Inhibition of Tumors in Mice Models by a Synthetic Ceramide-analog AD2725

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Abstract

A synthetic analog of ceramide (AD2725) elevated ceramide levels in MDA-MB-435 breast cancer cells inducing apoptosis and resulted in cell death. In animal studies, using a model of xenograft breast cancer cancer cells in nude mice, treatment with AD2725 resulted in a significant reduction of the tumor volume and weight, and a significantly prolonged survival of the treated mice. The analog also significantly elevated INF gamma and IL-12 cytokine levels and reduced the level of IL-10 in the plasma. These data indicate that use of suitable synthetic analogs that most probably elevate the ceramide levels may have beneficial anti-cancer therapeutic effects.

Keywords: Ceramide; Sphingolipids; Apoptosis; Cytokine; Breast cancer

Introduction

Apoptosis is recognized as an important mechanism by which cytotoxic agents induce tumor cell death [1,2]. One mechanism is using ceramide, as an intracellular, pro-apoptotic signaling molecule [3-5]. It is now clear that ceramide plays a central role in both apoptotic and mitogenic pathways [6,7]. Thus, generation of ceramide in cells initiates apoptosis and cell death while reduction of intracellular ceramide or increasing sphingosine-1-phosphate levels leads to drug resistance [8-12]. Ceramide is often generated in response to chemotherapeutic agents or radiation via hydrolysis of sphingomyelin [11,13]. Although the mechanism remains unclear, ceramide causes a disruption of mitochondria, leading to cell death by apoptosis [11,14-17]. Ceramide also reduces the vascular network that feeds the tumor [18]. In this study we used a synthetic ceramide-analog AD2725 to investigate the effects of chemotherapy while achieving an elevation of ceramide in the tumor cells. This study demonstrates that using a non-natural ceramide-like analog caused an elevation of intracellular ceramide level in MDA-MB-435 breast cancer cells, leading to their death by apoptosis and significantly prolonged the survival of tumor bearing mice.

Material and Methods

AD2646: (2R,3R)-2-(N-tetradecylamino)-3-(4-nitrophenyl)-1,3-propanediol; AD2673: (2R,3R)-2-amino-3-(4-tetradecanoylamino-phenyl)-1,3-propanediol, were synthesized by Dagan et al. [19] AD2725: (2S,3R)-2-(N-tetradecylamino)-3-phenyl-3-propanol, was synthesized as follows: One mille mole (2S,3R)-2-amino-3-phenyl-3-propanol was reacted with tetradecanal (1.2 mmol) in methanol/0.05 N acetic acid, 9:1 for 15 min and sodium cyanoborohydride (NaCNBH3) reacted with tetradecanal (1.2 mmol) in methanol/0.05 N acetic acid, 9:1 for 15 min and sodium cyanoborohydride (NaCNBH3) was added in portions during 1 h. The mixture was stirred overnight at room temperature, evaporated to dryness and the residue dissolved in dichloromethane/methanol, 2:1. Dilute HCl was added and following overtaxing, the upper phase was removed. The lower phase was washed two more times with dilute HCl and dried by adding MgSO4. The solvent was collected, evaporated to dryness and the product was purified by column chromatography using increasing concentrations of methanol in dichloromethane. Product is 550 μmol (55% yields).

Reagents

All chemical and solvents used were of analytical grade. Chemicals were purchased from Sigma Chemicals (St Louis, MO, USA). Thin layer chromatography plates were purchased from Whatman (Clifton, NJ, USA). Culture media and supplements were purchased from Beth Haemek, Israel.

Cell line

Breast cancer MDA-MB-435 cells were cultured at 37°C in an incubator with 5% CO2 in air in RPMI-1640 medium, supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 1% glutamate and 4.5 g/L glucose. Once the cells grew to about 80% confluence, the medium was removed, fresh medium was added and incubation was continued.

