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Inhibition of Breast Tumor growth in Mice After Treatment with Ceramide Analog 315

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Abstract

Aim—To evaluate the anti-cancer and anti-tumor activities of ceramide analog 315 in nude mice.

Methods—Nude mice (n=10) were injected bilaterally with 5×10^6 MDA-MB-231 cells on each side. Tumors were allowed to form for two weeks. The mice were then divided into two groups (n=5 in each group). The control group mice were injected with 25 μ L of DMSO (dimethyl sulfoxide) and the treatment group mice were injected with 10 mg/kg of analog 315 (in DMSO, 25 μ L volume) every day for a period of 3 weeks. Animal weights and tumors were measured every week for 3 weeks.

Results—At the end of the experimental period, control animals had retained excess fluid, and showed larger tumor sizes compared to the treated group (2.95 g vs. 1.67 g). A 45% reduction in tumor size and 80% decrease in tumor volume were observed in the treatment group. There was a significant increase in the weights of liver (10%) and spleen (19%) between the control and treated animals. Hematoxylin and Eosin (H&E) staining of MDA-MB-231 tumor sections revealed more acellular necrotic regions in tumors from the treatment groups compared to the ones from the control group. Ki67, a proliferation marker was higher in number in control tumor section (71.8 ± 12.8) compared to the treatment tumor section (37.4 ± 10.4), ($P < 0.001$). Photomicrographs showed metastatic tumor burden in kidney, lungs, and spleen collected from the control group mice bearing MDA-MB-231 tumors. Treatment group mice showed normal microscopic tissue architecture. Overall, our study demonstrated tumor growth inhibition and anti-metastatic effects for the novel ceramide analog 315.

Keywords

Ceramide; Tumor; MDA-MB-231; Nude Mice

Breast cancer is the leading cause of death in women. According to the American Cancer Society statistics, approximately 252,710 new cases of invasive breast cancer and 40,610

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breast cancer deaths were expected to occur among women in the United States in 2017 [1]. Also In 2017, 1,688,780 new cancer cases and 600,920 total cancer deaths were projected to occur in the United States [2]. Prostate cancer is the most common cancer among males (19%), followed by lung (14%) and colorectal (9%) cancers. Among females, breast (30%), lung (12%), and colorectal (8%) cancers are the most common, and lung (25%), breast (14%), and colorectal (8%) cancers are the leading causes of cancer death [3]. Triple negative breast cancer occurs in about 10–20% of diagnosed breast cancers [4] and is more likely to affect younger people, African Americans, Hispanics, and/or those with a BRCA1 gene mutation. Breast cancer cell lines have been used widely to study breast cancer biology, to screen new drugs, and to identify pathways leading to suppression of cancer growth and metastasis. The MDA-MB-231 cell line is triple negative (ER α /PR/HER2 negative) and resistant to hormone and endocrine therapies. Sphingolipid-signaling pathway is considered to be a novel anti-cancer target system. Sphingolipids, a class of lipids derived from serine and aliphatic acids, regulate the fluidity and mechanical stability of lipid bilayers [5]. However, numerous findings suggest that sphingolipids also play important roles in the regulation of cancer pathogenesis and development [6]. Ceramide serves as a central mediator in sphingolipid metabolism and signaling pathways, regulating many fundamental cellular responses [7]. Chemotherapeutic drugs and radiation therapy have been shown to increase intracellular ceramide levels. Exogenously treating cancer cells with short-chain ceramides also has been shown to lead to anti-cancer effects. Therefore, targeting ceramide-signaling pathway by activating ceramide down-stream receptors, inhibiting ceramide-metabolizing enzymes, or exogenously increasing the ceramide levels, comprise novel targets for cancer treatment. [8].

