BOVINE MILK EXOSOMES FOR THE TREATMENT OF MELANOMA

Thesis submitted in partial fulfillment for the Award of Degree

Doctor of Philosophy

By

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7 Summary and Conclusion

7.1 Summary

This thesis explores the use of bovine milk-derived exosomes as an innovative drug delivery system aimed at enhancing the therapeutic efficacy of DHA and HES in the treatment of melanoma. Melanoma, a highly aggressive form of skin cancer, poses significant health risks, particularly among Caucasians, with a lifetime incidence risk of 2.6%. The incidence of melanoma is rising, with approximately 325,000 new cases and 57,000 deaths reported in 2020. Current treatment regimens primarily rely on chemotherapeutic agents like dacarbazine (DTIC), which are associated with severe side effects, including liver toxicity and myelosuppression. Additionally, the parenteral administration route for DTIC can lead to discomfort and requires direct medical supervision.

The study highlights the limitations of existing chemotherapeutics, emphasizing the need for cost-effective and safer drug delivery systems. A significant number of new chemical entities fail during drug development due to poor water solubility and inadequate safety profiles; reports indicate that 40-45% of these compounds are poorly soluble. This scenario underscores the necessity for nanotechnology-based delivery systems, such as liposomes and nanoparticles, to enhance the bioavailability of poorly soluble drugs.

DHA, a semi-synthetic derivative of artemisinin known for its anticancer properties across various malignancies, including melanoma, faces challenges in therapeutic applications due to low oral bioavailability and a short half-life. This often necessitates multiple dosing, which can lead to dose-dependent side effects. To address these issues, various synthetic delivery systems have been developed, though they possess inherent drawbacks.

In contrast, exosomes, which are naturally derived lipid-based nanocarriers, offer a promising alternative. Isolated from bovine milk, these exosomes demonstrate favorable physicochemical

properties and easy accessibility. Previous research indicates their efficacy in delivering various anticancer agents, prompting their use in this study for the delivery of DHA and HES.

Hesperidin, a flavonoid found in citrus fruits, exhibits diverse pharmacological activities, including anticancer effects. However, like DHA, its therapeutic efficacy is limited by poor solubility and bioavailability. This study investigates the potential of exosomes to enhance the delivery and effectiveness of both DHA and HES.

In the research, exosomes were isolated and loaded with DHA using a sonication method, resulting in particles with a size range of 90 to 103 nm, a polydispersity index (PDI) between 0.119 and 0.123, and a zeta potential of -23 to -28 mV. *In vitro* assays confirmed the enhanced anticancer activity of DHA-loaded exosomes, demonstrating improved cytotoxicity and an increased apoptotic response in melanoma cell lines. Additionally, *in vivo* experiments indicated increased oral bioavailability, reduced toxicity, and inhibited metastasis.

For HES, exosomes were similarly loaded, with characterization revealing spherical particles of approximately 106 nm. Studies indicated that HES-loaded exosomes significantly improved therapeutic efficacy in both *in vitro* and *in vivo* models, suggesting a promising strategy for enhancing the effectiveness of hesperidin in melanoma treatment.

7.2 Conclusion

The findings of this thesis support the hypothesis that bovine milk-derived exosomes can serve as effective nanocarriers for delivering DHA and hesperidin HES, significantly enhancing their therapeutic potential against melanoma. This approach not only improves the solubility and bioavailability of these compounds but also reduces the toxicity and adverse effects commonly associated with conventional chemotherapy. The study establishes a foundation for further exploration of exosome-mediated drug delivery systems, emphasizing their potential to overcome the limitations of current melanoma therapies.

Future research is essential to validate the mechanisms of action of DHA and HES when delivered via exosomes, utilizing advanced techniques such as RT-PCR and Western blotting. Additionally, investigating the efficacy of these formulations in diverse animal models will be crucial for establishing their broader applicability and effectiveness in cancer treatment. Overall, the development of exosome-loaded therapies represents a promising avenue for advancing treatment strategies not only in melanoma but potentially in other malignancies as well. This innovative approach could pave the way for safer, more effective cancer therapies that improve patient outcomes.