Mice

Female CD1 nude mice (5-6 weeks old) weighing 20 g were purchased from the Harlan Laboratories, Israel. The animals were fed Purina chow and acidified water (pH 2.7) ad libitum, and maintained in an SPF animal facility at 21°C with 12 h cycling of light. The animal studies were approved by The Ethics Committee of the Hebrew University-Hadassah School of Medicine.

Determining of cellular protein concentration and cell number

Cells were harvested, washed with PBS, trypsinized, suspended in PBS and sonicated for 10 seconds with a Microson XL probe sonicator (Misonix) at 40% output power. For quantifying the protein, a sample

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(5 µl) was taken, interacted with Bradford reagent (250 µl) and the absorbance quantified at 595 nm. A culture sample was mixed with an equal volume of trypan blue, applied to a hemocytometer, and the numbers of live (unstained) and damaged or dead (blue) cells were determined.

**Cell viability analysis**

10^4 cells per 100 µl medium were seeded in each well in a 96-well plate, incubated overnight and varying concentrations ofceramide analogs in 100 µl medium containing 2 µl DMSO were added to the cultures. (The controls contained the same concentration of DMSO). An MTT solution was added and incubation was continued for 4 hours. The stop-solution [89% Isopropanol, 1% HCl (37%), 10% Triton-X-100] was then added and cell viability was determined at 570 nm.

**Apoptosis determined by DNA fragmentation**

1 × 10^6 MDA-MB-435 cells in 5 ml medium were seeded in a small flask and incubated for 24 h. The next day the medium was removed, new medium and 20 µM of AD2275 or AD2646 were added and incubation was continued for 24 h. On the third day, cells were harvested by trypsinization and the genomic DNA was extracted using the Sigma DNA extraction kit. Samples of DNA were then electrophoresed on a 1.5% agarose gel at 50 volts in TAE electrophoresis buffer for 2 hours. The DNA was detected by a UV-illuminator and pictures were taken.

**Apoptosis determined by measuring activation of Caspase 3**

Caspase-3 activity was assayed by Z-DEVD-R110 cleavage, using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes). Following incubation with the respective analogs, cells were collected, precipitated and washed with PBS. For each sample, 10^6 cells were lysed by incubating with 50 µl lysis buffer on ice for 30 min and cell debris was precipitated in a micro-centrifuge for 5 min at 5,000 rpm. The supernatants were incubated with 50 µM of the conjugated substrate Z-DEVD-R110 in a 100 µl reaction volume at room temperature for 30 min and the fluorescence of the released free R110 was measured at an excitation at 485 nm and emission at 535 nm using a FL600 microplate fluorescence reader.

**Quantification of cellular ceramide**

MDA-MB-435 cells were incubated for 3 hours in the absence or presence of the ceramide analog AD2275 and AD2646 (20 µM) and 15 µM pyrene decanoic acid (P10), as a solution in 1 µl DMSO per ml reaction mixture. The cells were trypsinized, collected and washed twice with PBS. 2 ml PBS were added and the cell suspension was sonicated in a Microson XL probe sonicator (Misonix) for 10 seconds. A sample was taken for quantifying the cell protein using the Bradford procedure. To the rest were added 2 ml of dichloromethane-methanol, 1:1, stirred and centrifuged. The lower phase was collected and the upper one retreated with 1 ml of dichloromethane-methanol, 3:1. The two dichloromethane phases were combined and the solvent was evaporated to dryness under a stream of air. The residue was dissolved in dichloromethane-methanol, 1:1 and applied to the concentrating zone of a TLC silica gel plate having markers of P10 and P10-ceramide. The latter was developed in a mixture of dichloromethane-methanol-ammonia, 95:5:0.5. The P10-ceramide spots (viewed with an ultraviolet lamp) were scraped, dichloromethane-methanol, 1:1, was added, stirred sonicated in bath sonicator and centrifuged. The fluorescence of P10-ceramide was recorded in a spectrophotometer (Perkin-Elmer LS5) using an excitation at 345 nm as an emission at 377 nm.

