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Cleavage mechanism and anti-tumor activity of 3,6-epidioxy-1,10-bisaboladiene isolated from edible wild plants

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ABSTRACT

A bisabolane sesquiterpene endoperoxide compound, 3,6-epidioxy-1,10-bisaboladiene (EDBD), was isolated from edible wild plants grown in the northern area of Japan, *Cacalia delphiniifolia* and *Cacalia hastata*, using a mutant yeast (*cdc2-1 rad9*Δ). It showed cytotoxicity at IC_{50} = 3.4 μM and induced apoptosis against the human promyelocytic leukemia cell line HL60 through a new stable rearrangement product (1) when in the presence of $FeSO_4$. This conversion mechanism is different from another sesquiterpene endoperoxide lactone compound, dihydroartemisinin (DHA), which is an anti-malarial drug. The cytotoxicity of EDBD decreased in the presence of the ferrous ion chelating drug deferoxamine mesylate (DFOM), and this suggested that the structural change of the drug caused by Fe^{2+} may be responsible for its biological activities. EDBD induced apoptosis via phosphorylation of p38 mitogen-activated protein kinase (MAPK) in HL60 cells, and was detected by Western blot. EDBD resulted in an immediate increase in DCF fluorescence intensity in HL60 cells using DCFH-DA (2′,7′-dichlorofluorescin diacetate) assay. The in vitro reaction of EDBD with $FeSO_4$ also increased DCF fluorescence intensity in a dose dependent manner. These results showed that the biological activity of EDBD involves an unstable carbon-centered radical intermediate. Furthermore, there was no similarity between the JFCR39 fingerprints of EDBD and DHA (correlation coefficient on COMPARE Analysis γ = 0.158). EDBD showed anti-tumor effects against a xenograft of Lox-IMVI cells in vivo.

1. Introduction

Numerous natural compounds with anti-tumor activity for clinical use are expected to be discovered using the combination of unique natural sources and bioassay methods. Drugs derived from natural origins are vitally important in many treatment protocols, such as anthracyclines from microorganisms, vinca alkaloid and taxanes from plants, etc.[1–3] Although a potent and unique anthracycline (barminomycin, which shows 1000 times higher activity than adriamycin or daunomycin) was isolated from Actinomycetes,[4] most of those drugs have substantial side effects when administered to patients. Intensive research on low molecular weight compounds that target cell cycle regulatory proteins has led to the identification of many candidate compounds that are able to arrest proliferation and induce apoptosis in neoplastic cells, especially by re-activation and/or activation of the cell death pathway downstream of the defect.[5]

Recently, we have isolated a bisabolane sesquiterpene endoperoxide, 3,6-epidioxy-1,10-bisaboladiene (EDBD) from one of the edible wild plants in the northern area of Japan, *Cacalia delphiniifolia* (the Japanese name is Momijigasa) using a unique biotechnological assay.[6,7] Briefly, the Rad9 protein is a key adaptor protein in DNA damage checkpoint pathways of *Saccharomyces cerevisiae* and the mutant strain (*cdc2-1 rad9*Δ) can not grow at 28 °C after incubation at 37 °C for 6 h because the DNA damage checkpoint pathways is abnormal. However, when the strain is incubated with a modulator of cell cycle such as the clinical drugs hydroxyurea (Fig. 1A)[8] or mycophenolic acid (Fig. 1B)[9] at 37 °C, it can grow at 28 °C as normal and showed a growth zone of the mutant strain

Abbreviations: EDBD, 3,6-epidioxy-1,10-bisaboladiene; ART, artemisinin; DHA, dihydroartemisinin; DFOM, deferoxamine mesylate; γ, correlation coefficient on COMPARE Analysis; PDA, Photo Diode Array; MAPK, mitogen-activated protein kinase; CDDP, *cis*-diamminedichloroplatinum (II) (cisplatin); MPA, mycophenolic acid; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; HE, dihydroethidium.

