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ACTION	STATUS	ID	TITLE	SUBMITTED	DECISIONED
	ADM: Paixão Maioli, Maria Lúcia ADM: Sant'Anna, Daniel • Immediate Reject (11-Oct-2021) *Archiving completed on 10-Jan-2022* view decision letter Contact Journal	AABC-2021-1137	Solubilization effectivity of indomethacin in binary and ternary systems following the addition of hidroxypropyl-β-cyclodextrin and/or N-methylglucamine *Files Archived*	15-Aug-2021	11-Oct-2021
	ADM: Paixão Maioli, Maria Lúcia ADM: Sant'Anna, Daniel • Accept (14-Feb-2021) *Archiving completed on 15-Aug-2021*	AABC-2020-1850.R1	The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes	30-Jan-2021	14-Feb-2021

ACTION	STATUS	ID	TITLE	SUBMITTED	DECISIONED
	view decision letter Contact Journal		*Files Archived*		
a revision has been submitted (AABC-2020-1850.R1)	ADM: Paixão Maioli, Maria Lúcia ADM: Sant'Anna, Daniel • Minor Revision (21-Jan-2021) • a revision has been submitted *Archiving completed on 15-Aug-2021* view decision letter Contact Journal	AABC-2020-1850	The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes *Files Archived*	30-Nov-2020	21-Jan-2021

andang miatmoko <andang-m@ff.unair.ac.id>

Annals of the Brazilian Academy of Sciences - Decision on Manuscript ID AABC-2020-1850

1 message

Igor Luis Kaefer <onbehalfof@manuscriptcentral.com> Thu, Jan 21, 2021 at 10:30 AM
Reply-To: kaefer@ufam.edu.br
To: andang-m@ff.unair.ac.id

21-Jan-2021

Dear Dr. Miatmoko:

Manuscript ID AABC-2020-1850 entitled "The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes" which you submitted to the Annals of the Brazilian Academy of Sciences, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into https://mc04.manuscriptcentral.com/aabc-scielo and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You may also click the below link to start the revision process (or continue the process if you have already started your revision) for your manuscript. If you use the below link you will not be required to login to ScholarOne Manuscripts.

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You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text.

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When submitting your revised manuscript, you will be able to respond to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewer(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to the Annals of the Brazilian Academy of Sciences, your revised manuscript should be submitted BEFORE 20-Feb-2021. If it is not possible for you to submit your revision by this date, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Annals of the Brazilian Academy of Sciences and I look forward to receiving your revision.

Sincerely,
Dr. Igor Luis Kaefer
Editor-in-Chief, Annals of the Brazilian Academy of Sciences
kaefer@ufam.edu.br

Associate Editor
Comments to the Author:

Dear Dr. Miatmoko, Andang

Thank you for submitting the above manuscript (AABC-2020-1850) to Annals of the Brazilian Academy of Sciences, We have now received the Reviewer reports, and I must inform you that the manuscript is acceptable for publication after minor revision.

Please find below the Referee comments on your paper

Thank you for your interest in submitting to AABC

Yours sincerely
Marcello Iacomini
Associate Editor

Comments of Reviewer 1

The short communication (AABC-2020-1850), entitled "The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes", probes the cellular uptake and cytotoxicity of low permeable Ursolic acid (UA) on HeLa and Huh7it cells using niosomes composed of span 60 and cholesterol. The results showed that the addition of chitosan increased particle sizes and ζ-potentials, with the improvement in cytotoxicity being observed in HeLa cells, but not in the Huh7it cell line. The article is concisely written and the results well documented. Yet, further explanation is needed, with respect to this "cell selectivity".

Entire Scoresheet:
Reviewer: 1

Recommendation: Minor Revision

Comments:
The short communication (AABC-2020-1850), entitled "The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes", refers to the development of UA niosome with chitosan layers. This system increased cytotoxicity in HeLa cells, but it was less sensitive for Huh7it cells. The results are well documented, but further explanation is needed, with respect to this "cell selectivity".

