA or alter the pH of the tumor microenvironment, thereby influencing the efficacy of RIBOTAC21-BA.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Based on the revisions, the authors have addressed all the comments, making this paper suitable for publication.

Reviewer #3

(Remarks to the Author)
After carefully reading the authors' revisions in response to the series of questions I raised previously, I think that Shi et al. have addressed all of my concerns regarding this article. It is an excellent article and, in my opinion, it meets the requirements for publication. I don't have any further questions. My suggestion is accept.

Comments: I would publish this as is in Nature Communications as for this journal and what it has published it is above the bar

1. What are the noteworthy results?

The development of an in vivo photo activated RNA degrader, demonstrating on-target effects and with the promise to minimize off-targets.

Response: Thanks for your affirmation and encouragement to us.

2.Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

this is an advance over the original pioneering robotac approach, including phototrigegred decaging and activation in mouse models.

Response: Thanks for your affirmation and encouragement to us.

3.Does the work support the conclusions and claims, or is additional evidence needed?

Yes, there is biochemical and cellular data that support the mouse studies, as well as pharmacodynamic studies.

Response: Thank the reviewers for their recognition of our work.

4.Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

None, i think this is fine as is.

Response: Thanks for your affirmation and encouragement to us.

5.Is the methodology sound? Does the work meet the expected standards in your field?

Yes, it does in general but it would be nice if there was some more characterization such as proton NMR for the compounds in the SI; there is generally MS only now.

Reply: Thanks for the reviewer's valuable comments. In fact, the NMR characterization data for all the synthesized compounds without cRGD peptides has already been provided. Since RIBOTAC21-BA and RIBOTAC21 have a large molecular weight, complicated structure, and too many protons, we have previously conducted ^{1}H NMR characterization for them, but the spectra are quite complex and cannot clearly reflect the structural information. Hence, we did not list them in supporting information. Fortunately, we believe that the high-resolution mass data are able to confirm them.

6. Is there enough detail provided in the methods for the work to be reproduced?

Yes, easily.

Reply: Thanks for the reviewer's affirmation.

Reviewer #2:

Comments: In the manuscript entitled "Tumor Microenvironment-activated Robotic Ribonuclease Targeting Chimeras for Precise Tumor Treatment" by Zhang et al., the study presents a tumor microenvironment-activated RiboTAC (TaRiboTAC) strategy designed to address the off-target limitations of conventional RiboTACs in RNA degradation therapies. A pre-miRNA-21 nano-degrader (RIBOTAC21-BA) was developed to selectively target and degrade pre-miR-21 in tumors by activating ribonucleases in response to tumor-specific conditions such as low pH and elevated H2O2 levels. Interestingly, the use of a near-infrared (NIR) probe to selectively activate the RiboTAC molecule significantly increases the selectivity of the proposed approach. This method effectively enhanced the radiotherapy response in lung cancer models, highlighting the potential of TaRiboTACs for precise and efficient cancer treatment.

Response: Thanks for your affirmation and encouragement to us.

Major Comments:

1. A similar study was published recently by the Jiang lab in JACS (J. Am. Chem. Soc. 2024, 146, 15815−15824). That study also features activation in response to cellular conditions, including H2O2 and bioorthogonal activation, which significantly reduces the novelty of this research.

Reply: Thanks for the reviewer's comments. During the process of drafting this work, we also noted this amazing work published in JACS. Although there are some similarities between our work and theirs, the system we developed herein is theoretically more precise for tumor treatment because the degradation of target RNA requires weak acid and hydrogen peroxide dual activation in tumor microenvironment, so our system has great biological safety. More importantly, the target RNA in our work is closely associated with the radiotherapy resistance. Taking advantage of our system, the radiotherapeutic susceptibility of malignant tumors can be greatly improved, which shows a great potential for clinical application. We have cited this JACS work in reference 32.

2.The large molecular weight and tendency of such big molecules to aggregate could limit their application as potential therapeutics.

Reply: Thanks for the reviewer's good suggestion. I fully agree with your opinions and concerns. To be honest, our current work is mainly focusing on the development of a new RNA degradation tool as a proof of concept to improve the accuracy and efficiency of tumor therapy. The large molecular weight and aggregating tendency of current probes indeed limit their application as potential therapeutic agents. To overcome these potential problems, we are currently optimizing the structure of probes and improving the water solubility, and the relevant results will be reported later.

3.To confirm that the cleavage occurs at the same binding site, a competitive cleavage assay with the non-activated compound and the original binder should be performed.

Reply: Thanks for the reviewer's valuable comments. To confirm that the cleavage occurs at the same binding site, a competitive cleavage assay containing RIBOTAC21-BA and Pre-miR-21 binder (compound **11**) were also conducted. Meanwhile, to evaluate the selectivity of RIBOTAC21-BA toward pre-miR-21, several Cy3 labeled RNAs as controls, e.g. pre-miR-21BP, miR-501, and miR-4761, were also treated with RIBOTAC21-BA, respectively. As shown in Figure S47, the degradation of Pre-miR-21 was significantly inhibited if the assay was pre-treated

to Pre-miR-21 binder (Group IV) compared with the ones without treatment of binder (Group III), indicating that the degradation of Pre-miR-21 occurs at the same binding site. Besides, for other control RNAs, no obvious degradation was observed, strongly demonstrating that RIBOTAC21-BA can be used for specific degradation of Pre-miR-21.

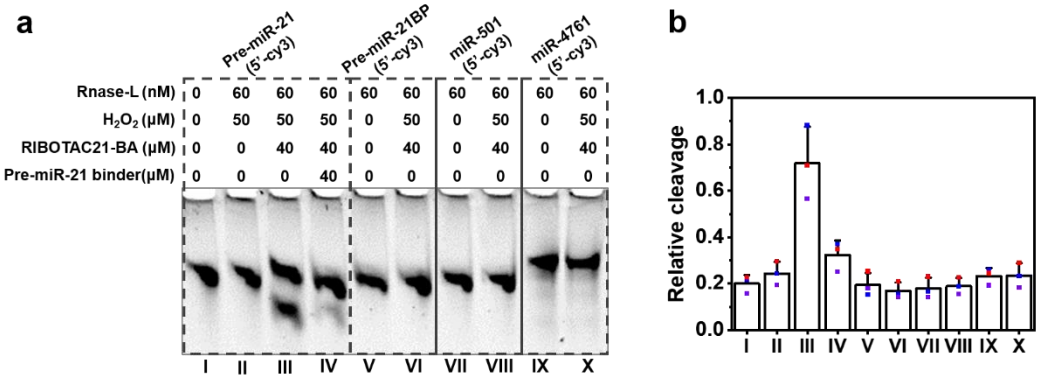


Figure S47. (a) Representative SDS-PAGE gel images of 5'-cy3 labeled RNAs (Pre-miR-21, Pre-miR-21BP, miR-501, and miR-4761) in aqueous solutions (pH=5.5) under different treatment conditions at 25°C for 12 h. Pre-miR-21 binder was compound **11**. (b) Quantification of miRNA degradation in a. n = 3 independent samples, data denote the mean ± standard deviation.

