Iranian Journal of Pharmaceutical Research
Volume 22 · Issue 1 · e131758

OPEN PEER REVIEW

Hyaluronic Acid-modified Liposomes for Ursolic Acid-targeted Delivery Treat Lung Cancer Based on p53/ARTS-mediated Mitochondrial Apoptosis

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SUBMITTED	REVISED	ACCEPTED	DOI
14 Sep 2022	8 Feb 2023	20 Feb 2023	10.5812/ijpr-131758

About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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Contents

1 **Original submission** Referee 1, Referee 2, Associate Editor 2
2 **Revision 1** Associate Editor 4
Authors' reply to reviewers 5
Reviewer and editor comments 13
3 **Revision 2** 14
Authors' reply to reviewers 15
Reviewer and editor comments 17

1 Original submission

3 comments · 30 Sep 2022 – 4 Oct 2022

Referee 1 Reviewer

REJECT 30 Sep 2022

Dear AE,
Targeted liposomes are desired nanoplatforms for the delivery of anti-cancer agents, and some active molecules can be introduced as promising alternatives to conventional treatments. Accordingly, a targeted nanoliposomal formulation was developed in this original research; however, the present study lacks novelty. In a recent publication titled "Dual stimuli-responsive ursolic acid-embedded nanophytoliposome for targeted antitumor therapy," a similar formulation was designed and developed with the same active agent and targeting moiety. Furthermore, some points are listed below that require your consideration:
- The authors applied western blotting to suggest an apoptotic pathway and evaluated the overexpression of some markers. However, to quantify western blot bands, the results should be provided in triplicate with the same exposure time (Fig. 6). Also, the whole blots must be provided as supplementary materials.
- The authors have not clarified the details of some methods (e.g., western blotting), and therefore, their experiments are not reproducible and are hard to evaluate. In scheme 1, membrane extrusion was noted as a development step, while there is no relevant description in the method section.
- Statistical analysis was not performed appropriately. The t-test was mentioned as the statistical tool, which is not acceptable for the study's comparisons. Besides, the statistical significance symbols in the figures' bar charts seem to be incorrect (Fig. 6). Also, *** was indicated as $P < 0.0001$, which is not correct, and the sample replicates are unknown.
- The experiments lack a control group with the empty formulation.
- The manuscript was written in poor English and contains several writing errors of all types (grammar, spelling, punctuation, etc.).
Overall, the manuscript is not appropriate for publication in IJPR. It may be resubmitted after major modifications if all the above-mentioned points can be considered and addressed.

Referee 2 Reviewer

MAJOR REVISION 30 Sep 2022

Dear AE,
This manuscript needs major revision.
The paper "Hyaluronic Acid-modified Liposomes for Ursolic Acid-targeted Delivery Treat Lung Cancer Based On p53/ARTS-mediated Mitochondrial Apoptosis" aimed to design liposomes for targeted delivery of ursolic acid (UA) in non-small cell lung cancer (NSCL). The purpose of the paper is made clear by explaining the context and background in the Introduction. After the issues surrounding current therapeutics for NSCL are stated, UA as a part of Traditional Chinese Medicine and hyaluronic acid (HA)-modified liposomes for resolving the lack of specific targets of UA are introduced. HA-modified liposomes are prepared through the thin-film hydration method. The paper then describes the characterization of liposomes, which confirmed that the nanoparticles had a size of ~ 200 nm, suitable stability, sustained release, and greater cellular uptake and cytotoxic activity. In addition, the mechanism by which the formulation was able to induce apoptosis was investigated. There are several issues that should be addressed:

- Regarding the originality of the research, line 69, which states "the underlying mechanisms in which UA acts against NSCLC", is not acceptable, as there are recent studies on the antitumor mechanisms of UA in NSCLC that should be mentioned (such as "Antitumor Effects of Ursolic Acid through Mediating the Inhibition of

STAT3/PD-L1 Signaling in Non-Small Cell Lung Cancer Cells" by Kang et al., published in Biomedicines (volume 9, issue 3, March 13, 2021)).
- For the interpretation of results, p-values should be reported (e.g., when comparing the drug release of free-UA and Lipo/UA).
- Sufficient details are not provided for several methods described in the paper. For example, in the thin-film hydration method (HA-Lipo-UA preparation), the dispersion medium used for the hydration of the lipid film, the lipid-to-drug ratio, and the manufacturer's name and address for the rotary evaporator are not stated clearly. Furthermore, in line 106, "The liposomes solution was diluted at a ratio of 1:10, 1:50 and 1:250", the diluent should be mentioned.
- Abbreviations should be introduced in parentheses upon their first mention. There are a few abbreviations that are not defined in the text, such as "DSPE" and "GADPH".
- The whole manuscript should be revised to correct punctuation errors in in-text citations and errors in title capitalization. Moreover, an in-text citation is not provided for Figure 1E.