Ceramide is involved in the induction of apoptosis and growth arrest in breast cancer. In our previous studies, we have synthesized and evaluated 51 ceramide analogs and performed structure-activity relationship studies yielding the following information:

1) Extension of the conjugated system in the ceramide backbone and/or side-chain increases the potency; 2) Phenyl imine group modification on the side-chain (especially ortho-substituted phenyl) enhances the anti-cancer potency; 3) 1-Position modification results in GCS (Glucosylceramide Synthase) inhibition; and 4) Ceramides can induce apoptosis and inhibit cell growth and proliferation in *in vitro* studies [9–12]. Despite recent advances in the treatment of cancer, triple negative breast cancer is very aggressive and difficult to treat [4], posing an important clinical challenge due to unresponsiveness to endocrine- and chemotherapy. Thus, human triple negative breast cancer cell line MDA-MB-231 was selected as the cell model for this study. Our main goal in this study was to investigate whether our lead compound *in vitro*, ceramide analog 315, ((S)-2-(2-hydroxybenzylideneamino)-3-hydroxy-N-tetradecylpropanamide), would show similar *in vivo* outcomes.

Synthesis of Analog 315

Analog 315 was synthesized as part of our ongoing efforts toward the synthesis of a potent ceramide anti-cancer drug. For the synthesis, Boc-L-serine 1 (Aldrich, USA) was coupled with amine 2 (tetradecyl amine, TCI, Japan) to get the desired dipeptide 3. EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, Bachem, Germany) and HOBt (1-hydroxy

benzotriazole, Aldrich, USA) were used as coupling reagents. N-methyl-morpholine was used as a base. The Boc group was cleaved using acidic conditions (in TFA, trifluoroacetic acid, Alfa Aesar, USA) to give the free amine 4, which was further reacted with salicylaldehyde (Acros Organic, China) to produce analog 315 [11,13].

Animal Studies

Ten female nude mice (Nu/Nu) aged between 7 and 8 weeks (Charles River Laboratories, USA) were housed in sterile cages and maintained in pathogen-free aseptic rooms with 12-hours light/dark schedule. Mice were fed with autoclaved food pellets and water ad libitum. All experiments were carried out in accordance with the protocol approved by the Xavier University of Louisiana's Institutional Animal Care and Use Committee. The animals were monitored daily for signs of distress, decreased physical activity, or any other behavioral changes, and were weighed every week.

Tumor xenograft studies

Human breast cancer MDA-MB-231 cells were purchased from Cell Biolabs (San Diego, CA) and were grown according to standard protocols. MDA-MB-231 cells were harvested with Phosphate Buffered Saline (PBS)/Ethylene Diamine Tetraacetic Acid (EDTA) solution, counted, and washed twice in PBS. Mice were anesthetized using Ketamine/Xylazine mixture. Mammary fat pad tumors were established by injecting 5×10^6 MDA-MB-231 cells in 150 μ L of PBS–Matrigel mixture (1:2) orthotopically and bilaterally into the mammary fat pads (2 tumors/mouse). Tumors were allowed to reach approximately 300 mm³ before starting treatments. The five control mice were injected intraperitoneally with 25 μ L of dimethyl sulfoxide (DMSO), while the five animals in the treatment group were injected with 25 μ L of 10 mg/kg (body weight) of analog 315 in DMSO, daily, for a period of three weeks. Tumor volumes were determined weekly by measurement with a caliper and calculated by the formula: $(W^2 \times L)/2$.

Necropsy procedures

At the end of 30 days (a total of seven weeks after inoculation), the mice were sacrificed. The care and treatment of experimental animals were in accordance with institutional guidelines. The whole animal body was examined. Tumors, livers, lungs, kidneys, spleens and hearts were removed, isolated from surrounding tissues, blotted dry and weighed. They