4. To confirm the selectivity of this approach in reducing only miR-21, a miRNome sequencing and RNA sequencing experiment should be conducted to assess whether the affected RNA is related solely to miR-21.

Reply: Thanks for the reviewer's insightful comments. To further verify the selectivity of our probes on miR-21, RNA sequencing experiment was also performed. Figure S54 shows that co-incubation of RIBOTAC21-BA with A549 cells can lead to the degradation of several small RNAs, including miR-21-5p, miR-3620-3p, miR-3144-5p, miR-6277-3p, etc. By contrast, the degradation degree of miR-21-5p (with the smallest p-value) is remarkably higher than others. The transcriptome analysis indicates that the transcription change of mRNAs can efficiently inhibit the tumor cell growth and improve the radiation sensitivity (Figure S55). Overall, these results strongly demonstrate that RIBOTAC21-BA possess excellent specificity for miR-21-5p.

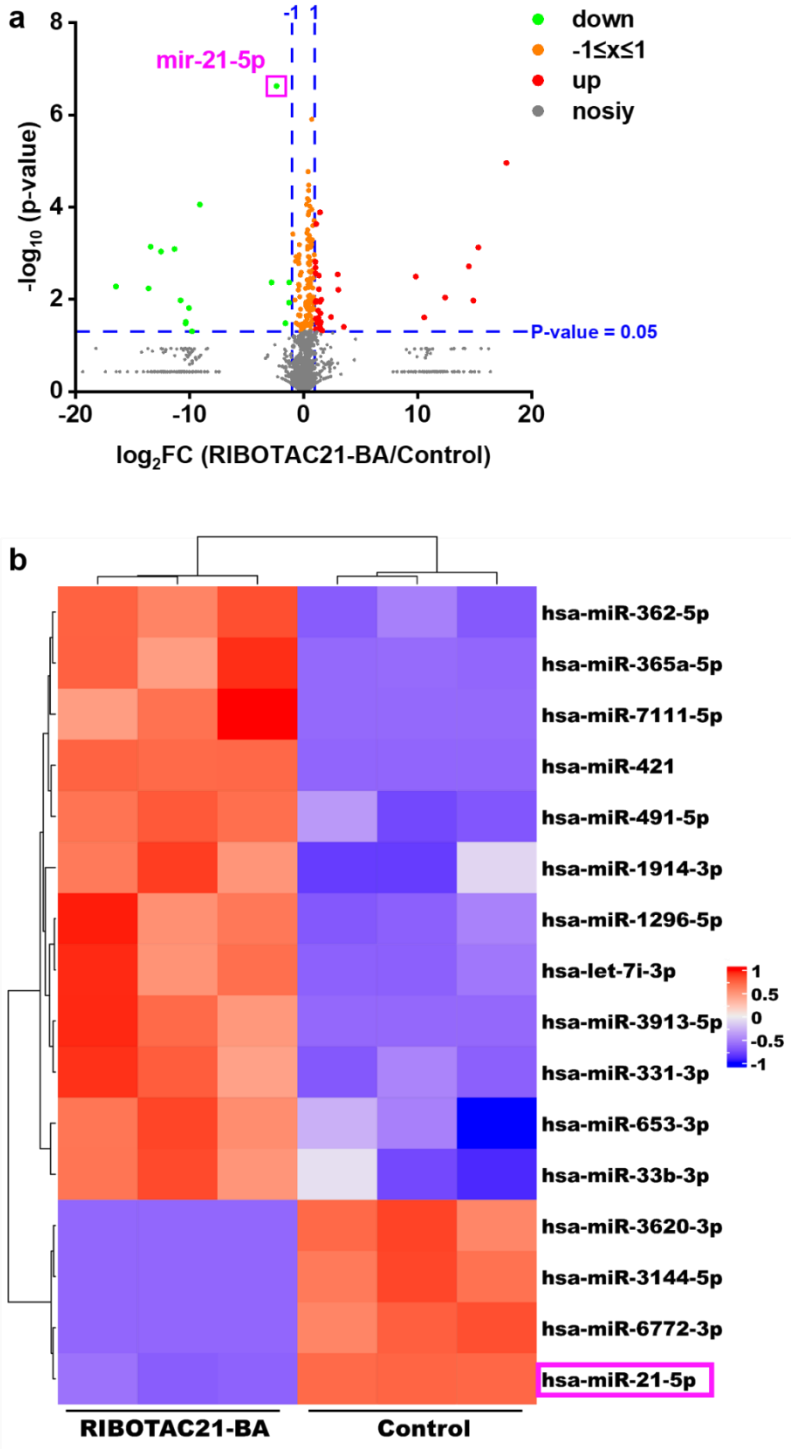


Figure S54. Small RNA sequencing analysis of A549 cells co-incubated with RIBOTAC21-BA (50 nM) for 48 hours. (a) Volcano plot of differential microRNAs. (b) Clustering heatmap of differential microRNAs. n = 3 independent samples.