Associate Editor

MAJOR REVISION 4 Oct 2022

Your manuscript has been reviewed and some points are noted for your consideration. Based on the reviewers' comments, we ask you to provide the complete blot images from the western blot experiments (the bands in triplicate and imaged using the same exposure time). Furthermore, please consider whether you can add the empty formulation as a control group to your experiments and improve the manuscript's statistical analysis and language based on the comments. We will consider your response and revisions if you can address the reviewers' comments properly.

2

Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor,

I'm sorry that the revised version of our manuscript has been affected due to COVID-19. Thank you for your kind letter regarding the decision on our manuscript on October 5th, 2022. With this cover letter, we submit a revised version of our manuscript entitled "***Hyaluronic Acid-modified Liposomes For Ursolic Acid-targeted Delivery Treat Lung Cancer Based On p53/ARTS-mediated Mitochondrial Apoptosis***" for your consideration for publication in Iranian Journal of Pharmaceutical Research.
First of all, we wish to thank you and the reviewers for the hard work and great comments as well as the recognition of the promising and significance of our work. We have carefully re-organized the structure of the manuscript and revised our manuscript to improve its quality based on the comments from the reviewer, and the point-by-point responses are appended to this letter. We hope that our responses have thoroughly addressed the issues raised by the reviewers.

Many thanks to you in advance for your kind consideration of our manuscript.

Yours sincerely,

Qi Chen
Ningbo Yinzhou No.2 Hospital

Point-by-point Response to the Editors' Comments

We highly appreciate editor for valuable comments and suggestions. We have carefully revised our manuscript as editorial and ethical corrections. Below is our point-by-point response to the comments.

> Associate Editor's Comments:

Associate Editor 1:

1.Upon the reviewers' comments, we ask you to provide the whole blot images of western blot experiments (the bands in triplicate and imaged by the same exposure time).

Answer: Thank for the comments. We have provide triplicate whole blot images as Fig. S2.

