DHL-TauZnNa, a newly synthesized α-lipoic acid derivative, induces autophagy in human colorectal cancer cells

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Abstract. In recent years, several antioxidant substances have been found to have an antiproliferative effect on various types of carcinomas. α-lipoic acid (ALA) induces apoptosis in several types of cancer cell lines, but it is difficult to apply α-lipoic acid in clinical use as it is easily oxidized and unstable. Recently, we succeeded in synthesizing the α-lipoic acid derivative sodium N-[6,8-dimercaptooctanoyl]-2-aminoethanesulfonate zinc complex (DHL-TauZnNa), which has highly stable antioxidant effects. We investigated whether DHL-TauZnNa elicits its antiproliferative effects in vivo and in vitro by inducing apoptosis, autophagy or cell cycle arrest, and we analyzed the expression of proteins related to these phenomena and their phosphorylation in HT-29 human colon cancer cells. Subcutaneously administered DHL-TauZnNa treatment applied daily for 41 days significantly inhibited tumor growth by 43% in a xenograft mouse model (P=0.0271). DHL-TauZnNa significantly reduced cell viability over that of controls in the trypan-blue exclusion test in a time- and dose-dependent manner (P<0.05). DHL-TauZnNa increased the proportion of cells in S phase and decreased that of cells in G0/G1 phase in the cell cycle analysis of HT-29 cells. Although DHL-TauZnNa did not increase caspase-3/7 activity and DNA fragmentation in flow cytometry analysis, it increased the expression of microtubule-associated protein light chain 3-II. Autophagosomes and autolysosomes were observed by electron microscopy in the cytoplasm of HT-29 cells treated with DHL-TauZnNa. These results suggest that DHL-TauZnNa inhibited the proliferation of HT-29 cells through the mechanisms of G2/M cell cycle arrest and autophagy but not that of apoptosis. The newly synthesized ALA derivative DHL-TauZnNa may be expected to become a novel cancer therapeutic strategy through its induction of autophagy.

Introduction

Colorectal cancer is the third most frequently diagnosed cancer worldwide, accounting for more than 1,000,000 cases and 600,000 deaths every year (1). 5-fluorouracil (5-FU), oxaliplatin and irinotecan are considered the global standard of chemotherapy for the treatment of colorectal cancer and have contributed to the improvement in overall survival of colorectal cancer patients (2). These medicines have several biological activities: they inhibit DNA synthesis, DNA replication, or growth signals (3).

In recent years, serum reactive oxygen species levels have been reported to be elevated in proportion to tumor invasion and have shown a significant positive correlation with tumor size in colorectal cancer patients (4). Clinical trials to apply antioxidants to cancer therapy have already been carried out (5,6). α-lipoic acid (ALA), vitamin C and curcumin are known as antioxidants with an antiproliferative effect on cancer cells (5-7). As the mechanisms of their antiproliferative effect, induction of apoptosis and activation of mitogen-activated protein kinases (MAPKs) are reported, but there is as yet no consensus as to the true mechanism.

ALA is one of the most popular antioxidants and is the coenzyme of the intramitochondrial enzyme complex in vivo. ALA induces apoptosis in several cancer cell lines but not in normal human colonocytes (5,8,9), and it is difficult to apply ALA in clinical use as it is easily oxidized and unstable. Recently, we succeeded in synthesizing the ALA derivative sodium N-[6,8-dimercaptooctanoyl]-2-aminoethanesulfonate zinc complex (DHL-TauZnNa), which has highly stable antioxidant effects (Fig. 1).

The aim of the present study was to clarify whether DHL-TauZnNa has an antiproliferative effect on colorectal cancer cells and if so, to elucidate the mechanism of this effect.

Materials and methods

Reagents. The ALA derivative DHL-TauZnNa was provided by Oga Research, Inc. (Osaka, Japan) (Fig. 1). ALA was...
purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture. The HT-29 human colorectal cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HT-29 cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd.) to which were added 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution containing 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2.5x10^-4 mg/ml amphotericin B (Gibco-BRL Life Technologies, Rockville, MD, USA) in a humidified atmosphere of 5% CO2 at 37°C.