were also thoroughly checked in order to search for any metastases. Tissues were stored in 10% neutral-buffered formalin and submitted to Tulane University Medical Center, Department of Pathology for paraffin embedding and slide preparation followed by Haematoxylin and Eosin staining.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed on 10% neutral buffered formalin fixed paraffin-embedded tumor samples. The sections mounted on slides were deparaffinized in xylene, dehydrated in ethanol, and rinsed in water. Antigen retrieval was carried out with Diva decloaker for 30 min in a steamer before incubation with 3% hydrogen peroxide for 5 minutes. After washing with PBS, sections were blocked by incubation in background sniper for 10 minutes, followed by overnight incubation with primary antibody, Ki67 (prediluted) (NeoMarkers, USA). After overnight incubation with the primary antibody, slides were washed with PBS and incubated for 30 minutes with biotinylated secondary antibody (Vector Labs, USA), rinsed in PBS and then incubated with ABC reagent (Vector Labs, USA) for 30 minutes. The stain was visualized by incubation in 3,3-diaminobenzidine (DAB) and counterstained with Harris Hematoxylin. Internal negative control samples incubated with either non-specific rabbit IgG, or blocking solution instead of the primary antibody showed no specific staining. Slides were dehydrated and mounted with Permount (Fisher, USA). Slides were visualized using a Nikon OPTIPHOT microscope, and randomly selected bright field microscope images (magnification, x 200) were captured by Nikon Digital Sight High-Definition color camera (DS-Fi1) using NIS-Elements BR software.

Statistical analysis

Data are expressed as mean $\pm$ SD. $P < 0.05$ was considered significant. The mean and SD were calculated using Microsoft Excel or GraphPad Prism 5 software. Statistical significance was determined by Student's t-test.

Results

Animals were weighed on a weekly basis. During the experimental period, the control animals were shown to have higher weights ($P < 0.01$) and were heavily bloated compared to the treated animals. At necropsy, it was observed that the control animals had fluid filled cavity and tumors were heavier and larger in size ($2.95 \pm 0.93\text{g}$ vs. 1.67 ± 0.94 , $P < 0.01$). Approximately 45% reduction in tumor weight was observed at sacrifice. After two weeks of treatment, the tumor volume was reduced in the treatment group by 60% (1000.79 ± 221.23 vs 364.40 ± 296.74). After 4 weeks of treatment, the tumor volume was reduced by 80% in the treatment group (2044.83 ± 386.85 vs 420.42 ± 74.78). When the whole-body weights were adjusted to tumor weights, there were no differences in whole body weights between the control and treated animals. There was a significant difference in weights of liver (10%) and spleen (19%) between the control and treated animals. Animal study results are shown in Figures 1–4 and Table 1.

Hematoxylin and Eosin (H&E) staining of MDA-MB-231 tumor sections revealed more acellular necrotic regions in tumors from the treatment groups than the ones from the control group. The immunohistochemistry was performed for Ki67, a proliferation marker that was higher in number in control tumor section (71.8 ± 12.8) compared to the treatment tumor section (37.4 ± 10.4), $P < 0.001$. Examination of sections from various organs revealed metastatic tumor burden in the kidneys, lungs, and spleens collected from the control group mice. Importantly mice treated with the ceramide analog did not show any metastases in the above mentioned organs and only exhibited normal microscopic tissue architecture (Figures 5 and 6).