INDIAN INSTITUTE OF TECHNOLOGY
(Banaras Hindu University)

Ph.D. EXAMINATION

Recommendation on Ph.D. Thesis

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Ref. No.: IIT(BHU)/TEC/Ph.D.Evl./PH/1364

1. Name of Candidate : Mr. Dulla Naveen Kumar
2. Registered for Ph.D. Degree in : Pharmaceutical Engg. and Tech.
3. Department/School : Pharmaceutical Engg. and Tech.
4. Title of Thesis : Bovine Milk Exosomes for The Treatment of Melanoma
5. Details of Examiner : Prof. Subash Chandra Gupta

Please give your **specific recommendation** by ticking ( ) any one of the following, with signature underneath and enclose your **detailed report** on separate sheet(s) with your signature, name and address.

Please specify the Category under which the Thesis should be placed.

EXAMINER'S REPORT

1. The thesis **be accepted** for the award of the Ph.D. degree : Yes

OR

2. ~~The thesis **is acceptable** for the award of the Ph.D. degree subject to the clarification of **certain points at the time of Viva-Voce.**~~
(Please enclose the points) :.....[ ]

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~~The thesis **is not acceptable in the present form** but **may be accepted** subject to **modification/clarification/ revision.**~~ (Please enclose you suggestions for modification etc. desired). After modification the **thesis should be referred back to me for final assessment.** :.....[ ]

OR

4. ~~The thesis **be rejected.**~~
(Please enclose your comments.) :.....[ ]

Place: Guwahati

Date: 29.12.24

(Signature of Examiner)

Name & Address of the Examiner

Dr. Subash Chandra Gupta
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& Associate Dean (*Research*)
All India Institute of Medical Sciences, Guwahati
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Enclosure: Detailed Report on separate sheets including list of points for clarification

Detailed Report on the Thesis

Name of Candidate: Mr. Dulla Naveen Kumar
Department/School: Pharmaceutical Engg. and Tech.
Title of Thesis: Bovine Milk Exosomes for The Treatment of Melanoma

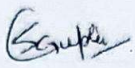
The primary goal of this Ph.D. thesis was to develop bovine milk-derived exosomal formulations loaded with the drugs dihydroartemisinin and hesperidin. These formulations were administered in the model systems of melanoma which is a severe form of skin cancer. Dihydroartemisinin has been used for the treatment of malaria while hesperidin is most commonly used for blood vessel conditions such as hemorrhoids, varicose veins, and poor circulation. Melanoma is mostly manageable especially after the development of checkpoint inhibitors such as ipilimumab (yervoy), nivolumab (opdivo) and pembrolizumab (keytruda). However, these drugs are expensive and can not be afforded by low middle income population. Dihydroartemisinin and hesperidin were repurposed for melanoma in this thesis. Drug repurposing is a novel concept for minimizing the process of drug development as well as reducing the associated cost. The aim, objectives and scope of the work presented in this thesis is well defined. The literature review is relevant to the work. The experiments are properly designed and the work is original. The data is properly presented and the results support the conclusion. The candidate has published 2 first author papers from his thesis and multiple other articles. The candidate has also been a part of 2 book chapters. Overall, the thesis is acceptable for the award of Ph.D. degree.

Questions to be asked during viva-voce

1. The exosomes from bovine milk were isolated using the ultracentrifugation. The purity of exosomes was assessed by western blotting and further characterized using multiple assays. However, the discussion lacks transparency regarding the efficiency and reproducibility of the isolation process, as well as critical details such as the yield of exosomes obtained, which are essential for evaluating the practicality and scalability of this methodology. What was the specific yield? Was the yield consistent across different experiments? Methodologies to control precision loading of dihydroartemisinin and hesperidin should have better discussed.
2. It is not clear at all where the candidate will begin if he wishes to continue the work from this thesis.
3. What was the basis for the targeting of melanoma by the exosome isolated from bovine milk?
4. What was the basis of using B16F10 cell lines and not cell lines of human origin? More than one cell line should have been used for key experiments to examine if the effects of test agents were cell line specific.
5. Does DHA affect food intake and body weight of mice?
6. Were positive and negative controls used for the data presented in Fig 5.12?
7. Were the effects of exosomes loaded dihydroartemisinin and hesperidin compared?
8. The clinical utility of exosome mediated drug delivery should have been better discussed.
9. The thesis should have been thoroughly reviewed for possible grammatical errors including typo (see NACL on page 50).
10. The lanes of the blots presented in Figure 5.27*A* should have been labelled.

11. The expansion of non-standard abbreviations should have been avoided. Tables such as the one presented on page 21 should have been numbered; this table should have included examples of drugs that are successfully repurposed for human use such as thalidomide and sildenafil.