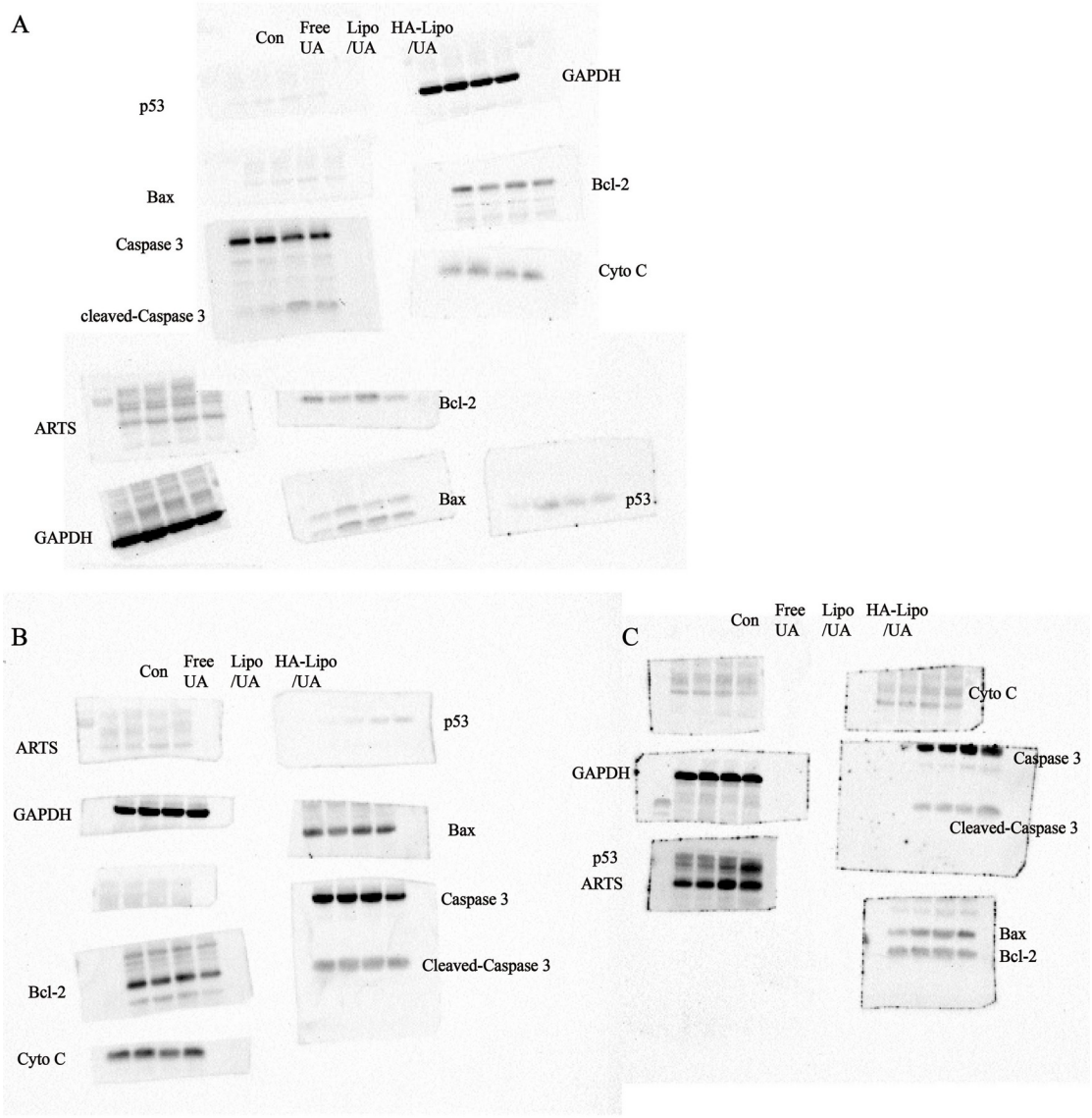


Fig. S2 Triplicate whole blot images. Channel labels are as following: con, free UA, Lipo/UA and HA-Lipo/UA (left to right).

2.Furthermore, please consider if you can add the empty formulation as a control group to your experiments ;

Answer: Thank for the comments. We proved by experiment that the blank carrier was non-toxic with used concentrations by MTT assays (Fig. S1). Therefore, we think empty formulation was similar to blank group.

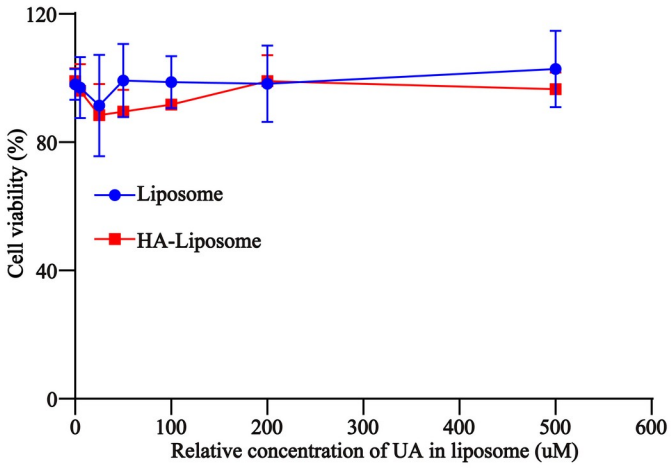
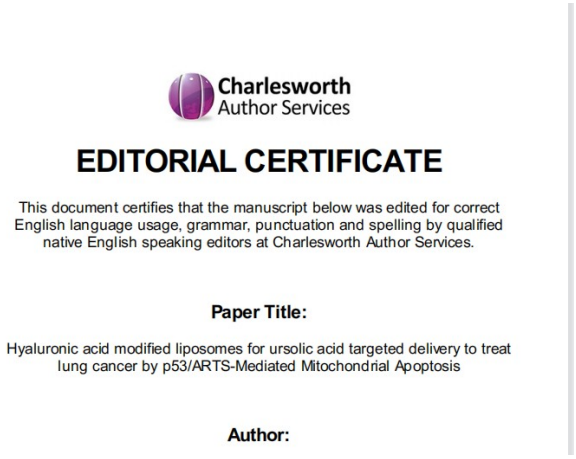


Fig. S1 Cell cytotoxicity test of blank carriers.

3.Please improve the manuscript's statistical analysis and language based on the comments.

Answer: Thank for the comments. Our manuscript edited the English text by Charlesworth Author Services. Statistical analysis was conducted using the GraphPad Prism software and performed by ANOVA. The Student's t-test was used to compare two group, and and $P < 0.05$ was considered statistically significant.



Charlesworth Author Services

EDITORIAL CERTIFICATE

This document certifies that the manuscript below was edited for correct English language usage, grammar, punctuation and spelling by qualified native English speaking editors at Charlesworth Author Services.

Paper Title:

Hyaluronic acid modified liposomes for ursolic acid targeted delivery to treat lung cancer by p53/ARTS-Mediated Mitochondrial Apoptosis

Author:

Point-by-point Response to the Reviewers' Comments

We highly appreciate both the reviewers for their valuable comments and suggestions. We have carefully revised our manuscript as suggested, and added new data. Below is our point-by-point response to the comments.

