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A novel formulation significantly increases the cytotoxicity of flaxseed orbitides (linusorbs) LOB3 and LOB2 towards human breast cancer MDA-MB-231 cells

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Flaxseed orbitides (linusorbs) are a family of N to C linked bioactive cyclic octa-, nona-, and decapeptides present in flaxseed oil. They are highly hydrophobic and thermally stable. Our previous studies showed that [1–9-NαC]-linusorb B3 (LOB3) and [1–9-NαC]-linusorb B2 (LOB2) exhibited cytotoxic effects towards human breast cancer HER2-subtype Sk-Br-3 cells at a concentration of ~400 μM. However, this high concentration significantly limits their potential clinical applications. In the current study, we developed a novel polyethylene glycol-based formulation for linusorbs and showed that both LOB3 and LOB2, especially LOB3, exhibited strong cytotoxicity towards human breast cancer triple-negative-subtype MDA-MB-231 cells at low nanomolar concentrations.

1. Introduction

As a popular nutraceutical food, flaxseed is a rich source of ω-3 fatty acids, phytoestrogen, and dietary fiber and low in carbohydrates (Parikh et al. 2018; Giacomino et al. 2013; Kajla et al. 2015). Consumption of flaxseed has been shown to be beneficial in preventing cardiovascular disease, diabetes, obesity and cancer (Bassett et al. 2009; Khalesi et al. 2015; Hutchins et al. 2013; Mohammadi-Sartang et al. 2017; Thompson et al. 2005; Mason and Thompson 2014). Various bioactive components including flaxseed orbitides (linusorbs) have been isolated and characterized (Bommareddy et al. 2010; Gui et al. 2012; Mabrok et al. 2012; Okinyo-Owiti et al. 2014; Kajla et al. 2015). Linusorbs are a family of highly hydrophobic and thermally stable cyclic octa-, nona-, and decapeptides present in flaxseed oil (Burnett et al. 2015; Okinyo Owiti et al. 2014). [1–9-NαC]-linusorb B3 (LOB3, Fig. 1A), which is a cyclic nonapeptide, is the most abundant as well the most studied linusorb. Previous studies have shown that LOB3 possesses immunosuppressive, antioxidative, antimalarial and anticancer activities (Drygała et al. 2009; Wieczorek et al. 1991; Zou et al. 2018; Bell et al. 2000; Okinyo-Owiti et al. 2015). [1–9-NαC]-linusorb B2 (LOB2, Fig. 1B), which is the second most abundant linusorb in flaxseed oil, also possesses immunosuppressive, antioxidative and anticancer activities (Witkowska et al. 2004; Zou et al. 2018; Okinyo-Owiti et al. 2015). Our studies showed that both LOB3 and LOB2 exhibit strong cytotoxic effects towards human breast cancer HER2-subtype Sk-Br-3 cells at a concentration of 400 μg/mL (~400 μM), however, it is impractical to research a serum level of 400 μg/mL for LOB3 and LOB2 to elicit their anticancer function *via* oral administration (Okinyo-Owiti et al. 2015). Therefore, we screened formulation conditions and identified a polyethylene glycol (PEG)-based formulation that significantly improved the anticancer activities of LOB3 and LOB2. Herein, we report out study results.