Animals. Male BALB/c nu/nu mice weighing 18-21 g (Kyudo Co., Ltd., Saga, Japan) were used in all animal experiments. The mice were maintained at 21°C on a 12-h light-dark cycle and given free access to water and standard laboratory chow. This study was approved by the Animal Studies Committee of Oita University, Japan, and was performed according to the National Institutes of Health Standards of Animal Care.

Animal experimental protocol. All mice (n=30) were allowed to acclimate to the unit for 1 week before any manipulations were carried out. Each experimental group consisted of 10 mice. HT-29 tumor cells (3x10^6) suspended in 0.2 ml of sterilized PBS buffer were injected subcutaneously (s.c.) into the back region of each mouse. The mice were then divided into 3 treatment groups 24 h later: the control group, which received 1 mol/l NaCl s.c. once a day for 42 days; the 0.1 mg DHL-TauZnNa group, which received 0.1 mg/kg body weight DHL-TauZnNa s.c. once a day for 42 days; and the 1 mg DHL-TauZnNa group, which received 1.0 mg/kg body weight DHL-TauZnNa s.c. once a day for 42 days. This day was defined as day 1 of the experiment. Tumor size was measured every 2 to 3 days by means of a vernier caliper, and tumor volume was estimated according to the following formula: Tumor volume = π/6 x L x W^2, where L is the greatest dimension of the tumor and W is the dimension of the tumor in the perpendicular direction (10). Animals were sacrificed under anesthesia 42 days after treatments, and blood and tissue samples were harvested for analysis. The tumor tissue samples were stored at -80°C until examination.

Cell viability. Trypan blue exclusion test was performed to examine the effects of DHL-TauZnNa, ALA and ZnCl2 on cell viability of HT-29 cells. DHL-TauZnNa and ALA were diluted in cell culture medium and regulated to final concentrations of 0.25, 0.5 and 1 mM. Cells (1x10^6) cultured in a 25-cm² flask were treated with either ALA or DHL-TauZnNa at different concentrations (0.25, 0.5 or 1 mM) for 24, 48 and 72 h at 37°C in a 5% CO2 incubator. We collected the trypanized cells that adhered to the flask. The cells were washed and resuspended in PBS. We added 10 µl of 0.4% trypan blue stain (Invitrogen, Eugene, OR, USA) to the 10-µl suspension. We placed this mixed liquor on Countess chamber slides (Life Technologies Japan Ltd., Tokyo, Japan) and counted the viable cells with an automated cell counter (Life Technologies Japan) (11).

Analysis of the cell cycle distribution. Cells (1x10^6) were incubated with 0.5 mM DHL-TauZnNa for 48 h, harvested by trypsinization, and fixed with 1% paraformaldehyde followed by 70% ice-cold ethanol. The fixed cells were washed in PBS and added to propidium iodide (PI)/RNase staining buffer (BD Pharmingen, Oxford, UK). The cell cycle distribution was analyzed by a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) according to the manufacturer's instructions (12). Results are displayed as histograms and are expressed as the percentage of cells in each phase of the cell cycle.

Analysis of apoptosis by caspase-3/7 activity. Caspase-3/7 activities of the HT-29 cells and tumors that were treated with ALA or DHL-TauZnNa were detected using a Caspase-Glo® 3/7 Assay kit (Promega Corp. Madison, WI, USA) according to the manufacturer's protocol. Cells (1x10^6) were incubated in a 1.5-ml tube for 24 h at 37°C in 5% CO2 and treated with 0.5 mM DHL-TauZnNa and 0.5 mM ALA for 3, 12 and 24 h. Cells and tumor extracts were added to 100 µl caspase-3/7 reagent and incubated at room temperature in a dark place for 30 min. Fluorescence of the samples was measured with a GloMax® 20/20 Luminometer (Promega Corp.).

Analysis of apoptosis by flow cytometry. Cells (1x10^6) treated with 0.5 mM DHL-TauZnNa were incubated for 6, 24 and 48 h, harvested by trypsinization, and fixed with 1% paraformaldehyde followed by 70% ice-cold ethanol. Apoptosis was analyzed using the APO-BrdU™ kit (Becton-Dickinson) according to the manufacturer's instructions (13). Cells were analyzed using a FACS Calibur flow cytometer (Becton-Dickinson) using at least 10,000 cells/sample. The results are displayed as histograms.