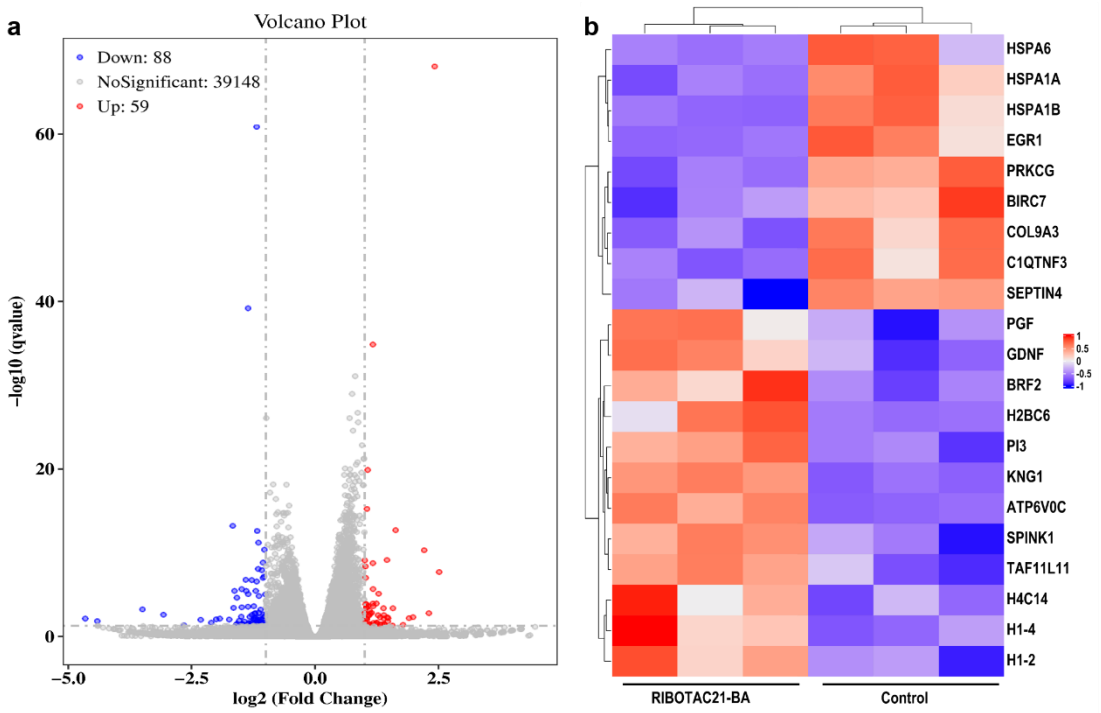


Figure S55. (a) Volcano plot and (b) clustering heatmap of transcriptome analysis of A549 cells co-incubated with RIBOTAC21-BA (50 nM) for 48 hours. n = 3 independent samples.

5. The effect of H_2O_2 on RNase L oligomerization with caged and non-caged RIBOTAC21 compounds should be added as a control.

Reply: Thanks for the reviewer's great suggestions. To study the effect of H_2O_2 on RNase L oligomerization, we further carried out the control experiments with caged and non-caged RIBOTAC21. As shown in Figure S46, the oligomerization of caged RIBOTAC21-BA only occurred in the presence of both RNase L and H_2O_2 (III), but non-caged RIBOTAC21 could be induced to form oligomerization by RNase L no matter with (V) or without (IV) H_2O_2 indicative of no effect on the activity of RNase L.

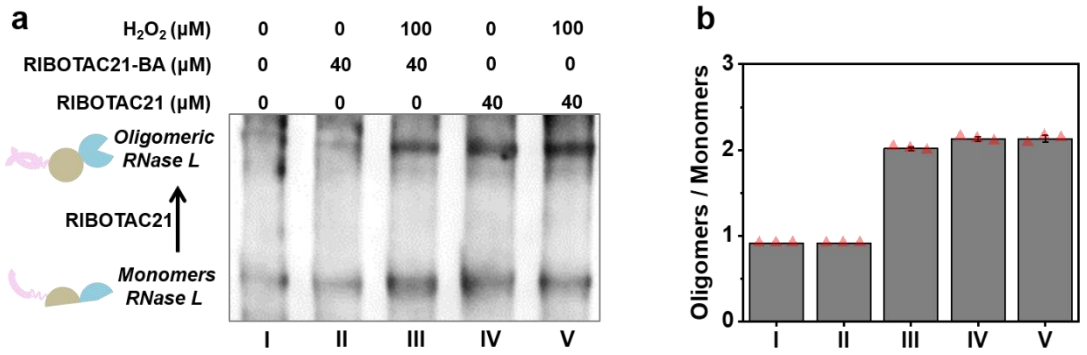


Figure S46. Representative PAGE gel images (a) and quantitative analysis (b) of the RNase L oligomerization induced by RIBOTAC21-BA+H_2O_2 or RIBOTAC21. n = 3 independent samples, data denote the mean ± standard deviation.

6. Figure 4e lacks clarity regarding the band being observed. If miR-21 is being cleaved, a hydrolyzed miR-21 construct should be used to map the cleavage and serve as a control. Additionally, the observed miR-21 cleavage appears relatively low in this assay, and the quantification of the VI seems difficult to understand.

Reply: Thanks for the reviewer's great comments. As shown in Figure 4e, the reason that the cleavage of miR-21 is relatively low here in VI assay may be due to the neutral buffer used, RIBOTAC21-BA can't be fully activated. To quantify the degradation of VI in Figure 4e, we further measured the grayscale value of the corresponding band generated by degradation of pre-miR-21. In contrast to the control assay (II), around half of total amount of miR-21 was cleaved (Figure 4f). However, if both weak acidic and hydrogen peroxide present in the assays, RIBOTAC21-BA shows a significantly high degradation efficiency for target RNA (Figure 4h).

Reviewer #3:

1. In Figure 3a, after treating A549 and BEAS-2B cells with 5 μM RIBOTAC21-BA, the fluorescence detection results provided by the authors show that the fluorescence intensity in A549 cells is significantly higher than in BEAS-2B cells. However, this does not fully indicate that the binding ability of RIBOTAC21-BA to A549 cells is higher than to BEAS-2B cells, as the authors did not test whether there were differences in the pH of the culture media between the two cell types. One possibility is that the tumor cells lower the pH of the culture media compared to normal cells (because of different metabolic characteristics between tumor cells and normal cells), thereby causing RIBOTAC21-BA to produce fluorescence.