Reviewer 1:

Dear editor,

Targeted liposomes are desired nanoplatforms for the delivery of anti-cancer agents and some active molecules can be introduced as promising alternatives for the conventional treatments. Accordingly, a targeted nanoliposomal formulation was developed in this original research; however, the present study lacks novelty. In a recent publication titled "Dual stimuli-responsive

ursolic acid-embedded nanophytoliposome for targeted antitumor therapy" a similar formulation was designed and developed with the same active agent and targeting moiety. Furthermore, some points are listed below that require your consideration:

1. The authors applied western blotting to suggest an apoptotic pathway and evaluated some markers' overexpression. However, to quantify western blot bands, the results should be provided in triplicates with the same exposure time (Fig. 6). Also, the whole blots must be provided as supplementary materials.

Answer: Thank for the comments. We have provide triplicate whole blot images as Fig. S2.

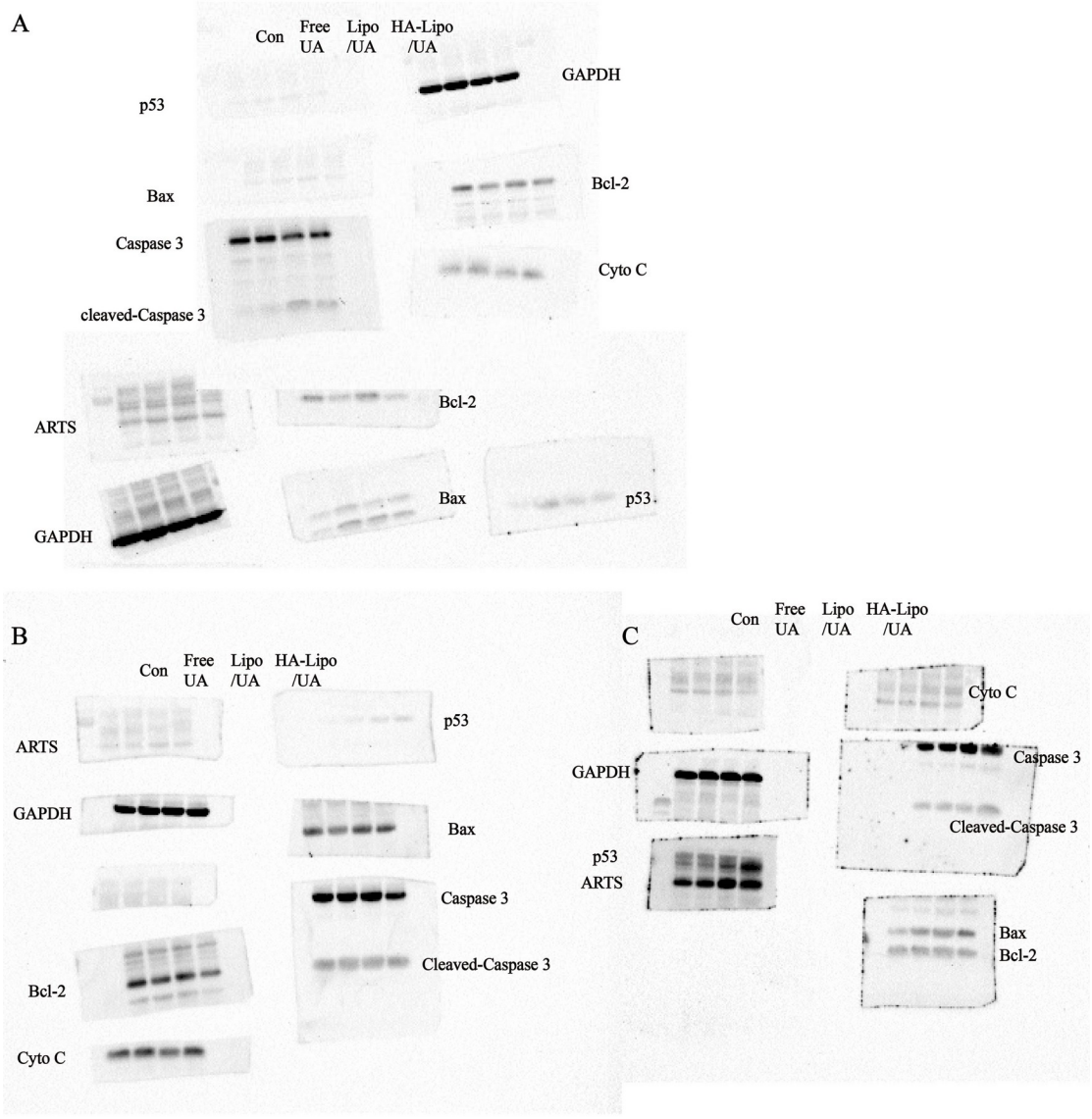


Fig. S2 Triplicate whole blot images. Channel labels are as following: con, free UA, Lipo/UA and HA-Lipo/UA (left to right).