Western blot analysis. Cells were treated in 0.5 mM DHL-TauZnNa for 6 or 24 h, homogenized with Mammalian Protein Extraction Reagents (M-PER®; Pierce Biotechnology, Rockford, IL, USA), and sonicated 3 times on ice for 10 sec each with a 1-min interval between each sonication using a Tomy UD-200 sonicator (Tomy, Tokyo, Japan). Lysates were added to 2-mercaptoethanol and boiled for 5 min at 100°C. Proteins (75 µg/sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 1% non-fat dry milk in Tris-buffered saline (TBS) with 0.1% Tween 20 and incubated with primary antibodies against microtubule-associated protein light chain 3 (LC-3) and β-actin (both from Abcam PLC, Cambridge, UK) for 24 h at 4°C in a refrigerator. The membranes were washed with 0.1%
Tween 20 in TBS and then incubated with anti-mouse and anti-
rabbit secondary antibodies (Immuno-Biological Laboratories
Co., Ltd., Fujioka, Japan) at room temperature for 60 min.
Proteins were visualized with ECL Western blot analysis
detection reagent (GE Healthcare UK Ltd., Buckinghamshire,
UK). Images were analyzed with ImageJ software (NIH
Image, Bethesda, MD, USA).

Electron microscopy. Cells were treated with 0.5 mM
DHL-TauZnNa for 6 or 24 h, and non-treated cells were
immersed in 2.5% glutaraldehyde and 2% paraformaldehyde
in 0.1 M cacodylate buffer, pH 7.4 (Karnovsky's fixative), at
4°C for 2 h and postfixed in cacodylate-buffered 2% osmium
tetroxide and 0.5% potassium ferrocyanide (pH 7.4) at 4°C for
2 h. The cells were then dehydrated in a graded ethanol series
and embedded in epoxy resin. Ultrathin sections (80-85 nm)
were stained with uranyl acetate and lead citrate and examined
with an H-7650 transmission electron microscope (Hitachi,
Tokyo, Japan).

Phosphorylated protein analysis. Cells (1x10⁶) were treated
with 0.5 mM DHL-TauZnNa for 6 or 24 h. Protein lysates
were obtained from samples collected at each time point
using a cell lysis kit (Bio-Rad Laboratories, Hercules, CA,
USA). Phosphorylated extracellular signal-regulated kinase
1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), Akt and p38
MAPK were detected using the Bio-Plex® phosphoprotein
assay (Bio-Rad Laboratories) and the Phosphoprotein Testing
reagent kit (Bio-Rad Laboratories) according to the manufac-
turer's instructions (14). The proteins were analyzed with the
Bio-Plex® Suspension Array system (Luminex 100, Bio-Rad
Phosphoprotein Testing reagent kit).

Statistical analysis. We conducted at least three or more inde-
pendent experiments for all experiments. Data are shown as
means ± standard deviation (SD). Data were analyzed by the
Mann-Whitney U test for single comparisons and by analysis
of variance (ANOVA) followed by Scheffé's post hoc test for
group pairs for multiple comparisons. A value of P<0.05 was
considered to be statistically significant. StatView Version 5.0
(Abacus Concepts Inc., Berkeley, CA, USA) was used for all
statistical analyses.

Results

Examination of the tumor growth of the implanted HT-29
colon cancer cells. We initially evaluated the effects of
DHL-TauZnNa administration (0.1 and 1.0 mg/kg body
weight) on tumor growth in a xenograft model in which
HT-29 cells were injected into the subcutis of nude mice.
On day 42 of the experiment, the control group had a mean
tumor volume of 3001 mm³, whereas the 0.1 mg and 1.0 mg/
kg body weight DHL-TauZnNa groups had mean tumor
volumes of 1278 and 1680 mm³, respectively. Subcutaneously
administered DHL-TauZnNa (0.1 mg/kg body weight) applied
daily for 42 days significantly inhibited tumor growth by
43% (P=0.0271) (Fig. 2A). All mice completed the treatment
period, and there were no deaths during the treatment.