**In vivo studies**

CD1-Nude mice (5-6 week old) were irradiated with a total body irradiation (TBI) of 400 cGy and one day later injected with MDA-MB-435 cells.

Four types of experiments were performed:

1. Breast cancer cells (4 × 10^6 cells in 0.1 ml medium) were injected intradermally into the left side of the flank and the next day the mice were injected i.p. with the analog AD2725.

2. Breast cancer cells (4 × 10^6 cells in 0.1 ml medium) were injected intradermally into the left side of the flank and one week later the mice were injected i.p. with the analog AD2725.

3. Breast cancer cells (5 × 10^6 cells in 0.2 ml medium) were injected intravenously into the lateral tail vein and two hours later the mice were injected i.p. with the analog AD2725.

4. Breast cancer cells (5 × 10^6 cells in 0.2 ml medium) were injected intravenously into the lateral tail vein and the mice were given the analog AD2725 in the drinking water, starting day 0.

The tumor size was measured twice per week and the tumor volume was calculated from the formula: length × width^2 × π/6. Mice injected with saline or drunk regular water were used as controls. At the end of experiment, mice were sacrificed with CO2; the tumors on the skin were removed and weighed. All organs of the mice were tested for presence of metastases.

**Cytokine assay**

The levels of cytokines in the plasma were assayed by ELISA assay. ELISA reagents were purchased as Opt. EIA Cytokine ELISA sets from BD Biosciences (San Diego, CA, USA) and the respective cytokines were analyzed according to the manufacturer’s protocol as previously described [20].

**Statistical analysis**

Most results are based on experiments run in triplicates at least twice, except where indicated. Statistical analyses were performed by Student’s t-test (except where indicated) and the results were considered statistically significant when p<0.05.

**Results**

**Cytotoxic effects of the sphingolipid analogs**

MDA-MB-435 cells (0.1 × 10^6 cells/ml, 100 µl/well) were grown overnight in RPMI-1640 without FCS in 96-well plates, then incubated for 24 hours with increasing concentrations of three ceramide analogs AD2646, AD2673 and AD2725. The viability of cells were determined by the MTT assay, all the synthetic drugs exhibited cytotoxicity toward breast cancer cell line MDA-MB-435 based on a dose respond manner (Figure 1B), the estimated IC50 of AD2725 was 12.5 µM. Trypan blue staining results further showed that all the 3 synthetic analogs of ceramide severely damaged the breast cancer cells. AD2725 as a ceramide analog was used for a further study in vivo (average of 2 experiments, each one in triplicates).

**Induction of apoptosis by the analogs**

**Caspase-3 analysis:** MDA-MB-435 cells (1 × 10^6 cells/ml) were incubated for 3 hours with 20 µM AD2725, the cells were collected and induction of apoptosis was analyzed by the Caspase-3 activity procedure. As shown in Figure 1C, AD-2725 increased the Caspase-3 activity approximately 2.5 times, which is considered statistically significant when p<0.05.
activity 4-5 fold relative to the control in which the cells were grown without the analogs (Figure 1C).

**DNA laddering:** In a small flask 2 x 10^5 of MDA-MB-435 cells/ml were seeded, 20 µM each of AD2725 and AD2646 were added and incubation for 24 hours. Cells were trypsinized and harvested; the genomic DNA was extracted, quantified and electrophoresed. Figure 1D shows the DNA electrophoresis on 1.5% agarose gel. DNA laddering was seen after 24 hours analogs treatment and without in control. In parallel experiments (not shown), the DNA laddering was not appeared after 6 hours when the concentration of the analogs was 20 µM, but when the concentration of AD2725 raised to 80 µM, considerable DNA fragments laddering was observed after 5 hours and was strengthened after 7 hours.