2. The authors have not clarified the details of some methods (e.g., western blotting) and therefore, their experiments are not reproducible and hard to evaluate. In scheme 1, membrane extrusion was noted as a development step, while there is no relevant description in the method section.

Answer: Thank for the comments. We have verified the method in details.
(1) Western blotting part:and transferred onto a polyvinylidene fluoride membrane. The membrane were blocked with 5% skim milk and then incubated with diluted primary antibodies (1:1000) in 5% skim milk with TBST at 4℃ overnight. The membrane were washed with TBST and incubated with a secondary antibody. Then the proteins were imaged by a ChemiDoc™ XRS
(2) HA-Lipo-UA preparation part: Finally, liposomes underwent ultrasound using an ultrasonic processor (HENGMIN-R1200, Hengmin, China) for 5 min at 100 W at room temperature and the liposomes were extruded (Avanti miniextruder, Avanti, USA) through a polycarbonate membrane with 200 nm pores.

*3.Statistical analysis was not performed appropriately. T-test was mentioned as the statistical tool which is not acceptable for the study's comparisons. Besides, statistical significance symbols of the figures' bar charts seem to be incorrect (Fig. 6). Also, *** was indicated as P < 0.0001 which is not correct and the sample replicates are unknown.*

Answer: Sorry for the confusing description and we have verified it. *** was indicated as P < 0.001. Statistical analysis was conducted using the GraphPad Prism software and performed by ANOVA. The Student's t-test was used to compare two group, and and P < 0.05 was considered statistically significant. In Fig. 6, we re-performed the statistical analysis and corrected statistical significance symbols.

