RESEARCH ARTICLE

Phloridzin docosahexaenoate, a novel polyphenolic derivative, is cytotoxic to canine osteosarcoma D17 cells

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Abstract
Canine osteosarcoma (OSA) is the most common form of bone cancer diagnosticated in dogs and is highly metastatic. There has been limited advancement in discovering an effective treatment for OSA in the last few decades. The major drawback of the currently used chemotherapeutic drugs is their side effects. In this preliminary study, we investigated the efficacy of using a novel food-derived drug, phloridzin docosahexaenoate (PZ-DHA), in the treatment of canine OSA *in vitro*. PZ-DHA was selectively cytotoxic to canine OSA D17 cells, while normal human liver cells (WRL68) were more resistant. We also found that PZ-DHA had enhanced cellular uptake in D17 cells compared to its precursors and in WRL68 cell line. This study provides preliminary evidence that PZ-DHA needs to be further assessed as a safe and efficacious new drug in the treatment of both canine and human OSA.

Keywords: Phloridzin, docosahexaenoic acid, cancer, cytotoxicity, cellular uptake, dog

1. Introduction

Canine osteosarcoma (OSA) is the most common form of canine bone neoplasia in which large and giant breeds are most at risk (Morello et al., 2011). The current standard course of treatment includes amputation (mainly for appendicular OSA), or a limb-sparing surgery, followed by chemotherapy, most frequently using carboplatin, cisplatin, or doxorubicin (Szewczyk et al., 2015). Moreover, in recent years, other treatment options for canine OSA, such as immunotherapy, targeted therapy, and radiation therapy, have been proposed. Appendicular OSA is highly metastatic; when treated with amputation alone, dogs can face metastasis rates of up to 88% (Straw et al., 1991). Chemotherapy treatments increase the survival chances of dogs with OSA, and have some success in slowing, but not necessarily decreasing, the rate of metastasis (Selmic et al., 2014; Straw et al., 1991; Sánchez-Céspedes et al., 2020). A drawback in the use of chemotherapeutic drugs is their toxicity, causing adverse effects in up to 48-76% of dogs (Selmic et al., 2014). The negative effects caused by the toxicity of the current chemotherapeutic drugs and their inability to prevent metastasis illustrate the need for safe and efficacious new drugs for the treatment of canine OSA.

Phloridzin docosahexaenoate (PZ-DHA), a novel polyphenol fatty acid ester derivative, is synthesized through lipase-catalyzed acylation of a flavonoid precursor, phloridzin (PZ), with an omega-3-fatty acid, docosahexaenoic acid (DHA), to enhance the bioavailability and biological activities of flavonoids (Ziaullah et al., 2013). Previously, we have shown that PZ-DHA is selectively cytotoxic to neoplastic cells and had the potential for treating multiple human cancer types with the comparable antiproliferative ability to chemotherapeutic drugs (Arumuggam et al., 2017; Fernando et al., 2016; Mantso et al., 2018; Nair et al., 2014). The antiproliferative effects of PZ-DHA cause cell cycle arrest of hepatocellular carcinoma cells and triple-negative breast cancer (TNBC) cells (Fernando et al., 2016; Nair et al., 2014). Furthermore, intra-tumoral injection of PZ-DHA showed suppressive effects on the growth of xenografted TNBC cells in non-obese diabetic severe combined immune-deficient mice (Fernando et al., 2016). When administered at sub-cytotoxic levels, PZ-DHA was able to reduce TNBC progression *in vitro* and *in vivo* (Fernando et al., 2019).

The objectives of this study were to (1) determine the dose-dependent cytotoxicity of PZ-DHA, PZ, its aglycone phloretin (PT), and DHA using cultured canine OSA cells (D17) and normal human hepatic cells (WRL68); and (2) measure the cellular uptake of PZ-DHA, PZ, PT, and DHA using the above cell lines.

2. Materials and Methods

PZ-DHA was synthesized as described in Ziaullah et al. (2013). DHA was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). PZ and PT were purchased from Sigma-Aldrich (Oakville, ON, Canada). D17 cells (ATCC® CCL-183™) and WRL68 cells (ATCC® CL-48™) were purchased from Cedarlane (Burlington, ON, Canada).