Reply: Thanks for the reviewer's valuable comments. It is well known that integrin proteins are highly expressed onto the membrane of tumor cells. To improve the specificity of our probes to cancer cells, a cyclic RGD peptide that can specifically bind to integrin proteins was introduced in the molecule structure. Thus, the uptake of probes in tumor cells is theoretically higher than that in normal cells. Since the fluorescence signal of RIBOTAC21-BA is responsive to the acidic condition, hence the tumor cells could be specifically lit up by probes. We also measured the pH value of the culture medium in A549 lung cancer cells and BEAS-2B cells, and found that the initial pH values of 1640 and DMEM were 8.4 and 7.98, respectively. However, the pH value of 1640 medium for A549 lung cancer cells was apparently decreased to 7.6 after 24 h culture, while the one of DMEM medium for BEAS-2B cells was increased to 8.1 (Figure S41). Furthermore, both BEAS-2B cells and A549 cells were treated to the same amount of RIBOTAC21-BA for 4 h at 37^{o}C. The culture medium was then replaced by fresh medium with pH 7.4 or 5.5 for further 30 min incubation followed by confocal imaging. The results in Figure S42 clearly show that the fluorescence of BEAS-2B cells is significantly weaker than the ones of A549 cells even in acidic medium (pH 5.5), again demonstrating that RIBOTAC21-BA has great binding ability to cancer cells.

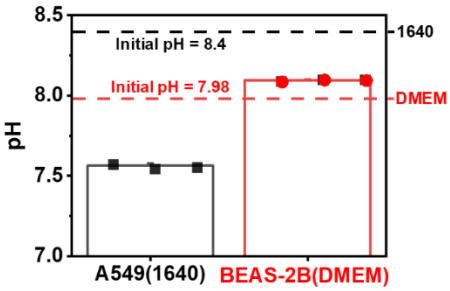


Figure S41. pH measurement of 1640 medium after 24 hours of A549 cells culture, and pH measurement of DMEM medium after 24 hours of BEAS-2B cells culture. The dashed lines represent the initial pH value of the medium. n = 3 independent samples, data denote the mean ± standard deviation.

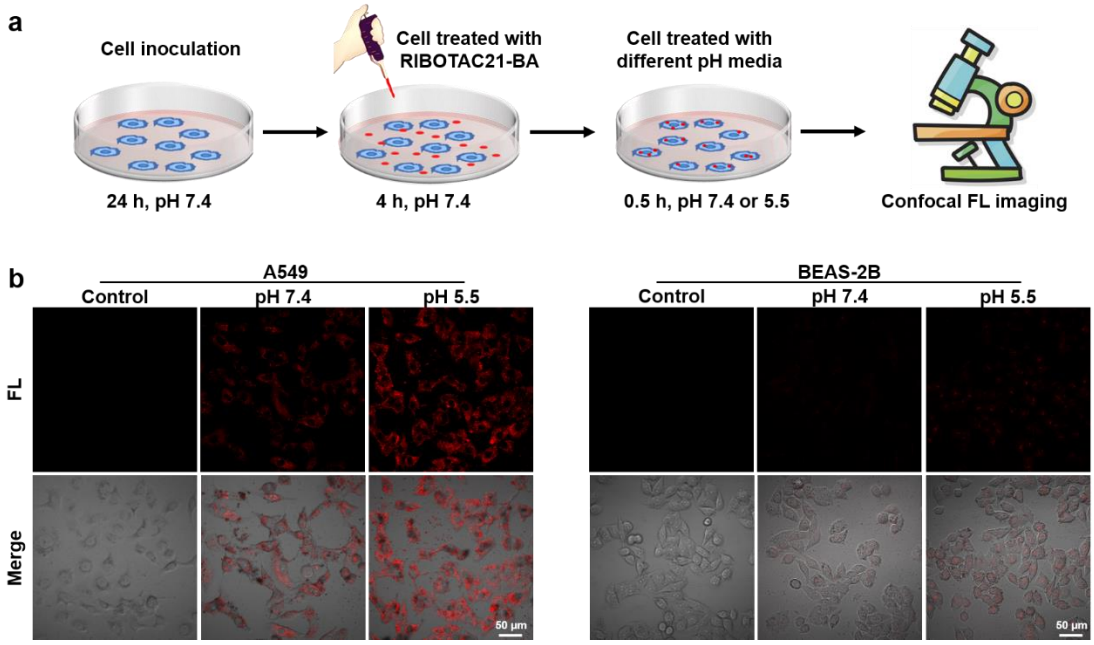


Figure S42. (a) Flow chart of RIBOTAC21-BA (5 μM) uptake experiment by tumor cells A549 and lung epithelial cells BEAS-2B. (b) A549 and BEAS-2B cells were cultured in medium containing RIBOTAC21-BA (5 μM) for 4 h, then the medium was sucked away and cultured in medium (pH =7.4 or 5.5) for 30 min, and then the fluorescence images of the cells were recorded by confocal. In the culture medium with pH=7.4, the fluorescence brightness of lung epithelial cells was significantly lower than that of tumor cells. In order to exclude the influence of the pH change of tumor cells on the fluorescence brightness, we further treated the cells with the culture medium with pH= 5.5, and the fluorescence brightness of tumor cells was still significantly higher than that of normal cells (scale bar = 50 μm).

2. Regarding the results presented in Figure 3d, I have a question: The authors added a cRGD peptide to RIBOTAC21-BA to achieve tumor targeting. Although RIBOTAC21-BA exhibited stronger fluorescence in the pH 5.5 culture system, this does not necessarily indicate that the binding capacity of RIBOTAC21-BA to tumors is enhanced under pH 5.5 conditions. What I mean is that under pH 5.5 conditions, the binding ability of RIBOTAC21-BA to tumors may actually weaken. So the authors need to demonstrate whether low pH affects the tumor-targeting ability of cRGD.

Response: Thanks for the reviewer's good question. The data in Figure 3d demonstrated that the fluorescence signal of the probes could be dramatically enhanced in weak acid condition except for the specific targeting of cancer cells through the binding between cRGD and integrin, supporting the potential of the probe for accurate and sensitive detection of tumor cells. Moreover, we also investigated whether low pH affects the tumor-targeting ability of the probes. Briefly, cancer cells were incubated with RIBOTAC21-BA in various medium with different pH (pH = 5.5, 6.5, and 7.4) for 4 h. The medium was then replaced by fresh medium (pH 5.5) and incubated for additional 30 min to fully activate the fluorescence of RIBOTAC21-BA in the cells. As shown in Figure S43, no significant fluorescence difference was observed for three groups of cells with different pH of cell medium, indicating that low pH condition does not affect the targeting ability of the probes.