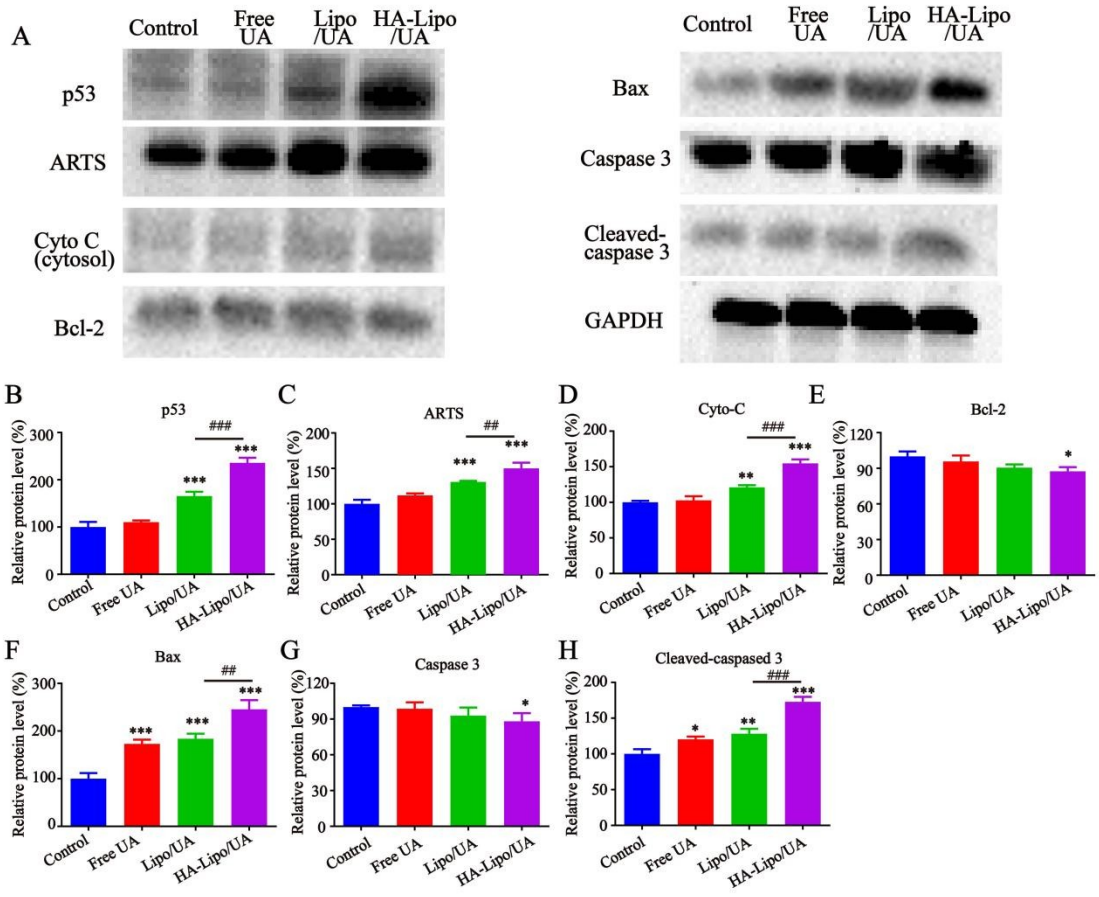


Fig. 6 Effects of different formulations on p53/ARTS induce mitochondrial apoptotic signalling.

p53, ARTS, Cyto C, Bcl-2, Bax, Caspase 3 and Gapdh protein expression in A549 cells detected by Western blot. Quantitative analysis of Western blotting bands including p53 (B), ARTS (C), Cyto C (D), Bcl-2 (E), Bax (F), Caspase 3(G) and Cleaved-caspased 3 (H). ***P < 0.001, ** P < 0.01, and * P < 0.05 compared to control group. $^{###}$P < 0.001 and $^{##}$ P < 0.01 compared to Lipo/UA group.

4. The experiments lack the empty formulation's control group.

Answer: Thank for the comments. We proved by experiment that the blank carrier was non-toxic with used concentrations by MTT assays (Figure S1). Therefore, we think blank carrier was similar to blank group and blank carrier group is not required. Some studies have also shown no blank carrier group was set in experimental.

Reference: 1. Tao J, Diao L, Chen F, Shen A, Wang S, Jin H, et al. pH-Sensitive Nanoparticles Codelivering Docetaxel and Dihydroartemisinin Effectively Treat Breast Cancer by Enhancing Reactive Oxidative Species-Mediated Mitochondrial Apoptosis. Molecular Pharmaceutics. 2021;18(1):74-86.

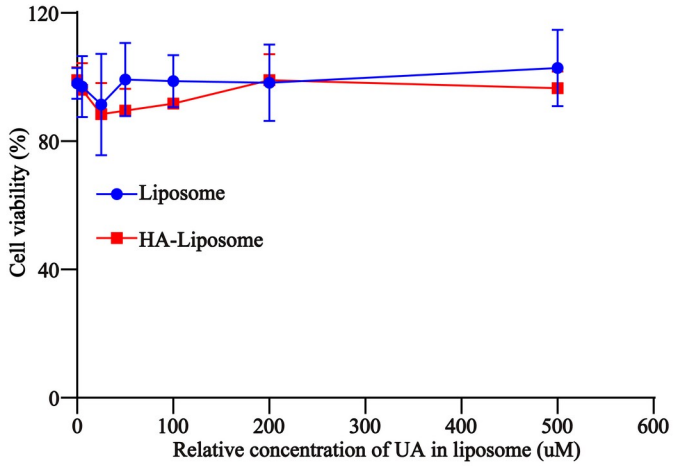
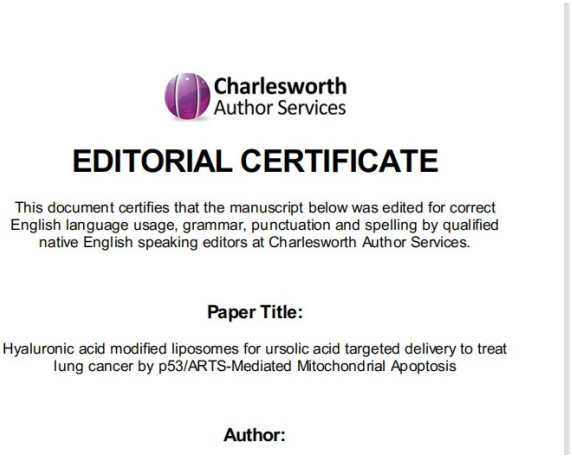


Fig. S1 Cell cytotoxicity test of blank carriers.

5.The manuscript was written in poor English and contains several writing errors of all types (grammatical, dictation, punctuation, etc.).

Answer: Thank for the comments. Our manuscript edited the English text by Charlesworth Author Services.