2. Investigations, results and discussion

It is well known that formulation plays a very important role in drug bioavailability, distribution, and activity. In our previous studies, dimethyl sulfoxide (DMSO) was used to dissolve the linusorbs. It was observed that linusorbs tend to aggregate during treatment, a property that is due to their hydrophobicity (Okinyo-Owiti et al. 2015). Poor dissolution likely led to weak cytotoxicity towards human breast cancer cells, requiring a high concentration (400 μg/mL) of LOB3 or LOB2 to exhibit cytotoxicity. Therefore, it is critical to identify better formulation conditions that can both increase aqueous solubility and decrease linusorb aggregation. In turn, the formulation would enhance linusorb cytotoxicity towards human breast cancer cells. After a wide range of screening, we identified a PEG-based approach that significantly increases the aqueous solubility of linusorbs. We first evaluated whether this formulation approach would exert any cytotoxic effect on breast cancer cells. As shown in Fig. 2, the PEG-based formulation solution exhibited a positively correlated time-dependent cytotoxic response towards both cell lines. For Sk-Br-3 cells, cytotoxicity increased from 12-15 % at 24 h of treatment to 35-40 % at 120 h of treatment, whereas for MDA-MB-231 cells, cytotoxicity increased from 5-7 % at 24 h of treatment to approximately 20 % at 120 h of treatment. Subsequently, we evaluated whether the formulation solution could improve linusorb cytotoxicity at nanomolar concentrations. For the Sk-Br-3 cells, LOB3 did not cause any significant change in cytotoxicity compared to the control (*i.e.* formulation solution), whereas LOB2 caused a marginal cytotoxicity increase (~3-6%) for the 96 h and 120 h treatments (Figs. 2A and 2B). Although the cytotoxicity increase is statistically significant ($p < 0.05$) for LOB2 concentrations of 25 nM, 50 nM, 100 nM and 200 nM at 96 h treatment and concentrations of 50 nM, 100 nM, 200 nM and 400 nM at 120 h treatment, it is highly unlikely to be biologically significant. Therefore, we concluded that neither LOB3 nor LOB2 could elicit

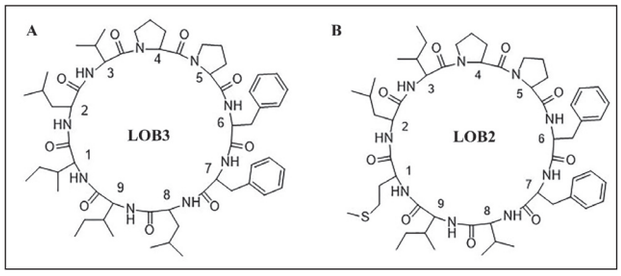

Fig. 1: Chemical structure of linusorbs: (A) [1–9-NαC]-linusorb B3 (LOB3) and (B) [1–9-NαC]-linusorb B2 (LOB2).

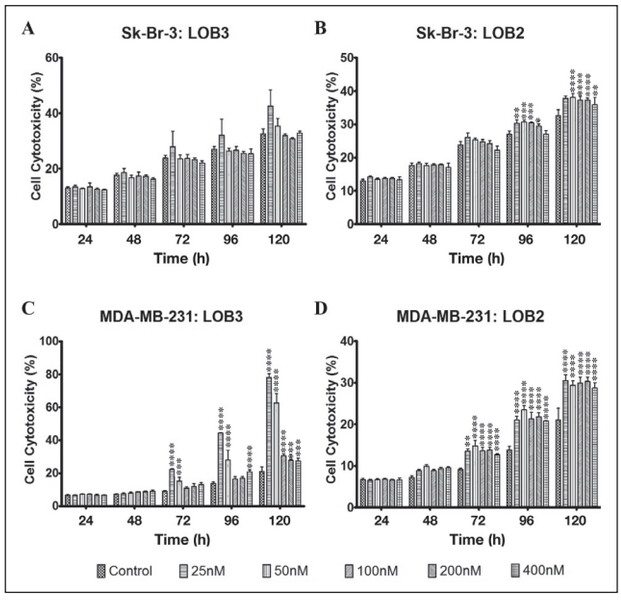

Fig. 2: Cytotoxicity of LOB3 and LOB2 towards human breast cancer Sk-Br-3 (A and B) and MDA-MB-231 (C and D) cells. The final concentrations of LOB3 and LOB2 used in treatments were 25 nM, 50 nM, 100 nM, 200 nM and 400 nM. The PEG-based formulation solution was used as a comparative control and the cell culture medium was used as a vehicle control. Significance differences are indicated by asterisks: $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$.