Name & Address of the Examiner: Prof. Subash Chandra Gupta, Department of Biochemistry, All India Institute of Medical Sciences, Guwahati, Silbharal, Changsari, District – Kamrup, Assam – 781101, India

Signature of Examiner: 

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Ph.D. EXAMINATION

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4. Title of Thesis : Bovine Milk Exosomes for The Treatment of Melanoma
5. Details of Examiner : Prof. Armin Gamper

Please give your **specific recommendation** by ticking ( ) any one of the following, with signature underneath and enclose your **detailed report** on separate sheet(s) with your signature, name and address.

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EXAMINER'S REPORT

Contd....2

1. The thesis **be accepted** for the award of the Ph.D. degree :.....[x]

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2. The thesis **is acceptable** for the award of the Ph.D. degree subject to the clarification of **certain points at the time of Viva-Voce.** (Please enclose the points) :.....[ ]

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3. The thesis **is not acceptable in the present form** but **may be accepted** subject to **modification/clarification /revisions.** (Please enclose your suggestions for modification etc. desired.) After modification the **thesis need not be referred back to me.** :.....[ ]

OR

The thesis **is not acceptable in the present form** but **may be accepted** subject to **modification/clarification/ revision.** (Please enclose you suggestions for modification etc. desired). After modification the **thesis should be referred back to me for final assessment.** :.....[ ]

OR

4. The thesis **be rejected.** (Please enclose your comments.) :.....[ ]

Place : Edmonton, AB, Canada

Date : January 1st, 2025

(Signature of Examiner)

Name & Address of the Examiner

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Encl. : (a) Detailed Report (see below)

Evaluation of Thesis submitted by Mr. Dulla Naveen Kumar

This is an outstanding thesis summarizing extensive work on the use of milk exosomes as delivery vehicle for cancer therapeutics. The reported findings not only contribute to the general field of pharmaceutical science by providing important insights into enhancing pharmaceutical properties of difficult to administer drugs by exosomal delivery. The findings also have great translational potential as the two investigated drugs, dihydroartemisinin and hesperidin, are repurposed drugs that previously had shown anti-cancer activity but poor physicochemical properties as cancer therapeutics without proper delivery strategies. Furthermore, the cancer model investigated, melanoma, is a prevalent disease with poor prognosis due to its high metastatic potential. More effective and less costly treatments are dearly needed.

In detail my analysis of the thesis is based on the following factors:

Originality and Contribution to the Field

As well described in the excellent introduction chapter, investigation on the use of milk exosomes as nanocarriers, despite their high biocompatibility, is relatively new and poorly explored. The use of bovine milk exosomes to repurpose the well characterized antimalaria drug dihydroartemisinin and the anti-hemoroidal drug hesperidin as cancer therapeutics for melanoma by administering them in exosomes is highly innovative. Because the safety profile of these two drugs is well documented and because they are relatively easy to purify / synthesize, the animal study could provide the base for subsequent clinical research to establish the efficacy of these exosome loaded drugs as cost-effective adjuvant cancer treatments. This very original study is thus a big contribution to the field of pharmaceutical sciences by evaluating the physicochemical properties of exosome loaded drugs and the field of biomedical research by determining the anticancer properties in a preclinical model. The impact is also documented by several publications in peer reviewed journals having resulted from the study presented in the thesis.

Presentation and Writing

The thesis is well structured and well written in a concise and precise style. The English is clear with correct grammar and very few typographical errors. Figures, tables, and lists (e.g. of abbreviations) are clear and well-presented. The formatting, including of the citations, is well done.

Clarity and Coherence

The arguments in the thesis are laid out clearly, are logical, and coherent. The thesis flows well, with each section logically leading to the next. It was a pleasure to read the document.

Research Methods

The student used a variety of methods ranging from physical characterization of the nanoparticles (exosomes) to animal models (mice and rat). The choice of methods to address the various questions was appropriate, sound and applied correctly. The research was done in a meticulous and careful manner with statistical significance in mind.

Critical Analysis

The thesis demonstrates the candidate's critical understanding of the relevant literature and the ability to critique his own work. He clearly now is an expert in the field and with the large repertoire of methods he acquired during his PhD work as well as the critical analysis he showed in the use of the techniques has proven his ability to carry out independent research.

Summary

Based on the outstanding text and figures provide to me, I recommend that the thesis is accepted without the need for clarifications or corrections.