Reviewer 2:

The paper "Hyaluronic Acid-modified Liposomes for Ursolic Acid-targeted Delivery Treat Lung Cancer Based On p53/ARTS-mediated Mitochondrial Apoptosis" aimed to design liposomal for targeted delivery of ursolic acid (UA) in non-small cell lung cancer (NSCL). The purpose of the paper is made clear through explaining the context and background in the Introduction. After stating the issues revolved around current therapeutics for NSCL, UA as a part of Traditional Chinese Medicine and hyaluronic acid (HA)-modified liposome for resolving the lack of specific targets of UA are introduced. HA-modified liposomes are prepared though thin film hydration method. The paper then describes the characterizations of liposomes which confirmed that the nanoparticles had a size of ~ 200 nm, suitable stability, sustained release and greater cellular uptake and cytotoxic activity. In addition, the mechanism in which the formulation was able to induce apoptosis was investigated. There are several issues that should be addressed:

1.Regarding to the originality of the research, line 69 which states 'the underlying mechanisms in which UA acts against NSCLC" is not acceptable; as there are recent studies on the antitumor mechanisms of UA in NSCLC which should be mentioned (Such as "Antitumor Effects of Ursolic Acid through Mediating the Inhibition of STAT3/PD-L1 Signaling in Non-Small Cell Lung Cancer Cells" by Kang et al. published in Biomedicines (volume 9, issue 3, March 13, 2021)).

Answer: Thank for the comments and that is a good suggestion. we have verified it and the details are as follow.

Some studies have shown that UA can induce the apoptosis of tumor cells, and the mechanism by which UA acts against NSCLC through EGFR/JAK2/STAT3 signaling. We further explored the anti-lung cancer mechanism of UA.(18)

18. Kang DY, Sp N, Lee JM, Jang KJ. Antitumor Effects of Ursolic Acid through Mediating the Inhibition of STAT3/PD-L1 Signaling in Non-Small Cell Lung Cancer Cells. Biomedicines. 2021;9(3):297. doi:10.3390/biomedicines9030297. [PubMed:33805840].

2. For the interpretation of results p value should be reported (e.g. comparing the drug release of free-UA and Lipo/UA)

Answer: Thank for the comments and that's a good question. According to the references and our own understanding, we decided not to conduct p value of the drug release results.
The cumulative drug release is a dynamic process which suggested the release of drugs from different carriers. The comparison of UA release from free-UA or Lipo/UA were suggested the liposome could sustained release UA and It is not appropriate to select endpoints for statistical analysis. Qin (1) and Tao (2) the results of the drug release of nanoparticles was not statistically analyzed.
Reference：
1.Qin H, Teng R, Liu Y, Li J, Yu M. Drug Release from Gelsolin-Targeted Phase-Transition Nanoparticles Triggered by Low-Intensity Focused Ultrasound. Int J Nanomedicine. 2022;17:61-71. doi:10.2147/ijn.s341421. [PubMed:35023919].
2.Tao J, Diao L, Chen F, Shen A, Wang S, Jin H, et al. pH-Sensitive Nanoparticles Codelivering Docetaxel and Dihydroartemisinin Effectively Treat Breast Cancer by Enhancing Reactive Oxidative Species-Mediated Mitochondrial Apoptosis. Molecular Pharmaceutics. 2021;18(1):74-86.

3.Sufficient details are not provided for several methods described in the paper. For example, in the thin-film hydration method (HA-Lipo-UA preparation), the dispersion medium used for the hydration of lipid film, the lipid to drug ratio and the manufacturer's name and address for rotary evaporator are not stated clearly. Furthermore, in line 106 "The liposomes solution was diluted at a ratio of 1:10, 1:50 and 1:250" the dilutant should be mentioned.
Answer: Thank for the comments and we have verified it.

4.- Abbreviations should be introduced in parentheses upon their first mention. There are a few abbreviations that are not defined in the text. Such as "DSPE" and "GADPH".
Answer: Thanks for the comments and and we have verified it.

5.- The whole manuscript should be revised regarding to the punctuation errors of in-text citations and title capitalization. Moreover, Figure in-text citation is not provided for Figure 1E.
Answer: Sorry for the confusing description and we have verified it.

2 Revision 1

1 comment · 5 Feb 2023

Associate Editor

MAJOR REVISION 5 Feb 2023

The reviewer's comments are not addressed properly.

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor,

Thank you for your kind letter regarding the decision on our manuscript on February 5th, 2023. With this cover letter, we submit a revised version of our manuscript entitled "***Hyaluronic Acid-modified Liposomes For Ursolic Acid-targeted Delivery Treat Lung Cancer Based On p53/ARTS-mediated Mitochondrial Apoptosis***" for your consideration for publication in Iranian Journal of Pharmaceutical Research.

First of all, we wish to thank you and the reviewers for the hard work and great comments as well as the recognition of the promising and significance of our work. We have carefully re-organized the structure of the manuscript and revised our manuscript to improve its quality based on the comments from the reviewer, and the point-by-point responses are appended to this letter. We hope that our responses have thoroughly addressed the issues raised by the reviewers.