5-[3-(carboxymethoxy) phenyl]-3-(4,5-dimethyl-2-thiazolyl)2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) colorimetric

assay (Promega, Madison, WI, USA) was used to determine mitochondrial metabolic activity of cells treated with PZ-DHA, PZ, DHA, and PT. D17 and WRL68 cells were seeded in 96-well flat-bottom plates and treated with PZ-DHA, PZ, DHA, or PT at concentrations of 0 (control), 12.5, 25, 50, 75, or 100 µM. Treated cells were incubated for 24 h at 37°C. MTS/phenazine methosulfate (PMS) reagent (333 µg/ml MTS and 25 µM PMS) was then added to each well and further incubated for 3 h at 37°C. A microplate reader (Tecan Trading AG, Mannedorf, Switzerland) was used to measure the absorbance at 490 nm (Arumuggam et al., 2017).

D17 and WRL68 cells were seeded at a density of 1×10^5 cells/T-75 flasks, then cultured overnight to encourage adhesion. Adherent cells were treated with PZ-DHA, PZ, DHA, or PT at a sub-toxic concentration of 20 µM, and cultured at 37°C for 72 hours. The supernatant was then removed, and the cells were washed with cold PBS. TrypLE Express was used to harvest the cells, which were washed again with cold PBS. Cold acetone (3 mL) was used to resuspend the harvested cells before incubating them at 4°C overnight. This solution was centrifuged at 14,000 ×g for 15 min at 4°C and nitrogen gas was used to evaporate the acetone prior to collecting cell lysates. The cell pellets were reconditioned with methanol then filtered. A liquid chromatography/mass spectrometry (UPLC-ESI-MS) system was used to analyze the samples for the presence of PZ-DHA, PZ, DHA, and PT. Samples (2 µL) were injected into an Allure biphenol column at a flow rate of 0.3 mL/min. ESI negative mode was used with a nebulizer gas (N_2) temperature of 375°C, and a capillary voltage of 3000 V. Standard curves of PZ-DHA (R^2=0.99), PZ (R^2=0.99), DHA (R^2=0.98), or PT (R^2=0.97) made in methanol were used to determine the percentage cell uptake of the administered dose.

The significance of the main and the interaction effects of cell type (2 levels: D17, WRL68), compound (4 levels: PZ-DHA, PZ, DHA, PT), and concentration (5 levels: 12.5, 25, 50, 75, 100 µM) on metabolic activity (%) was determined using ANOVA of a 2×4×5 factorial design with 6 replications. The significance of the main and the interaction effects of cell type (2 levels: D17, WRL68) and compound (4 levels: PZ-DHA, PZ, DHA, PT) on cellular uptake (%) was determined using ANOVA of a 2×4 factorial design with 3 replications. The analysis was completed using the GLM Procedure of SAS (SAS 2014). For each response variable, the validity of model assumptions (normal distribution and constant variance assumptions on the error terms) was verified by examining the residuals as described in Montgomery (2020). Independence assumption was met through randomization of the treatment combinations. Since the highest order interaction effects (3-way for metabolic activity and 2-way for cellular uptake) were highly significant ($P < 0.01$), further multiple means comparison was completed, and letter groupings generated using the lsmeans statement of the GLM procedure with Tukey's adjustment at the 5% level of significance.

3. Results

The mean metabolic activity values of the 40 combinations of these three factors

shown in Figure 1 reveal that PZ-DHA reduced metabolic activity of D17 cells in a dose-dependent manner. The metabolic activity in D17 cells significantly decreased at concentrations of 50 µM and higher compared to that in WRL68 cells, which were more resistant to the cytotoxic effects of PZ-DHA. PZ-DHA had a significantly greater ability to inhibit the metabolic activity of D17 cells than either of its precursors, though DHA did show selective cytotoxic effects starting at 50 µM as well. PT exhibited a similar dose-dependent reduction in the metabolic activity of D17 cells; however, PT also caused detrimental effects on the survival of WRL68 cells.