cytotoxic effects towards the human breast cancer HER2-subtype Sk-Br-3 cells at a concentration in the nanomolar range. For the MDA-MB-231 cells, 25 nM of LOB3 enhanced cytotoxicity from approximately 10 to 22 % at 72 h treatment ($p < 0.001$), 15 to 45 % at 96 h treatment ($p < 0.0001$), and 20 to 80 % at 120 h treatment ($p < 0.0001$), respectively, compared to the control (Fig. 2C). In addition, cytotoxicity decreased dramatically as LOB3 concentration increased from 25 to 400 nM, and we suggested that aggregation of LOB3 might still induce this phenomenon. As shown in Fig. 2D, LOB2 increased cytotoxicity by approximately 5-8 % at 72 h treatment ($p < 0.01$), 8-10 % at 96 h treatment ($p < 0.0001$), and 10-12 % at 120 h treatment ($p < 0.0001$), respectively, compared to the control. Increased cytotoxicity caused by LOB2 was significantly lower than that caused by LOB3. Moreover, the increase in LOB2 cytotoxicity was not concentration dependent (Fig. 2D).

In conclusion, linusorbs LOB3 and LOB2 did not exert any cytotoxic effect towards human breast cancer HER2-subtype Sk-Br-3 cells at nanomolar concentrations. Prolonged treatment (> 72 h) increased cytotoxicities of LOB3 and LOB2 towards human breast cancer triple-negative-subtype MDA-MB-231 cells, and LOB3 was more potent than LOB2, especially at low nanomolar concentrations. Further studies are warranted to investigate how aggregation and aqueous solubility affect LOB3 cytotoxicity and whether the new formulation would also enhance cytotoxicity of LOB3 towards other triple-negative-subtype breast cancer cells at low nanomolar concentrations from both *in vitro* cell line and *in vivo* mouse xenograft model studies.

3. Experimental

3.1. Materials

All chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Pure LOB3 and LOB2 (>95%) were a research gift from Prairie Tide Chemicals Inc. (Saskatoon, SK, Canada). Human breast cancer HER2-subtype cell line Sk-Br-3 and triple-negative-subtype cell line MDA-MB-231 were purchase from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture media, McCoy's 5A Modified Medium and Leibovitz's L-15 Medium, were purchased from Thermo Fisher Scientific (Ottawa, ON, Canada). CytoTox Green Cytotoxicity Assay was purchased from the Promega Corporation (Madison, WI, USA).

3.2. Cell culture

Human breast cancer cell lines Sk-Br-3 and MDA-MB-231 were cultured in T-75 cell culture flasks in a Forma Series II 3110 Water-Jacketed CO_2 incubator (Thermo Fisher Scientific) at 37 °C under a humidified atmosphere. Cell line Sk-Br-3 was cultured in McCoy's 5A Modified Medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin under 5 % CO_2, and cell line MDA-MB-231 was cultured in Leibovitz's L-15 medium supplemented with 10 % FBS and 1 % penicillin under 0 % CO_2. Cell culture medium was changed every 2-3 days for each cell line.

3.3. Cytotoxicity assay

The Sk-Br-3 and MDA-MB-231 cells were plated in 96-well plates at 8,000 cells per well with the final culture volume of 300 µL and allowed to grow overnight for attachment before being treated with LOB3 or LOB2 at final concentrations of 25 nM, 50 nM, 100 nM, 200 nM and 400 nM. The PEG-based formula was used as a comparative control and cell culture medium was used as a vehicle control. CytoTox Green reagent was added to wells during treatments. Following manufacturer's recommended protocols, cytotoxicities of LOB3 and LOB2 were continuously monitored for 120 h at an interval of 24 h. Cytotoxicity was calculated using the following equation.

$$Cytotoxicity\,(\%) = \frac{\mathrm{OD}\,(\text{experiment}) - \mathrm{OD}\,(\text{vehicle control})}{\mathrm{OD}\,(\text{maximum cell death}) - \mathrm{OD}\,(\text{vehicle control})} \times 100\%$$

3.4. Statistical analysis

Statistical analysis of the results was conducted using a one-way ANOVA using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Significance differences are indicated by asterisks: $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$.

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