**Quantification of cellular ceramide:** As shown in HL-60 cell with ceramide analogs by Dagan et al. [19], incubation of MDA-MB-435 cells with AD2725 showed elevated cellular ceramide (Figure 2). This was measured by the conversion of pyrene-dodecanoylsphingosine (P12) to pyrene dodecanoylsphingosine (P12-ceramide). In the current study, 6 x 10^6 MDA-MB-435 cells were incubated with 50 µM of P12 for 3 hours in the absence or presence of 20 µM of AD2646 or AD2725. The lipids were extracted, applied to a thin layer chromatography plate, and was developed in a mixture of chloroform–methanol–ammonia 95:5:0.5 (by volume). The P12-ceramide spots were visualized with a UV-lamp, and then were scraped, the lipid was extracted and the fluorescence was quantified in a spectrophotometer. In Figure 2, the values of ceramides are presented as the fluorescent units divided by the cellular protein, indicating that AD2646 resulted in a 2.3 fold increase and AD2725 in a 2.6 fold P12-ceramide increased compared to the control.

**Effect of AD2725 on the growth of MDA-MB-435 Xenografts:** CD1-Nude mice were irradiated with 400 cGy TBI and following one day, each mouse was injected i.d. into one flank with 4 x 10^5 MDA-MB-435 cells in a volume of 0.1ml. After one day the mice were injected i.p. with 5 mg/kg of AD2725 in 10% CE saline daily. A control group (n=8) of mice was similarly treated with saline. As seen in (Figure 3A) after 25 days of daily injections, the tumors in AD2725 injected mice were significantly smaller than that of the controls. In another experiment the mice were injected i.d. with 4 x 10^5 MDA-MB-435 cells and when the tumors were palpated after a week, the ceramide analog AD2725 was injected i.p. for another 6 weeks. Also in these experiments there was a significant inhibition of the tumor growth, measured up to 25-50 days (Figure 3B).

**Survival of nude mice injected i.v. with human breast cancer cells and treated with AD2725:** CD1-Nude mice, 6 mice in each group, were irradiated with 400 cGy, and one day latter injected intravenously with 5 x 10^6 MDA-MB-435 cells and AD2725 (10 mg/kg) was administrated by intraperitoneal injections after two hours, and then continue 60 injections, starting day 0, 6 days per week. Figure 4A shows that all the treated mice by AD2725 survived till 80 days while the control mice started to die on 43 days and 2 mice of 6 died till 80 days. In another experiment, 8 mice in each group were injected with 5 x 10^6 breast cancer cells i.v. and the ceramide analog AD2725 was administered in the drinking water (10 mg/kg, starting day 0). The mice were followed up for 100 days. Figure 4B shows that mice treated with AD2725 in daily drink did not lose weight and survived more than 100 days. On the other hand 5 of the 8 mice in the control group died (Figure 4B).

**Ceramide analog AD2725 effect on cytokine profile:** Cytokine concentrations of IL-12, IFN-γ and IL-10 were assessed by ELISA in sera from each group of mice. A significant reduction of IL-10 was seen in AD2725 treated mice compared to the vehicle control (p<0.05). IFN- and IL-12 were significantly elevated in AD2725 treated mice compared to control (p<0.05) (Table 1).

**Discussion**

The accumulation of intracellular ceramide as a result of a) treatment by ceramide analogs in vitro [21], b) modification of endogenous ceramide metabolism [22], c) inhibition of the conversion of ceramide [23] and d) the induction of de novo ceramide synthesis [24] cause cancer cell death through apoptosis. Our results and those of others illuminate the potential therapeutic utility of ceramide to cancer. In this study, we show that the application of an analog of ceramide AD2725 has a cytotoxic effect on MDA-MB-435 breast cancer cells and 5 µM of AD2725 may reduce the number of cancer cells. These results are in agreement with other previous studies showing that ceramide can effectively induce apoptosis in tumor cells [25,26]. Moreover, in this study we show that AD2725 exhibited mitochondrial toxicity (IC50 12.5 µM) leading to cell death, we proposed that the mechanism of cell death is because of the accumulation of ceramide in mitochondria. The mechanism of ceramide induced apoptosis has been well established, the elevated ceramide in mitochondria is responsible for mitochondria dysfunction including loss of electron potential, cytochrome C release and prerequisites for caspase activation [27,28].