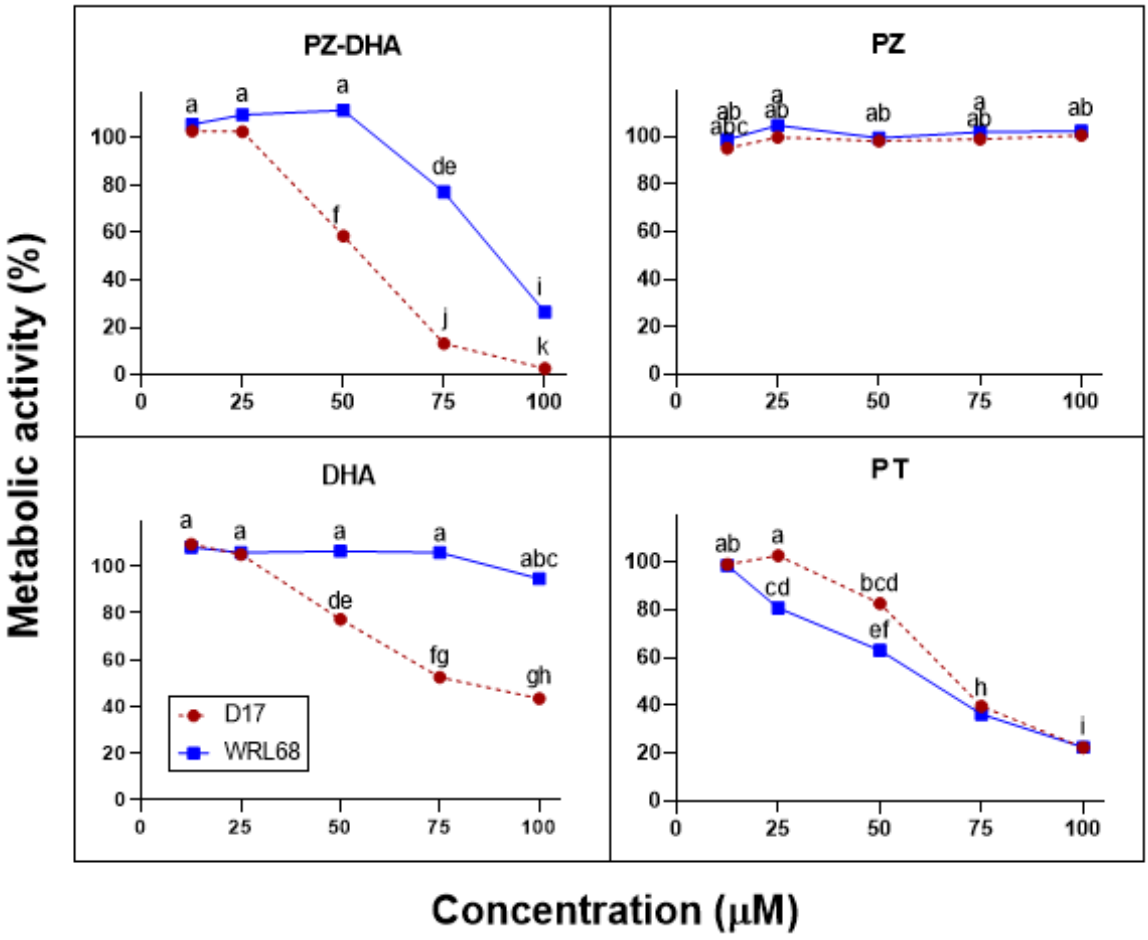

Figure 1. Interaction plot of cell type and concentration within each panel representing compound on metabolic activity (%). Means sharing the same letter are not significantly different.

The cellular uptakes of PZ-DHA, PZ, DHA, and PT into D17 and WRL68 cells are shown in Figure 2. PZ-DHA had significantly higher cellular uptake (20- to 30-fold greater) compared to its precursor compounds. There was also significantly enhanced uptake of PZ-DHA into D17 cells over WRL68 cells.