Additional Questions:
Does the manuscript contain new and significant information to justify publication?: Yes

Does the Abstract (Summary) clearly and accurately describe the content of the article?: Yes

Is the problem significant and concisely stated?: Yes

Are the methods described comprehensively?: Yes

Are the interpretations and conclusions justified by the results?: Yes

Is adequate reference made to other work in the field?: Yes

Is the language acceptable?:

Please rate the priority for publishing this article (1 is the highest priority, 10 is the lowest priority): 3

Length of article is: Adequate

Number of tables is: Adequate

Number of figures is: Adequate

Please state any conflict(s) of interest that you have in relation to the review of this paper (state “none” if this is not applicable).:

Rating:

Interest: 2. Good

Quality: 2. Good

Originality: 3. Average

Overall: 2. Good

andang miatmoko <andang-m@ff.unair.ac.id>

Annals of the Brazilian Academy of Sciences - Decision on Manuscript ID AABC-2020-1850.R1

1 message

Igor Luis Kaefer <onbehalfof@manuscriptcentral.com> Mon, Feb 15, 2021 at 8:03 AM
Reply-To: kaefer@ufam.edu.br
To: andang-m@ff.unair.ac.id

14-Feb-2021

Dear Dr. Miatmoko:

It is a pleasure to accept your manuscript entitled "The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes" in its current form for publication in the Annals of the Brazilian Academy of Sciences. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Annals of the Brazilian Academy of Sciences, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Igor Luis Kaefer
Editor-in-Chief, Annals of the Brazilian Academy of Sciences
kaefer@ufam.edu.br

Associate Editor
Comments to the Author:
Dear Miatmoko,

I am pleased to confirm that your Manuscript number AABC-2020-1850.R1 has been accepted for publication in Annals of the Brazilian Academy of Sciences, having read your response to the Reviewer, I consider that you have dealt satisfactorily with the points raised

Thank you for submitting your work to this journal.
With kind regards,

Associate Editor
AABC

Entire Scoresheet:

Dear Editor,

Many thanks for the email. We really appreciate all comments to improve our manuscript. Below are the answers addressed for the reviewer's comment.

Reviewer's comments:

The short communication (AABC-2020-1850), entitled "The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes", refers to the development of UA niosome with chitosan layers. This system increased cytotoxicity in HeLa cells, but it was less sensitive for Huh7it cells. The results are well documented, but further explanation is needed, with respect to this "cell selectivity".

Answer:

Thank you for the comment. In this study, HeLa and Huh7it cells had different sensitivity for cytotoxic study of UA and niosomes since each cell type has specific biological characteristics devoted for certain functions (Gersdorff et al., 2006). We have added the sentence into page 5 line number 3-5 (highlighted).

Ref:

VON GERSDORFF K, SANDERS NN, VANDENBROUCKE R, DE SMEDT SC, WAGNER E, OGRIS M. 2006. The Internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther 14: 745–753. https://doi.org/10.1016/j.ymthe.2006.07.006

The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes

Abstract

This study evaluated the cellular uptake and cytotoxicity of low permeable Ursolic acid (UA) on HeLa and Huh7it cells using niosomes composed of span 60 and cholesterol. The results showed that the addition of chitosan increased particle sizes and ζ-potentials. The UA niosomes with chitosan layers had higher cytotoxicity in HeLa cells than without chitosan, however, there was no improvement observed for Huh7it cells. Moreover, chitosan layers improved the cellular uptake, which clathrin-mediated endocytosis may determine the cellular transport of UA niosomes. In conclusion, the addition of chitosan improved cellular uptake and cytotoxicity of UA niosomes in the HeLa cells.

Introduction

Ursolic acid (UA) is a pentacyclic triterpene which promotes anti-cancer activity in humans (Ali et al., 2019). They revealed the role of UA in accelerating liver proliferation, restoring biochemical and histological functions in liver cells damaged by hepatocarcinoma, and protecting the integrity of hepatocytes against liver damage.

The use of UA in anti-cancer therapy has drawbacks related to its poor permeability and water solubility. Lawsone, an anti-cancer drug that demonstrates poor solubility and permeability, is reported to induce high cytotoxicity when in the form of niosomes (Barani et al., 2018). The increased cytotoxic effect was due to the internalization effect of the niosomes in MCF-7 breast cancer cells which was superior to that of the free form and the resulting sustained-release effect.

Research conducted by Song et al. (2014) shows that the mechanism transporting niosomes into cells is active and linked to the endocytosis pathway. Chitosan, a natural polysaccharide (Szymańska and Winnicka, 2015) and a cationic polymer, has been shown to increase cell uptake (Li et al., 2013; Zhang et al., 2013). The cationic polymer charge significantly affects cellular absorption *in vitro* (Li and Ju, 2017) due to the electrostatic interaction between cells and positively charged cationic polymers.