Discussion

Ceramide analog 315 has been previously shown to be anti-proliferative and cytotoxic to human breast cancer cells *in vitro* [12]. This compound has an imine functional group in the ceramide side-chain, and an amide functional group replacing the allyl alcohol group in the ceramide backbone [9]. In the present study, treatment with ceramide analog 315 was shown to result in a significant reduction in tumor volume and tumor weight *in vivo* using nude mice xenograft. Measurement of tumor size is important in preclinical animal studies when assessing responses to cancer treatment. In longitudinal studies, sequential measurements of tumor volume with a non-invasive method are essential. Current standard technique for subcutaneous tumor xenograft measurement is by caliper, where tumor volume is calculated using the formula $(W^2 \times L)/2$. In this study, we demonstrate that the treatment with analog 315 exhibited an anti-tumorigenic effect. These results are in agreement with previous studies showing that certain ceramides can effectively induce apoptosis in tumor cells [14,7], and inhibit tumors *in vivo* [15]. Our studies also showed that analog 315 exhibited mitochondrial toxicity leading to cell death (Data not shown). In addition, Analog 315 also showed anti-cancer activity in the treatment of chemo-resistant cancers *in vitro and in vivo* (Manuscript in preparation). The mechanism of ceramide-induced apoptosis has been well established, showing the elevated ceramide levels in mitochondria as the cause of mitochondrial dysfunction including loss of electron potential, and cytochrome C [16]. In our studies, the ceramide treated mice did not lose weight and did not show any signs of suffering from drug toxicity. None of the treated mice showed any metastases in internal organs, whereas 3 out of the 5 control mice developed distant metastases in visceral organs. Visceral metastasis in triple negative breast cancer has been reported by other investigators [17]. In the light of these results, we have shown that administration of ceramide analog 315 results in cancer cell apoptosis, tumor growth inhibition, and reduced metastasis to visceral organs in mice bearing triple negative breast cancer xenograft. Furthermore, analog 315 showed potent anti-tumor activity without causing significant body weight loss in the treated mouse xenograft model. Therefore, analog 315 may be a promising drug candidate.

Our goal is to revolutionize breast cancer treatment regimens by replacing them with ones that are more effective and less toxic.

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Figure 1. Animal Weights

Mice were inoculated with 5 million MDA-MB 231 cells. After 3 weeks of tumor development they were separated into two groups. The control group mice were injected with DMSO while the treated group mice were injected with 10mg/kg (body weight) of analog 315, daily for three weeks. Significant weight difference was observed at 3 and 4-week time point.

Figure 2. Tumor Weights

Mice were inoculated with 5 million MDA-MB 231 cells. After 3 weeks of tumor development they were separated into two groups. The control group mice were injected with DMSO while the treated group mice were injected with 10mg/kg of analog 315, daily for three weeks. Animals were sacrificed at 7 weeks postinoculation. Tumor weights were recorded. There was significant difference $P<0.001$ in tumor weights

Figure 3. Tumor Volume

Mice were inoculated with 5 million MDA-MB 231 cells. After 3 weeks of tumor development, they were separated into two groups. The control group mice were injected with DMSO while the treated group mice were injected with 10mg/kg of analog 315, daily for 3 weeks. Tumor volume was recorded twice a week. There was significant difference $P<0.001$ in tumor volume after treatment

Figure 4. Final Average Weight of mice after adjusting tumor weights

Mice were inoculated with 5 million MDA-MB 231 cells. After 3 weeks of tumor development they were into two groups. The control group mice were injected with DMSO while the treated group mice were injected with 10mg/kg of analog 315, daily for 3 weeks. No significant weight difference was observed at 4-week time point after adjusting the tumor weights.

Figure 5.

A–C. Tissues were isolated and fixed in 10% formalin, paraffin embedded sectioned and stained with Hematoxylin & Eosin. IHC was also done for Ki67 proliferation marker in the tumor sections

Figure 6.

A–C Photomicrograph shows the metastasis in the kidney, spleen and lungs collected from control mice bearing MDA-MB-231 tumors. Arrows indicate metastatic breast cancer cells surrounded by normal tissue. The photomicrograph of treatment mice shows normal microscopic architecture of all three tissues

Table 1

Raw weights of the tissue after necropsy

Average Weight (g)	Liver	Kidney	Lungs	Heart	Spleen	Tumor
Control	1.94+/-0.56	0.38+/-0.03	0.16+/-0.02	0.12+/-0.01	0.21+/-0.04	2.96+/-0.93
Treated	1.75+/-0.34	0.37+/-0.03	0.15+/-0.03	0.12+/-0.01	0.17+/-0.03	1.67+/-0.94
% Difference	10%*	2.60%	1%	0%	19%*	44%*

Control and treated mice were sacrificed, tissues were isolated and blotted dry on a tissue paper. They were weighed and weights were recorded. Values are an average of five samples $\pm$ SD.

* P<0.001