A new liposomal nanocarrier for co-delivery of gedunin and p-glycoprotein siRNA to target breast cancer stem cells

Mohan Singh Rana\textsuperscript{a}, Meran Keshawa Ediriweera\textsuperscript{b}, Umapiyatharshini Rajagopalan\textsuperscript{a}, Desiree Nedra Karunaratne\textsuperscript{c}, Kamani Hemamala Tennekoona\textsuperscript{a} and Sameera Ranganath Samarakoon\textsuperscript{a}

\textsuperscript{a}Institute of Biochemistry, Molecular Biology and Biotechnology, Cumaratunga Munidasa Mawatha, University of Colombo, Colombo, Sri Lanka; \textsuperscript{b}Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka; \textsuperscript{c}Department of Chemistry, Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka

\textbf{ABSTRACT}

Gedunin is a secondary metabolite found in neem tree. Since the first discovery of this compound, its bio-active properties have been continuously evaluated. However, the low hydrophobicity of gedunin decreases its bioavailability and pharmacokinetic profile. In the present investigation, a new liposomal nanocarrier for co-delivery of gedunin and P-glycoprotein (P-gp) siRNA [siRNA coated liposomal gedunin (Lipo-Ged-siRNA)] was developed to improve the anti-proliferative activity of gedunin. Characteristics of prepared Lipo-Ged-siRNA demonstrated promising effects. Lipo-Ged-siRNA showed greater anti-proliferative effects (IC\textsubscript{50} 8.5 \textmu g/mL) followed by pure gedunin (IC\textsubscript{50} 40.2 \textmu g/mL) in breast cancer stem cells (bCSCs). Immunofluorescence analysis demonstrated reduced expression of P-gp following exposure to Lipo-Ged-siRNA. Furthermore, Lipo-Ged-siRNA affected the expression of \textit{ABCB1}, \textit{Cyclin D1}, \textit{Bax}, \textit{p53}, and \textit{surviving} genes in bCSCs.

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\textbf{CONTACT}

Sameera Ranganath Samarakoon sam@ibmbb.cmb.ac.lk

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1. Introduction
Cancer stem cells (CSCs) are a small group of cells which initiate tumor development and metastasis (Susa et al. 2010). CSCs have been reported to overexpress P-gp that largely contribute to their drug resistance (Susa et al. 2010). Liposome have been reported as effective nanocarrier for targeted drug delivery (Bianco et al. 2007; Sanvicens and Marco 2008; Dong et al. 2012; de Figueiredo-Rinhel et al. 2019). Co-delivery of drug resistant gene specific siRNA as selective inhibitors with a selected drug using suitable liposomal formulations has been reported to exert favorable pharmacokinetics (Meng et al. 2013). Azadirachta indica, neem, is a phytochemically diverse plant (Narender et al. 2008; Ashfaq et al. 2016; Nguyen et al. 2019). Gedunin, a secondary metabolite found in the neem tree, has shown promising anti-cancer activities in several in-vitro models (Patwardhan et al. 2013; Nwokwu et al. 2017). Poor solubility of gedunin in aqueous environment limits its bioavailability and the pharmacokinetic profile (Nwokwu et al. 2017). Therefore, in the current study we designed and developed a new liposomal nanocarrier for co-delivery of gedunin and P-gp siRNA, hypothesizing that nano-encapsulation of gedunin may enhance its biological effects in bCSCs.