Against this background, research was carried out on cell uptake and in vitro cytotoxicity of UA niosomes with chitosan coating on HeLa and Huh7it cells (Guo et al., 2019;·Purnamasari et al., 2019).

Materials and Methods

In this study, UA niosomes (Nio-UA) was composed of Span 60, Cholesterol, UA at a molar ratio of 6:4:1, respectively. A solution of UA, Span 60, and Cholesterol was mixed in a round bottom flask, then the organic solvents was evaporated until a thin lipid film was formed.

This film was hydrated using Phosphate buffered saline solution pH 7.4. Furthermore, a solution of 0.005% w/v chitosan was added for producing Nio-UA-CS.

The particle size and ζ-potential were measured by means of dynamic and electrophoresis light scattering method, respectively, by the use of a Delsa™ Nano C Particle Analyzer at 25°C.

A cytotoxicity test was performed using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on HeLa and Huh7it cells after incubating Nio-UA and Nio-UA-CS for 48 hours. The absorbance of MTT was measured at λ_{ex}: 475 nm and λ_{em}: 500-550 nm using a GloMax-Multi Microplate Multimode Reader (Promega).

Furthermore, the cell uptake test was carried out on HeLa cells after incubating the UA niosomes labeled Coumarin-6 (Cou-6= 10 μg/mL). In order to determine the mechanism of cell uptake, pre-incubation was carried out with endocytosis specific inhibitors, namely; 200 μM of genistein and 450 mM of sucrose. Pre-incubation was performed on sucrose for one hour and on genistein for 30 minutes at 37ºC. After pre-incubation, the media was replaced with 10 μg/mL of coumarin 6-labeled UA niosomes in the medium and incubated for two hours at 37°C. The the fluorescence intensity of Coumarin-6 in the cells was measured at λ_{ex}: 475 nm and λ_{em}: 500-550 nm using the Glomax Microplate Reader. The cell uptake was also evaluated by using a fluorescence microscope.

For statistical analysis, significance was indicated by $P<0.05$ by using one-way analysis of variance followed by a least significant difference test.

Results

The data shown in Table 1 indicates that the particle size of Nio-UA-CS was larger than that of Nio-UA. Similarly, for niosomes labeled with coumarin, the size of Nio-UA-CS-Cou6 was greater than that of Nio-UA-Cou6. The cytotoxicity results show that the IC_{50} Nio-UA was higher than that of IC_{50} Nio-UA-CS in HeLa cells (Fig 1A). Thus, chitosan coating can

increase the cytotoxicity of UA niosomes with regard to HeLa cells; however, the Huh7it cells were less sensitive to UA than HeLa cells as shown in Figure 1B.

Observations were further conducted using a fluorosense microscope after HeLa cells had been treated with UA niosomes for two hours. It can be seen in Figures 2 that the Coumarin-6 intensity of the Nio-UA-CS was higher than that of Nio-UA. Moreover, the appearance of cells in the treatment of Nio-UA indicates that Nio-UA slightly entered the cell and still mainly remained in the membrane, while Nio-UA-CS appear evenly across the cell membrane and were accumulated highly inside the cells. The Figure 2 also show that after genistein pretreatment as transport inhibitor of Nio-UA, the cells had less intense fluorescence than that of sucrose pretreatment. On the contrary, the cells treated Nio-UA-CS had a higher intensity than that without the addition of the inhibitor, and with the addition of sucrose, these cells had relatively lower intensities than that of genistein addition.

To determine the cellular uptake mechanism of the niosome, a test was carried out involving the addition of specific endocytosis inhibitors i.e. sucrose and genistein. As in Figure 3, with the addition of genistein or sucrose as an endocytosis inhibitor of the caveolae or clathrin pathway, respectively, the Nio-UA demonstrated relatively similar levels than the Nio-UA without inhibitor. The contrast results were produced by the Nio-UA-CS, which without inhibitor, it had significant lower levels than Nio-UA-CS with the genistein.