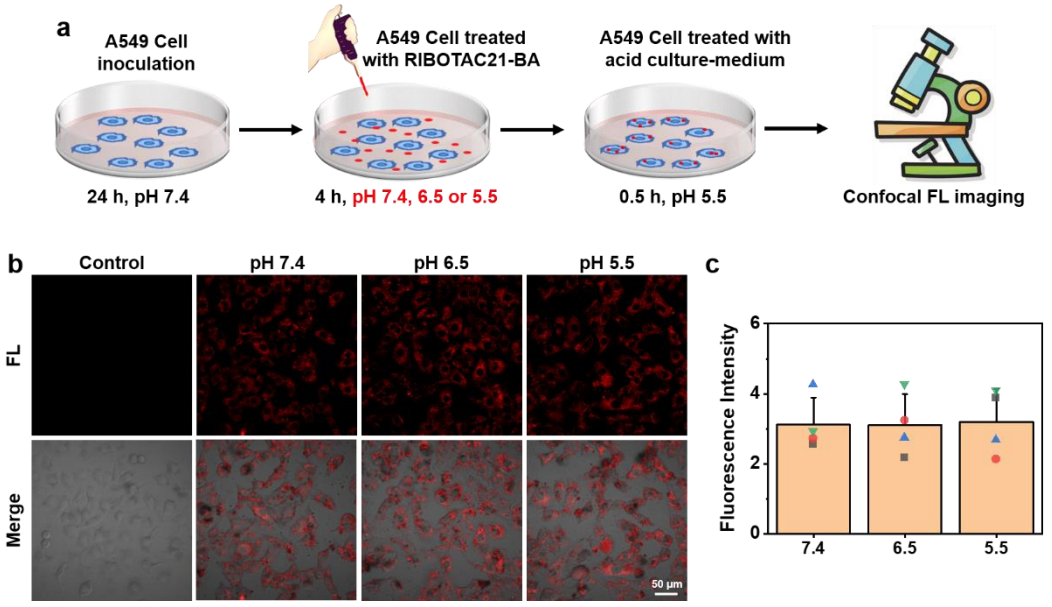


Figure S43. Uptake of RIBOTAC21-BA (5 μM) in A549 cells in the medium with different pH. (a) Experimental flow chart. (b) A549 cells were incubated with RIBOTAC21-BA in different pH medium for 4 h, and then replaced by fresh medium (pH = 5.5) for 30 min followed by fluorescence imaging. (c) Fluorescence quantification in Figure b. n = 4 independent samples, data denote the mean ± standard deviation.

3. The authors should include an additional set of experiments in Figure 4, selecting several unrelated miRs as substrates (preferably with sequences similar to pre-miR-21) to verify the specificity of the miR-21 binder and whether RIBOTAC21-BA exhibits any off-target effects.

Response: Thanks for the reviewer's great suggestions. To further verify the specificity of RIBOTAC21-BA to Pre-miR-21, Three unrelated miRNAs including Pre-miR-21BP, miR-501, and miR-4761 were parallelly treated to RIBOTAC21-BA followed by SDS-PAGE gel imaging. As shown in Figure S47, no degradation was detected for three control miRNAs except for Pre-miR-21, revealing that RIBOTAC21-BA exhibits no off-target effect.

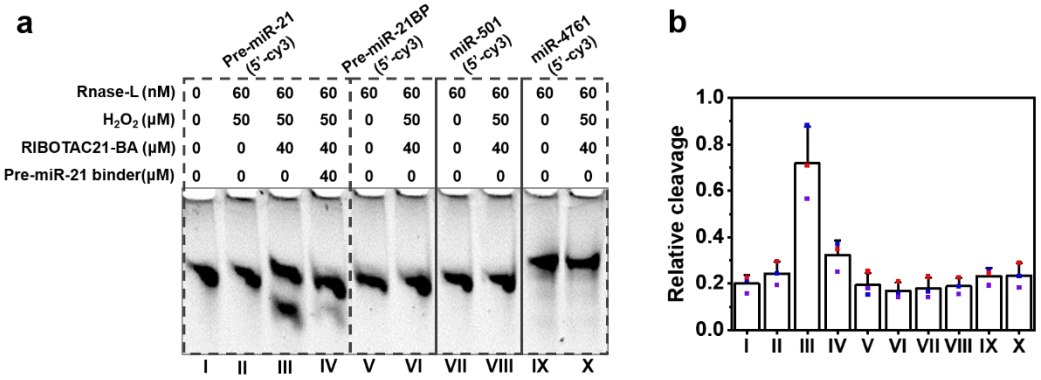


Figure S47. (a) Representative SDS-PAGE gel images of 5'-cy3 labeled RNAs (pre-miR-21, Pre-miR-21BP, miR-501 and miR-4761) in aqueous solutions (pH = 5.5) under different treatment conditions at 25°C for 12 h. Pre-miR-21 binder was compound **11**. (b) Quantification of miRNA degradation in a. n = 3 independent samples, data denote the mean ± standard deviation.

4. In Figures 5d and 5e, why did the authors choose to amplify miR-21-5p instead of directly performing quantitative analysis on pre-miR-21 using PCR? Even so, the authors should also include a set of real-time fluorescence quantitative PCR results rather than simply presenting semi-quantitative PCR plus agarose gel results.

Response: Thanks for the reviewer's great comments. To double confirm that RIBOTAC21-BA specifically degrades endogenous miR-21 in A549 cancer cells, we further performed real-time fluorescence quantitative PCR assays, and the results in Figure S49 clearly show that RIBOTAC21-BA can specifically degrade the endogenous miR-21 in A549 cancer cells but not the ones in BEAS-2B cells. However, for RIBOTAC21 whose targeting ability of miR-21 is always on, the endogenous miR-21 both in A549 and BEAS-2B can be efficiently degraded, again revealing the excellent biosafety of RIBOTAC21-BA in living system.