Examination of the cell viability of HT-29 colon cancer
cells. DHL-TauZnNa at concentrations of 0.25, 0.5 and 1 mM
significantly reduced cell viability when compared with the
cell viability of the controls as detected by the trypan-blue
exclusion test at 48 and 72 h, respectively (P<0.05) (Fig. 2B).
Both ALA and DHL-TauZnNa reduced cell viability in a
dose- and time-dependent manner. The cytotoxic effect of
DHL-TauZnNa was stronger than that of ALA at the concen-
tration of 0.5 mM at 72 h (P<0.05). There were no statistically
significant differences in the antiproliferative effect or cyto-
toxic effect at concentrations of 0.25 and 1 mM.

Analysis of the cell cycle distribution. Cell cycle analysis by
flow cytometry showed an accumulation of HT-29 cells in
the G2/M phase after exposure to 0.5 mM of DHL-TauZnNa.
The percentages of cells in the G0/G1 and S phases following
treatment with DHL-TauZnNa were significantly decreased to
a greater extent when compared with these percentages in the
control cells (43.7±0.90 vs. 66.9±3.87%, P<0.0001; 17.3±3.04
vs. 23.4±2.15%, P<0.017, respectively), and the percentage
of cells in the G2/M phase was significantly increased to a greater
Analysis of apoptosis. Caspase-3/7 activity of the HT-29 cells following treatment with 0.5 mM DHL-TauZnNa was significantly lower than that of the control at 3, 12 and 24 h (each, P<0.05), whereas caspase-3/7 activity of the HT-29 cells treated with 0.5 mM ALA at 3 and 24 h was significantly higher than that of the control. In particular, the caspase-3/7 activity level of ALA at 24 h was ~1.7 times that of the control (P<0.05) (Fig. 4A). Analysis of DNA fragmentation by flow cytometry did not reveal evidence of apoptosis after exposure to 0.5 mM of DHL-TauZnNa (Fig. 4B).

Conversion of LC-3-I to LC-3-II. Treatment of DHL-TauZnNa for 6 and 24 h more significantly increased the expression of LC-3-II than that of LC-3-I, indicating that DHL-TauZnNa converted LC-3-I to LC-3-II in HT-29 cells (P<0.05). A significant increase in the expression of LC-3-II over that of LC-3-I was not shown in the control cells (non-treatment with DHL-TauZnNa) (Fig. 5).

Electron microscopy. HT-29 cells either treated with 0.5 mM DHL-TauZnNa or untreated were observed using electron microscopy. A large number of HT-29 cells with loss of membrane integrity and cells with autophagosomes or autolysosomes in the cytoplasm were observed after treatment with 0.5 mM DHL-TauZnNa for 6, 24 or 48 h as detected by flow cytometric analysis. Data are expressed as the mean of four independent experiments. (P<0.05 vs. control; error bars indicate SD).

Analysis of phosphorylated proteins associated with the MAPK cascade and phosphorylated p53 protein. Levels of...
ERK1/2 and p53 phosphorylated proteins were significantly increased to a greater extent in the HT-29 cells treated with 0.5 mM DHL-TauZnNa when compared with these levels in the control cells at 6 and 24 h (both, P<0.05). Furthermore, levels of JNK and p38 MAPK were significantly increased to a greater extent than levels in the control cells at 24 h (both, P<0.05). Phosphorylated Akt levels were not changed compared with those in the control at 6 and 24 h (Fig. 7).

Discussion

The present study is the first report of the anticancer action of DHL-TauZnNa, a newly synthesized ALA derivative, on HT-29 human colorectal cancer cells both in vitro and in vivo. Our results indicated that DHL-TauZnNa had both cytotoxic and antiproliferative effects on colorectal cancer cells, resulting in cell death and G2/M-phase cell cycle arrest. DHL-TauZnNa did not increase caspase-3/7 activity or DNA fragmentation, suggesting that DHL-TauZnNa did not induce apoptosis, unlike the action of ALA, but resulted in non-apoptotic cell death, autophagy, characterized by the appearance of cells with autophagic vacuoles as detected by electron microscopy, and elevation of LC3-II expression. In the future, DHL-TauZnNa may be a useful anticancer drug.

DHL-TauZnNa was derived by coupling ALA and taurine and then binding zinc to the thiol group. Although ALA is prone to oxidation, polymerization, and desulfurization and is hard to dissolve in water, DHL-TauZnNa is stable, difficult to polymerize and desulfurize and is water soluble. Furthermore, DHL-TauZnNa had potent hydroxyl radical scavenging activity greater than that of ALA in our study (data not shown).