Many thanks to you in advance for your kind consideration of our manuscript.

Yours sincerely,

Point-by-point Response to the Editors' Comments

We highly appreciate editor for valuable comments and suggestions. We have carefully revised our manuscript as editorial and ethical corrections.

Associate Editor 1:

The reviewer's comments are not addressed properly

Answer: Thank for the comments and we have carefully revised our manuscript as suggested.

Point-by-point Response to the Reviewers' Comments

We highly appreciate the reviewer for their valuable comments and suggestions. We have carefully revised our manuscript as suggested. Below is our point-by-point response to the comments.

Reviewer 1:

1. According to the comments of Reviewer 1, there is a recent paper titled 'Dual stimuli-responsive ursolic acid-embedded nanophytoliposomes for targeted antitumor therapy" with the same active agent and targeting moiety. This comment was not addressed. The novelty and significance of the work should be further clarified.

Answer: Thank for the comments and that is a good suggestion. We read carefully the Poudel paper of Dual stimuli-responsive ursolic acid-embedded nanophytoliposomes for targeted antitumor therapy. Firstly, Poudel was prepared stimuli-responsive ursolic acid-embedded nanophytoliposomes which prepared ursolic acid-loaded liposomes by ethanol injection method and then loaded poly-L-lysine and hyaluronic acid by electrostatic adhesion. In our study, the HA-lipo-UA of UA, lecithin, cholesterol, DSPE-PEG2000, and DSPE-PEG2000-HA were prepared

using a thin-film hydration method. Secondly, Poudel studied focus on the therapeutic effect of ursolic acid-embedded nanophytoliposomes on squamous cell carcinoma of head and neck, and did not involve mechanism exploration. Our research suggested HA-Lipo/UA exhibited significant inhibition of proliferation of A549 cells and through p53/ARTS-mediated mitochondrial apoptosis. Therefore, the two preparations are different in their preparation principles, materials and methods and HA-lipo-UA more conducive to later commercialization because of its simple preparation. Also our study clarified the efficacy and mechanism of HA-lipo-UA in lung cancer. We quote from this article and clarify our novelty and significance of this work. Details are as follows:

Poudel team reported dual stimuli-responsive UA-embedded nanophytoliposome, which through prepared ursolic acid-loaded liposomes by ethanol injection method and then coated poly-L-lysine and HA by electrostatic adhesion, target as well as are retained in the tumor and are enzymatically cleaved to release UA in the acidic pH of the tumor microenvironment and showed good activity of anti-head and neck squamous cell carcinoma. However, they did not explore its anti-tumor mechanism (17). Kang team research have shown that UA induces cell cycle arrest, apoptosis and anti-tumor angiogenesis. And UA exhibits anticancer activities by inhibiting MMP2 and PD-L1 expression through EGFR/JAK2/STAT3 signaling (18). Therefore, the anti-lung cancer mechanism of ursolic acid nanopreparations by inducing apoptosis remains to be further explored.

In this study, to overcome the lipophilic and non-targeted of using UA in LC therapy, a HA-mediated nanodrug delivery system was designed (Scheme 1). The lipophilic drug, UA, was embedded in a HA modified liposome, which is simple preparation process and easy to prepare. The enhanced bioavailability, sustained release and increased tumor target of UA could be obtained by HA modified liposome (HA-Lipo-UA). The targeting potential of the HA-Lipo-UA was investigated using in vitro studies. We investigated the antitumor activity of HA-lipo-UA against A549 cells and the molecular mechanism related to mitochondrial apoptosis. Our study may aid development of an efficacious strategy for tumor-targeting therapy.

17. Poudel K, Gautam M, Maharjan S, Jeong JH, Choi HG, Khan GM, et al. Dual stimuli-responsive ursolic acid-embedded nanophytoliposome for targeted antitumor therapy. Int J Pharm. 2020;582:119330. doi:10.1016/j.ijpharm.2020.119330. [PubMed:32298743].

18. Kang DY, Sp N, Lee JM, Jang KJ. Antitumor Effects of Ursolic Acid through Mediating the Inhibition of STAT3/PD-L1 Signaling in Non-Small Cell Lung Cancer Cells. Biomedicines. 2021;9(3):297. doi:10.3390/biomedicines9030297. [PubMed:33805840].

2.The in-text citations do not conform the Guide for Authors of this journal (the terminal punctuation should be placed after the parentheses).

Answer: Thank for the comments and we have verified it.

3.There are several unrevised writing errors (e.g. PBS is mentioned in line 111 and defined in line 119).

Answer: Sorry for this mistake and we have verified it, such as line 16, 121, 129, 155, 185, 278.

3 Revision 2

0 comments

No public comments in this round.