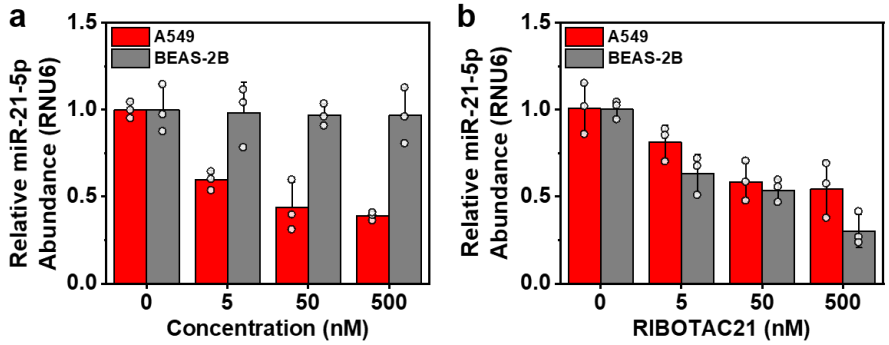


Figure S49. Effects of different concentrations of RIBOTAC21-BA (a) andRIBOTAC21 (b) on mature miR-21 levels in A549 and BEAS-2B cells as determined by RT-qPCR. n = 3 independent samples, data denote the mean ± standard deviation.

5. In Figures 5d-5g, since A549 and BEAS-2B are two different cell lines, the endogenous expression levels of miR-21-5p and PDCD4 are inherently different. Therefore, comparing the expression level differences of miR-21-5p and PDCD4 in the two cell lines after RIBOTAC21-BA treatment is not meaningful. The authors should compare the expression differences between

different treatment concentrations instead.

Response: Thanks for the reviewer's valuable comment and suggestion. Indeed, we already compared the expression variation between different treatment concentrations while studying the RIBOTAC21-BA's effect on endogenous expression of miR-21-5p and PDCD4. As shown in Figure 5d and 5e, the degradation of miR-21-5p in A549 cells gradually enhanced with the increasing concentrations of RIBOTAC21-BA used, whereas almost no miR-21-5p degradation was determined in BEAS-2B cells regardless of how much RIBOTAC21-BA is used (Figure S49a). The expression of downstream protein PDCD4 in A549 and BEAS-2B cells after the treatments of different concentrations of RIBOTAC21-BA were also evaluated through western blotting. It was consistently found that the expression of PDCD4 in A549 cells was gradually enhanced with the increase of RIBOTAC21-BA concentrations, but remained unchanged in BEAS-2B cells (Figure 5f and 5g).

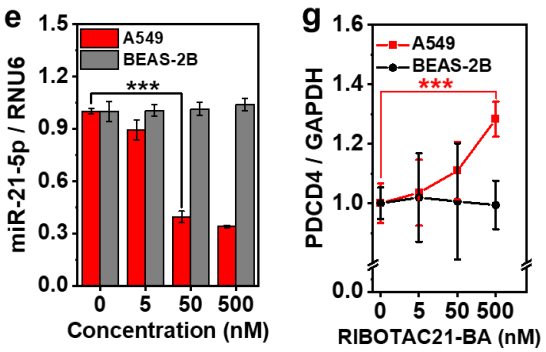


Figure 5. (e). The corresponding quantitative analysis of degradation of miR-21-5p both in A549 and BEAS-2B cells treated with different concentrations of RIBOTAC21-BA determined by agarose gel. (g). The corresponding quantitative analysis of expression of PDCD4 both in A549 and BEAS-2B cells treated with different concentrations of RIBOTAC21-BA determined by WB.

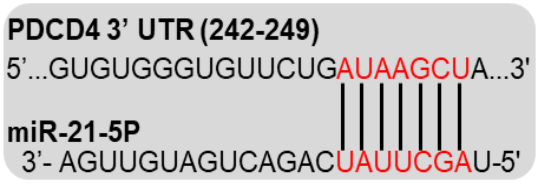
6. Please provide a reference for the statement "As programmed cell death 4 (PDCD4) is well known to suppress neoplastic transformation, cell proliferation, and metastasis, and it can be typically downregulated by miR-21 in cancer cells".

Response: Thanks for the reviewer's comment. Below references have been properly cited as 57-59 in the revised manuscript.

57. Matsuhashi, S. Manirujjaman, M. Hamajima, H. Ozaki, I. Control Mechanisms of the Tumor Suppressor PDCD4: Expression and Functions. *Int. J. of Mol. Sci.* **20**, 2304 (2019).
58. Vikhreva, P.N. Shepelev, M.V. Korobko, I.V. mTOR-dependent transcriptional repression of Pdcd4 tumor suppressor in lung cancer cells. *BBA-Gene Regul. Mech.* **1839**, 43-49 (2014).
59. Wang, Q. Yang, H.S. The role of Pdcd4 in tumour suppression and protein translation. *Biol. Cell.* **110**, 169-177 (2018).

7. If PDCD4 is a target gene regulated by miR-21-5p, please provide a schematic diagram in Figure 5 showing the binding sequence between miR-21-5p and the 3'UTR of PDCD4 mRNA.

Response: Thanks for the reviewer's great comment. Below is the binding sequence between miR-21-5p and the 3'UTR of PDCD4 mRNA, and it has been inserted into Figure 5a.



The relevant literature is listed below:

[1] Peng, T. et al. Exosomes from Hypoxia Preconditioned Muscle-Derived Stem Cells Enhance Cell-Free Corpus Cavernosa Angiogenesis and Reproductive Function Recovery. *Adv. Healthc. Mater.* 2402406 (2024). https://doi.org/10.1002/adhm.202401406.

[2] Li, P. et al. Role of microRNA-21 in radiosensitivity in non-small cell lung cancer cells by targeting PDCD4 gene. *Oncotarget.* **8**, 23675-23689 (2017).

[3] Asangani, I., Rasheed, S., Nikolova, D. et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* **27**, 2128–2136 (2008).

[4] Frankel, Lisa B. et al. Programmed Cell Death 4 (PDCD4) Is an Important Functional Target of the MicroRNA miR-21 in Breast Cancer Cells. J. Biol. Chem. **283**, 2, 1026 - 1033.

[5] Lu, Z. et al. MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. *Oncogene* **27**, 4373–4379 (2008).

[6] Wright, E.C. et al. MicroRNA-21 and PDCD4 expression during in vitro oocyte maturation in pigs. *Reprod. Biol. Endocrin.* **14**, 21 (2016).

8. In the section discussing the function of PDCD4, the authors state that "Numerous studies have proven that the elevation of protein PDCD4 can enhance the radiosensitivity of cancer cells" and cite references 57 and 58. However, in fact, these two references do not describe any correlation between PDCD4 and radiosensitivity.