Discussion

Following the research conducted into the increase in particle size and changes in the ζ-potential value, it can be argued that chitosan layers on niosomes induce this change (Aquila, unpublished data). The chitosan layer may be formed by electrostatic interactions increasing in particle size (Guo et al., 2003). In the niosome, ionic attraction occurs between the ammonium group of chitosan and the phosphate group of phospholipids or other negatively charged groups in lipids (Frank et al., 2020). This interaction also causes changes in the ζ-

potential of the niosome as shown in Table 1 where the ζ-potential value becomes positive after niosome coating.

In this study, HeLa and Huh7it cells had different sensitivity for cytotoxic study of UA and niosomes since each cell type has specific biological characteristics devoted for certain functions (Gersdorff et al., 2006). The decrease in the IC_{50} value of UA niosomes with chitosan coating compared to UA niosomes is in accordance with the research findings of Brkić Ahmed et al (2017) regarding the higher absorption of poly L-lysine silver nanoparticles than silver nanoparticles due to the interaction between positively charged poly L-lysine on the surface of the nanoparticles and the negatively charged HepG2 hepatoblastoma cell surface. In chitosan, the positive surface charge can increase the bio-nano interaction with negative surface charge of the cytoplasmic membrane resulting in an increase in cell uptake and cytotoxicity (Frank et al., 2020).

The photomicrographs of cellular uptake confirms that the niosome as a cell retrieval carrier accumulates on the surface during the cell uptake process (Kaksonen and Roux, 2018). Since the addition of genistein produces higher yields, it clearly affects cell uptake from Nio-UA-CS (Quagliariello et al., 2019). Hence, the entry of Nio-UA-CS was improved with the presence of genistein as the inhibitor of the caveolae-mediated endocystosis, but no significant difference was observed after addition of sucrose. From these results, it can be concluded that Nio-UA-CS undergoes cell uptake through the clathrin-mediated endocytosis pathway, while the mechanism of Nio-UA uptake may involve both mechanisms. This finding is in accordance with that of Quagliariello's research from 2019 that cell uptake from Butyric acid in liposomes occurs through clathrin-mediated endocytosis whose mechanism is initiated when the endocytic protein layer of the cytosol clusters on the plasma membrane. After the coating is formed, the uptake molecules are concentrated in the layer area and a bond occurs which results in the flat plasma membrane becoming a 'clathrin-layered hole' containing the uptake molecules. Furthermore, the process of constriction and cutting of the

neck membrane occurs with the result that the vesicles enter the cell (Kaksonen and Roux, 2018).

Conclusion

It can be seen from this study that the UA niosome with chitosan layers increased cytotoxicity in HeLa cells but it was less sensitive for Huh7it cells. The difference in cell uptake between Nio-UA and Nio-UA-CS should also be investigated at greater length in order to identify the effect of chitosan addition on cellular uptake pathways.

Acknowledgement

This study was funded by a Research on Excellence in Faculty (Penelitian Unggulan Fakultas, PUF) Grant Number 281/4N3.14/PT/2020 provided by Universitas Airlangga.

Contribution of authors to the work

Name of Authors	Contributions
Andang Miatmoko	Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing-original draft, writing-review & editing
Berlian Sarasitha Hariawan	Data curation, formal analysis, investigation,methodology, project administration, resources, writing original draft
Devy Maulidya Cahyani	Data curation, formal analysis, investigation, project administration, resources
Retno Sari	Conceptualization, data curation, formal analysis, supervision, validation, writing-review & editing

Aristika Dinaryanti	Investigation, methodology, resources, validation, visualization
Eryk Hendrianto	Investigation, methodology, validation, visualization

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expression depends on both cell line and polyethylenimine polyplex type. Mol Ther 14: 745–753. https://doi.org/10.1016/j.ymthe.2006.07.006

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Figure and Table Legends

Figure 1. Viability of HeLa (A) and Huh7it (B) cells in the presence of UA, Nio-UA and Nio-UA-CS treatment after incubation for 48 hours at various concentrations.

Figure 2. Photomicrographs of cellular uptake of HeLa cells after incubated with Nio-UA and Nio-UA-CS containing Coumarin-6 for two hours without and with addition of genistein and sucrose pre-treatment. Scale bar= 100 μm.

Figure 3. Coumarin-6 levels in HeLa cells after incubation with Nio-UA and Nio-UA-CS for two hours without and with addition of genistein and sucrose pre-treatment (*P<0.05).

Table 1. Physical characteristics of Nio-AU, Nio-AU-Cs, Nio-AU-Cou6 and Nio-AU-Cs-Cou6