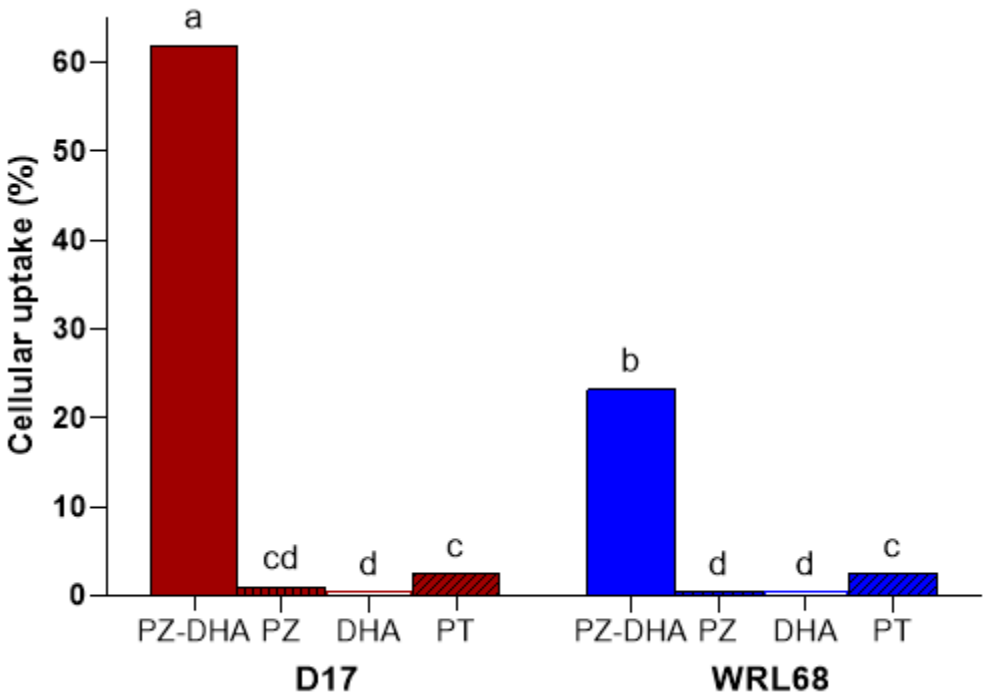

Figure 2. Bar graph showing the interaction effect of compound and cell type on cellular uptake (%). Means sharing the same letter are not significantly different.

4. Discussion

The dose-dependent cytotoxicity activity of PZ-DHA has been shown previously in human T-cell acute lymphoblastic leukemia (Arumuggam et al., 2017) and breast carcinoma (MDA-MB-231, MDA-MB-468, 4T1, MCF-7, and T-47D) cells (Fernando et al., 2016). Similar to current findings, DHA has also caused a significant decrease in cell viability of leukemia cells at 100 µM (Arumuggam et al., 2017). Flavonoids have been shown to be cytotoxic to a variety of different cancer cells, including breast, gastric, ovarian, cervical, and prostate cancers; depending on their structure, dose, and the type of cancer being treated, they can modulate different cell signaling pathways (Sak, 2014). DHA has antiproliferative effects on multiple cancer cell lines as well, causing cell cycle arrest at different stages and apoptosis depending on the cell type (Newell et al., 2017). Fernando et al. (2016) found similar dose-dependent cytotoxic effects of PT in mammary carcinoma (MDA-MB-231, MDA-MB-468, 4T1, MCF-7, and T-47D) cells. A study done by Wu et al. (2009) found that PT caused a significant reduction of cell viability in liver cancer cells but had no significant cytotoxic effects on normal human breast epithelial cells or breast cancer cells. They also found that PT triggered apoptosis by interfering with GLUT2, which is highly expressed in hepatic and malignant cells (Wu et al., 2009). This suggests that the cytotoxic effects of PT on WRL68 cells may be caused by its interference with GLUT2 receptors.

Fernando and colleagues have observed PZ-DHA was taken up by human (MDA-MB-231, MDA-MB-468, and MCF-

7) and mouse (4T1) mammary carcinoma and human non-malignant mammary epithelial cells (MCF-10A) (Fernando et al., 2020). DHA is a conditionally essential fatty acid, which is readily used within cells for membrane phospholipid structure (Hishikawa et al., 2017). Martin et al. (2000) found that DHA uptake was rapid in neurons, where the highest rate occurred one minute after treatment; DHA was also quickly metabolized and used in membranes, with over 90% incorporated in one minute. This rapid metabolism may explain DHAs apparent lack of presence in the cytoplasm due to the detection of compounds was performed 72 h after incubation. PT had significantly higher uptake than its glucoside PZ only in WRL68 cells. However, Gee et al. (2000) reported that another flavonoid, quercetin, had significantly lower uptake compared to two of its mono-glucosides. This can be attributed to the differences in the intestinal absorption of these compounds. PZ was mainly hydrolyzed into PT before being absorbed, whereas isoquercitrin can be absorbed before undergoing hydrolysis, indicating a difference in cellular uptake mechanisms, where isoquercitrin was more readily absorbed (Crespy et al., 2001). Acylation with DHA improved cellular uptake of PZ. This finding was supported by another study that showed increased lipophilicity of the flavonoid epigallocatechin gallate (EGCG) modified with DHA esters than EGCG alone (Zhong and Shahidi, 2011). The important implication from this finding was that acylation of flavonoids could enhance their bioavailability, therefore increasing their use within the cell.

5. Conclusion

The present study provides evidence that PZ-DHA is selectively cytotoxic to canine OSA D17 cells while sparing normal human hepatic cells (WRL68). Additionally, we also demonstrated enhanced cellular uptake of PZ-DHA into D17 cells. In conclusion, this study shows promising preliminary evidence for the potential application of PZ-DHA as a therapeutic agent in the treatment of cancer in dogs and warrants further research into the mechanisms of action of PZ-DHA using both *in vitro* and *in vivo* models.

Declaration of Competing Interest

The authors declare no conflict of interest.

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