Response: I appreciate the reviewer's question. The correlation between PDCD4 and radiosensitization was only mentioned in reference 57 and 58, but no detailed study was performed. Therefore, we searched and listed some more relevant literatures below, and some of them were cited as ref 60 and 61 in the revised manuscript. Below is some discussion on it in the relevant paper.

1. "We identify PDCD4 as an important ubiquitination substrate of SKP2. SKP2 promotes breast cancer tumorigenesis and radiation tolerance via PDCD4 degradation. Radiotherapy combine with SKP2-targeted adjuvant therapy may improve breast cancer patient survival in clinical medicine." [1]
2. "Finally, U87-MG cells regained radiosensitivity following PDCD4 overexpression."[2]
3. "Programmed cell death 4 (PDCD4) is critical in mediating apoptosis in GBM, and is downregulated by miR-21, which may mediate the resistance of glioblastoma cells against chemotherapy or radiation via its target genes PDCD4." [3]
4. "Moreover, PDCD4 knockdown reversed the radiosensitizing effects of S6K1 inhibition in NSCLC." [4]
5. "The findings suggested that miR-21 may mediate the resistance of glioblastoma cells against radiation via its target genes PDCD4 and hMSH2." [5]

[1] Li, C. et al. SKP2 promotes breast cancer tumorigenesis and radiation tolerance through PDCD4 ubiquitination. *Exp. Clin. Canc. Res.* **38**, 76 (2019).

[2] Guo, P. et al. Upregulation of miR-96 promotes radioresistance in glioblastoma cells via targeting PDCD4. *Int. J. Oncol.* **53**, 1591-1600 (2018).
[3] Wang, G. et al. Targeting strategies on miRNA-21 and PDCD4 for glioblastoma. *Arch. Biochem. Biophys.* **580**, 64-74 (2015).
[4] Wang, Y. Mei, H. Shao, Q. Wang, J. Lin, Z. Association of ribosomal protein S6 kinase 1 with cellular radiosensitivity of non-small lung cancer. *Int. J. Radiat. Biol.* **93**, 581-589 (2017).
[5] Chao, F. Xiong, H. Liu, W. Chen, Y. Zhang, J. MiR-21 Mediates the Radiation Resistance of Glioblastoma Cells by Regulating PDCD4 and hMSH2. *J. Huazhong U. Sci-Med.* **33**, 525-529 (2013).
[6] H. Ishinaga, Y. Okugawa, B. Hou, F. He, C. Yin, M. Murata, Y. Toiyama, K. Takeuchi, Journal of Radiation Research, 64 (2023) 668-676.

9. In the Instrumentation section, the authors did not mention the X-ray equipment and its supplier.
Response: Thanks for the reviewer's suggestion. The X-ray irradiator is RS-2000-PRO made by Radsource Technologies Co., LTD, which has been added in the instrument section of revised manuscript.

10. The results shown in Figures 5k-5n, the descriptions in the Results section, and the figure legends for them, do not correspond to each other, making it very difficult to understand these results. Additionally, the results in Figure 5L are quite confusing. What does the SER unit represent? What is the D0 (Gy) parameter? Why are the radiation doses listed under the D0 column different? Furthermore, in the results, the authors describe that "RIBOTAC21-BA has the lowest survival rate with a SER of 1.33," but in Figure 5L, the SER for the RIBOTAC21-BA group is shown as 1.30, and G0 (Gy) = 1.33.
Response: Thank the reviewer for pointing out our mistake. We have corrected the description of Figure 5k-5n in the Results section as well as Figure legends. We are so sorry for our unclear description. Some detailed explanations were properly added in the legend of Figure 5. SER unit represents the sensitivity enhance ratio. D0 (Gy) parameter represents the dose required for survival drop to 37% before exposure. Because the irradiation dose required to reduce cell survival to 37% varied in each group, the D0 was also different in each group. We apology for typing a wrong value. The SER value of RIBOTAC21-BA group has been corrected to be 1.30.

11. In the description of Figure 6, the authors mention that the tumor suppression rate is up to 74.3% on day 18 compared to the PBS group. The authors should specify which day's tumor volume in the PBS group they are comparing to, as by day 18, all the mice in the PBS group had already died.
Response: Thanks for your great comments. The tumor inhibition rate of 74.3% mentioned in the manuscript was calculated by comparing the tumors in the experimental group (RIBOTAC21-BA+X-ray) at 18 days with the one in the PBS group at 12 days. In fact, the mice were considered to be dead when the tumor volume exceeded 1098 mm^3, so the subsequent volumes of the tumors in PBS group were not recorded after 12 days. If the tumors of the experimental group (RIBOTAC21-BA+X-ray) on day 18 were compared to the ones of the PBS group on day 18, the tumor inhibition rate is theoretically higher than 74.3%.

12. Again in Figure 6, the authors should monitor the expression levels of miR-21 and PDCD4 in the tumors of the tumor-bearing mice to demonstrate that, in the in vivo experiment, RIBOTAC21-BA and X-RAY killed the tumors through the miR-21-PDCD4 axis.

Response: Thanks for the reviewer's valuable comment. To demonstrate that RIBOTAC21-BA and X-RAY killed the tumors through the miR-21-PDCD4 axis, we monitored the expression levels of miR-21 and PDCD4 in A549 tumors. As shown in Figure S50, compared to the control group, the miR-21 expression exhibited a 42% reduction after the treatment of RIBOTAC21-BA, accompanied by a significant increase of the programmed cell death protein PDCD4 expression. These results strongly reveal that the tumor suppression by RIBOTAC21-BA and X-ray mainly caused by the miR-21-PDCD4 axis.