2. Results and discussion
The agarose gel (2%) retardation assay showed that more siRNA has been loaded into liposomes (Lipo-Ged-siRNA) as the concentration of the liposomes increase (Figure S1), confirming that siRNAs are condensed into the prepared liposomes (Lipo-Ged). Characterization of particle size, polydispersity index (PDI) and zeta potential are presented in Table S2. The unloaded liposome with approximate size of 97.00 ± 9.11 nm, PDI of 0.45 ± 0.24 and a zeta potential value of +42 ± 7.55 mV was synthesized, characterized and used to prepare liposomal gedunin with 97.22 ± 7.12 nm size, PDI of 1.00 ± 0.24 and a zeta potential of 35.20 ± 4.57 mV and 80% encapsulation efficiency. siRNA coated liposomal gedunin displayed approximate size of 236.01 ± 44.80 nm, a PDI of 0.35 ± 0.15 and a zeta potential value of 41.30 ± 4.48 mV (Table S2). It was observed that in preparation of Lipo-Ged-siRNA, the polydispersity index (PDI) achieved was 0.35 ± 0.15, the lowest among all. Although, size of liposome in drug delivery is considered critical quality where PDI of 0.3 and below considered acceptable indicating homogenous population, FDA Guidance for Industries has not set criteria for acceptable PDI (Danaei et al. 2018). The liposomal surface charge is important attribute for its stability. Further when zeta potential is greater than ±30 mV, they become more stabilized electrically and prevent particle aggregation due to repulsion between adjacent particle load (Jain and Thareja 2019; Sopyan and Gozali 2020). Therefore, in our formulation, stearylamine was added to unloaded liposome to achieve better surface charge to provide stability and prevent particle aggregation. However, stability study needs to be conducted. For Lipo-Ged we have observed polydispersity of 1.00 as reported above. In another measurement, the polydispersity obtained was 0.87 with size of particle 468.40 nm. We chose the smaller particle size (97.22 nm) preparation for the study even though the PDI was high. We have observed in other preparations of nanoparticles (in other studies in our laboratory) that the
particle size, PDI, and zeta potential shows variations at each new preparation. Therefore, further studies are necessary to understand the heterogeneity of the particles based on the size.

Among the various formulations tested, Lipo-Ged-siRNA showed higher anti-proliferative effects followed by Lipo-Ged, paclitaxel, pure gedunin, and unloaded liposome (UL) in bCSCs (Figure S2 and Table S3). According to the microscopic images (Figure S3), a dose-dependent reduction in the formation of tumorspheres was evident following treatment with UL, Lipo-Ged, Lipo-Ged-siRNA, pure gedunin and paclitaxel, with Lipo-Ged-siRNA having the most potent effects (Figure S3). Immunofluorescence analysis demonstrated that untreated control has higher P-gp expression compared to the Lipo-Ged-siRNA treated bCSCs and appeared to be reducing when the concentration of the Lipo-Ged-siRNA increased from 1 μg/mL to 2 μg/mL, suggesting that Lipo-Ged-siRNA could decrease the expression of P-gp (Figure S4). BCSCs treated with Lipo-Ged-siRNA demonstrated a significant up-regulation in the expression of p53 (Figure S5). The expression of Bax was up-regulated at both doses tested. Survivin showed a significant down regulation following 1 μg/mL of Lipo-Ged-siRNA treatment, confirming that Lipo-Ged-siRNA can induce apoptosis. The gene P-gp demonstrated a significant down regulation at both doses tested. While regulating the normal cell cycle, the proto-oncogene Cyclin D1 has been reported to be overexpressed in a range of human cancers (Tobin et al. 2011). Cyclin D1 was significantly down regulated following exposure to 2 μg/mL of Lipo-Ged-siRNA (Figure S5) and its regulation at 1 μg/mL was not significant (Figure S5).

3. Conclusion

The enhancement of the anticancer activity of gedunin was attempted by the co-delivery of gedunin and P-gp siRNA. In conclusion, siRNA coated liposomal gedunin (Lipo-Ged-siRNA) nanoparticles were prepared and it was identified for the first time that (Lipo-Ged-siRNA) nanoparticles can exert anti-proliferative effects in bCSCs with the modulation of P-gp and Cyclin D1 and apoptotic related genes p53, Bax, and Survivin.

Disclosure statement

The authors report no conflicts of interest.

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ORCID

Mohan Singh Rana http://orcid.org/0000-0003-2728-7075
Meran Keshawa Ediriweera http://orcid.org/0000-0001-9393-9516
Data availability statement

Data available within the article.

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