The present study demonstrated that DHL-TauZnNa inhibited the proliferation of HT-29 cells by inducing autophagy but not apoptosis. Autophagic cell death has been considered a primary mechanism for tumor suppression (15). Chang et al (16) reported that cancer cell death was induced through activation of autophagy instead of apoptosis in vitro and showed antiproliferative effects in a xenografted mouse model. The most striking evidence for pro-autophagic chemotherapy to overcome resistance to apoptosis in cancer cells comes from the use of temozolomide, a pro-autophagic cytotoxic drug that has demonstrated therapeutic benefits in glioblastoma.

Figure 6. Electron microscopic examination. Electron microscopy evaluation of HT-29 cells after the treatment of (A and B) 0.5 mM DHL-TauZnNa or (C) non-treated cells. White arrows indicate autophagosomes, and white arrowheads indicate autolysosomes. NE, necrotic cell; Atp, autophagic cell.
patients and is in clinical trials for several types of apoptosis-resistant cancers (17). In addition, Ullman et al (18) reported that autophagy activates necrosis in apoptosis-deficient mouse embryonic fibroblasts. Gamrekelashvili et al (19) reported that necrosis may inhibit cancer recurrence by acquiring tolerance without inducing tumor immunity such as apoptosis. In our study, DHL-TauZnNa showed an antiproliferative effect associated with autophagy in HT-29 cells, which exhibit resistance to apoptosis and chemoresistance (20), suggesting that DHL-TauZnNa may be a new therapeutic strategy against cancer cells with resistance to apoptosis. In the present study, DHL-TauZnNa induced G2/M-phase cell cycle arrest. These actions are very important in cancer therapy since it is commonly known that G2/M arrest increases radiation sensitivity, and furthermore, it has been reported that autophagy also enhances radiation sensitivity (21). The combination of radiotherapy and DHL-TauZnNa may result in increased antiproliferative activity.

There are a variety of reports on the association of MAPKs and the induction of autophagy. ERK1/2, JNK and p38 MAPK are reported to be associated with inducing or inhibiting autophagy (22-24). Activation of ERK1/2 and/or JNK induces autophagic cell death (25,26). In HT-29 human colon cancer cells, ERK1/2 induces autophagy (27), and activation of JNK is associated with autophagy-mediated cell death (28). Silibinin, an antioxidant, induces autophagic cell death in human fibrosarcoma HT1080 cells via reactive oxygen species-p38 and c-Jun N-terminal kinase pathways (24). Phosphorylated proteins of the MAPK cascade, JNK, p38MAPK and ERK1/2, were enhanced by DHL-TauZnNa in the present study, and levels of both phosphorylated ERK1/2 and LC-3-II were increased following treatment with DHL-TauZnNa at 6 and 24 h. Therefore, phosphorylation of ERK1/2 may be a trigger in a mechanism of autophagic cell death induced by DHL-TauZnNa, a strong antioxidant.

DHL-TauZnNa did not induce apoptosis but produced autophagy. Majewski et al (29) reported that phosphorylated Akt inhibits caspase activity, resulting in the inhibition of apoptosis, whereas inhibition of the phosphorylation of Akt may increase autophagy (30). In our study, DHL-TauZnNa did not significantly alter the phosphorylation of Akt in HT-29 cells. Inhibition of apoptosis or progression of autophagy in the present study may not have been related to Akt. This study analyzed only the effect on HT-29 cells, and further study is necessary to clarify these issues.

In conclusion, induction of autophagy, but not apoptosis, by DHL-TauZnNa inhibited the proliferation of HT-29 cells in vitro and in vivo. This mechanism was associated with an increase in phosphorylated MAPKs, ERK1/2, JNK and p38. Elucidation of the mechanism of autophagic cell death initiation and knowledge of the pharmacokinetics and side effects of DHL-TauZnNa are necessary before DHL-TauZnNa can be used in the clinical setting in the future. Newly synthesized ALA derivative DHL-TauZnNa may be expected to become a novel cancer therapeutic strategy through its induction of autophagy.

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