In agreement with these data, our results show a significant inhibition of growth of the human breast cancer cells MDA-MB-435 in vivo as xenograft in nude mice. The mice that were injected i.v. with tumor cells and drank water with AD2725 did not lose weight and survived more than 100 days, whereas 60% of the control died within this period. Same as our previously study in TSU-Pr1 prostate cancer model [29], we found mice injected i.v. with MDA-MB-435 cells and treated i.p. with AD2725 showed a remarkable reduction of metastases in internal organs of the survivors, whereas all control mice died with developed metastases tumors (data not show). Measuring the cytokine...
Inhibition of subcutaneous growth of MDA-MB-435 tumor in CD1 NUDE mice. A: CD1 NUDE mice were injected intradermally into one flank with MDA-MB-435 cells, and next day the mice were injected i.p. with the analog AD2725 near the tumor site with 10 mg/kg of AD2725 in 10% Cremophor-ethanol (vehicle). B: MDA-MB-435 cells were incubated for 3 hours with 20 µM of AD2646 or AD2725 in the presence of 50 µM pyrene dodecanoic acid (P12). The cells were collected, the lipids extracted, applied to thin layer chromatography plates and the P-12-ceramide was quantified as described in materials and methods.

Figure 3: Inhibition of subcutaneous growth of MDA-MB-435 tumor in CD1 NUDE mice. A: CD1 NUDE mice were injected intradermally into one flank with 5 × 10^6 MDA-MB-435 cells and next day the mice were injected i.p. with the analog AD2725 near the tumor site with 10 mg/kg of AD2725 in 10% Cremophor, control group mice were injected by Cremophor-ethanol (vehicle). B: MDA-MB-435 cells (5 × 10^6 cells in 0.2 ml medium) was injected i.d. into one flank of mice, when the tumors were palpable after one week; the mice were injected daily i.d. near the tumor site with 10 mg/kg of AD2725 in 10% Cremophor and 10% ethanol. The tumor size was measured twice per week and the tumor volume was calculated from the formula: length × width^2 × π/6. Tumor sizes were evaluated as described in materials and methods.

Figure 4: Survival of CD1 nude female mice injected intravenously with MDA-MB-435 cells followed by intraperitoneal injections and daily water drinking with AD2725. A: MDA-MB-435 cells (5 × 10^6) were injected i.v. and 2 hours later the mice were treated with the analog AD2725. Daily i.p. injections of AD2725 were continued for 23 days, followed by 7 bi-daily injections. Survival of the mice was followed for 7 more weeks without injections. B: MDA-MB-435 cells (5 × 10^6 cells in 0.2ml medium) were injected i.v. into the lateral tail vein and the mice were given AD2725 in the drinking water, starting day 0.

Table 1: Cytokine levels in the plasma from untreated and AD2725 treated mice.

| In vivo treatment of Mice | Number of Mice | Cytokine evaluated | pg/ml Median | pg/ml Range |
|--------------------------|----------------|--------------------|--------------|-------------|
| AD2725                   | 4              | Plasma IL-10       | 10           | 10-660      |
| Vehicle                  | 4              | Plasma IL-10       | 372          | 19-820      |
| AD2725                   | 4              | Plasma IL-12       | 217          | 122-3800    |
| Vehicle                  | 4              | Plasma IL-12       | 15           | 10-1080     |
| AD2725                   | 6              | Plasma IFN gamma   | 115          | 10-240      |
| Vehicle                  | 7              | Plasma IFN gamma   | 10           | 10-185      |

In light of these results, we suggest that administration of ceramide analog AD2725 elevates ceramide in MDA-MB-435 breast cancer cells leading to apoptosis and growth inhibition of tumor. In addition, we propose that ceramide analogs AD2725 can react as immune-modulators and create a shift of Th2 to Th1. Th1 cells and Th1 associated cytokines can create anti-tumor response which leads to a suppression of tumor growth and induces cell death.

Declaration of Interest

The authors report no conflict of interest.

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