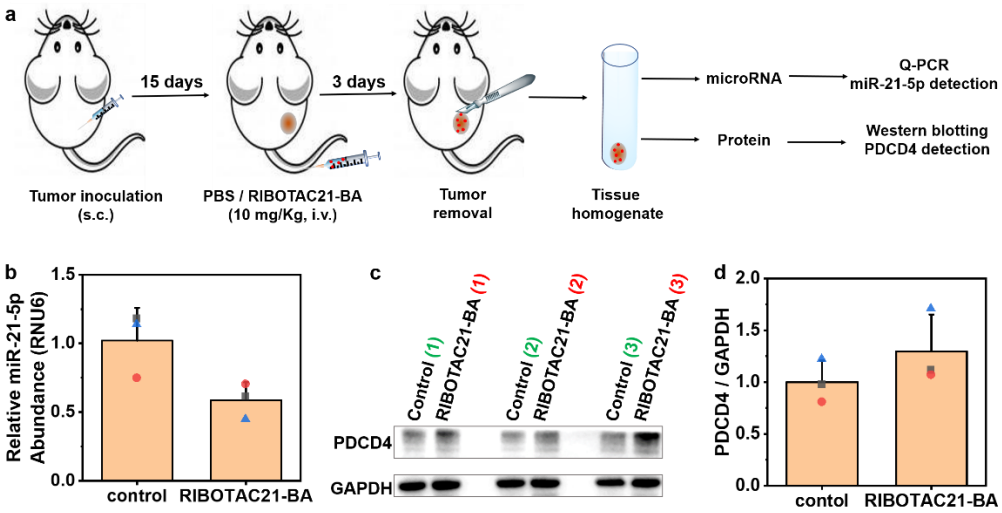


Figure S50. Effects of RIBOTAC21-BA (10 mg/kg, i.v.) on miR-21 and PDCD4 expression levels in A549 subcutaneous tumors-bearing mice. (a) Schematic diagram of the experimental procedure. (b) RT-qPCR analysis of the effect of RIBOTAC21-BA on mature miR-21 expression levels in A549 subcutaneous tumors. (c) Western blot analysis of the effect of RIBOTAC21-BA on PDCD4 levels in A549 subcutaneous tumors. (d) Quantification of PDCD4 protein levels as shown in (c). n = 3 independent samples, data denote the mean ± standard deviation.

13. In Supplementary Figure 46, the mice in the PBS group had already died after day 12, so why are there body weight data available after that point?

Response: Thanks for the reviewer's good question. In fact, when the tumor volume exceeded 1098 mm^3, we assumed that the mice were dead, so we did not further monitor the tumor volume of the mice at subsequent time points, but continued to measure their body weights. All mice were eventually euthanized at the end of the final experiments.

14. The numbering of the experimental result figures in the supplementary material provided by the authors is incorrect (starting from Figure 38 and Figure 39). In the main text, the authors reference Figure 38a and Figure 38b, but in the supplement, the numbering is Figure 38 and Figure 39.

Response: Thanks for pointing out our mistake. We have carefully gone through the whole manuscript and have properly corrected the numbering both in the main text and SI.

15. I am curious whether X-ray, as a high-energy radiation, could affect the stability of RIBOTAC21-BA or alter the pH of the tumor microenvironment, thereby influencing the efficacy of RIBOTAC21-BA.

Response: Thanks for your great comments. To study the stability of RIBOTAC21-BA under high-energy radiation of X-ray, HPLC analysis were utilized to evaluate it. As shown in Figure S39b, the HPLC profile of RIBOTAC21-BA had no obvious change even after 10 Gy irradiation, suggesting that X-ray has no effect on RIBOTAC21-BA. Moreover, to investigate whether X-ray irradiation alter the pH of the tumor microenvironment, BCECF AM, a commonly used fluorescent pH indicator, was used to monitor the pH change of tumor microenvironment with or without X-ray irradiation (6 Gy). The results in Figure S53 indicate that no pH change was determined in tumors after X-ray irradiation.

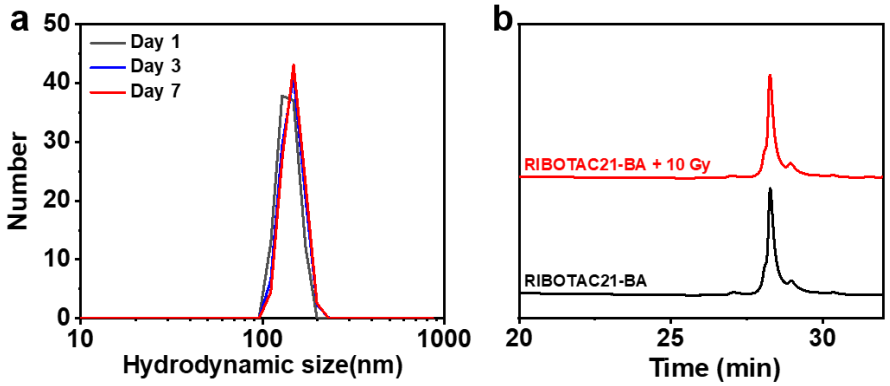


Figure S39. (a) The hydrodynamic size of RIBOTAC21-BA nanoparticles in PBS buffer (pH=7.4) over 7 days. (b) HPLC profiling of RIBOTAC21-BA before and after irradiation of 10 Gy X-ray.

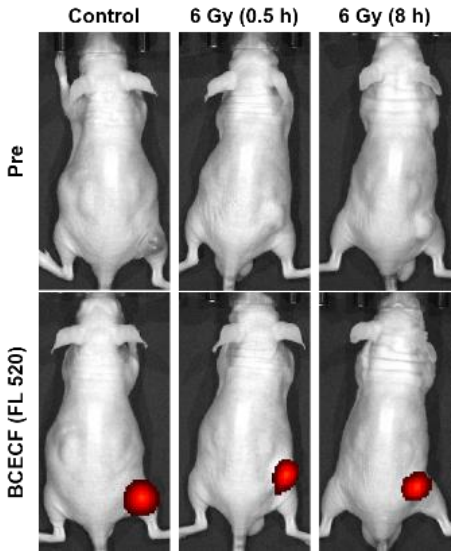


Figure S53. BCECF AM was used to monitor the pH change in tumors with or without X-ray irradiation (6 Gy). The pH detection reagent BCECF AM (5 μM, 50 μL) was injected into the tumors and fluorescence imaging was performed after 0.5 h (*Ex* = 480 nm, *Em* = 520 nm). No significant fluorescence change within tumors was determined, indicating that 6 Gy X-ray did not affect the pH value of the tumor microenvironment.

Reviewer #2 (Remarks to the Author):

Comments: Based on the revisions, the authors have addressed all the comments, making this paper suitable for publication.

Response: Thank you for your affirmation and encouragement.

Reviewer #3 (Remarks to the Author):

Comments: After carefully reading the authors' revisions in response to the series of questions I raised previously, I think that Shi et al. have addressed all of my concerns regarding this article. It is an excellent article and, in my opinion, it meets the requirements for publication. I don't have any further questions. My suggestion is accept.

Response: We sincerely appreciate your affirmation and encouragement.