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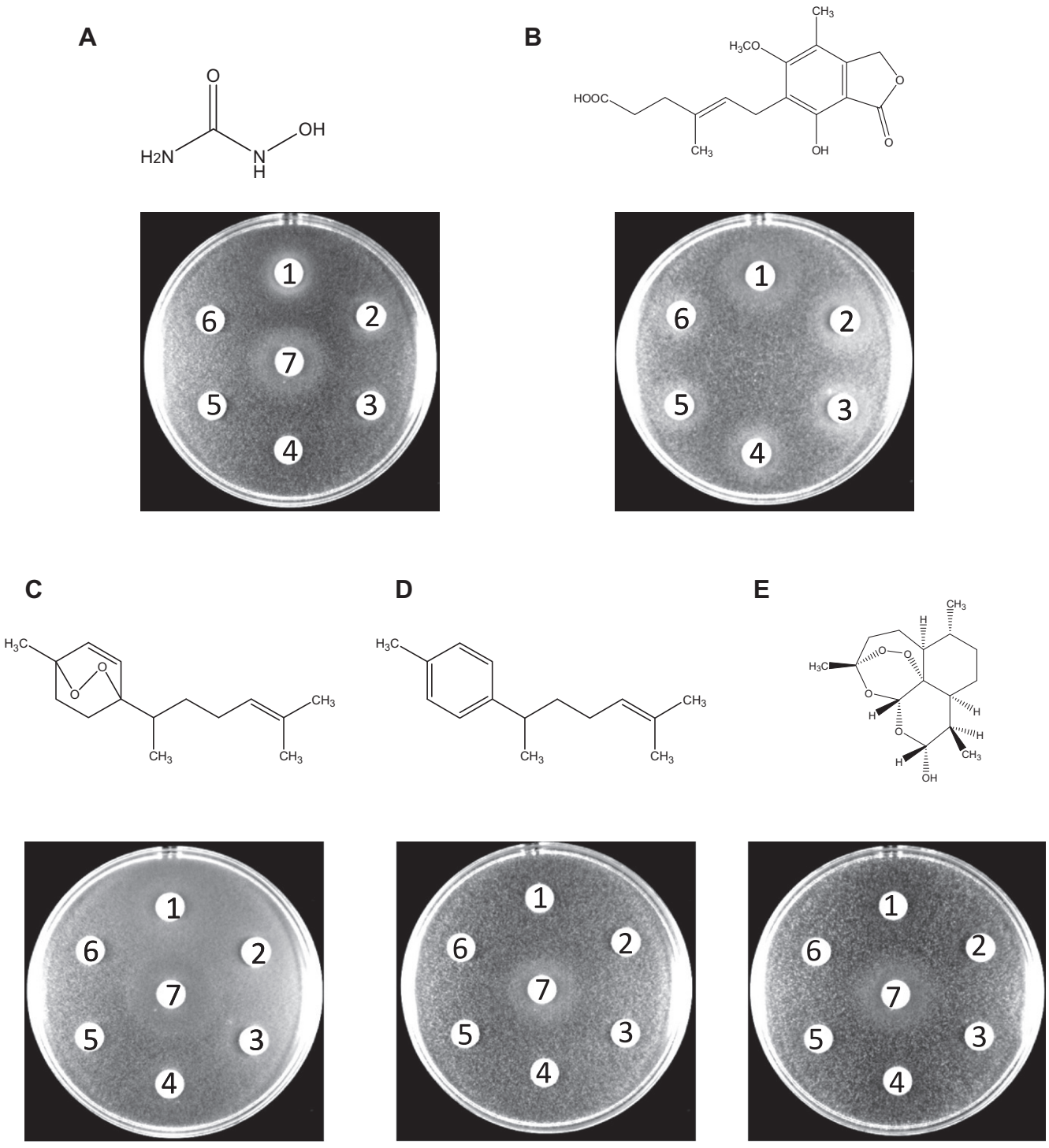

Figure 1. Growth restored activity of hydroxyurea, mycophenolic acid, 3,6-epidioxy-1,10-bisaboladiene (EDBD), α-curcumene and dihydroartemisinin (DHA) against the mutant yeast strain (*cdc2-1 rad9*Δ). Hydroxyurea (A) 1: 10 mg/disc, 2: 5 mg/disc, 3: 2.5 mg/disc, 4: 1.25 mg/disc, 5: 0.63 mg/disc, 6: 0.31 mg/disc, 7: Mycophenolic acid (50 μg/disc). Mycophenolic acid (B) 1: 100 μg/disc, 2: 50 μg/disc, 3: 25 μg/disc, 4: 12.5 μg/disc, 5: 6.3 μg/disc, 6: 3.1 μg/disc. EDBD (C) 1: 4 μg/disc, 2: 2 μg/disc, 3: 1 μg/disc, 4: 0.5 μg /disc, 5: 0.25 μg/disc, 6: 0.13 μg/disc, 7: Mycophenolic acid (50 μg/disc). α-Curcumene (D) and DHA (E) 1: 400 μg/disc, 2: 200 μg/disc, 3: 100 μg/disc, 4: 50 μg /disc, 5: 25 μg/disc, 6: 12.5 μg/disc, 7: Mycophenolic acid (50 μg/disc).

around a disc containing active compounds.[7] Using this system, we have already isolated borrelidin[10] and fredericamycin A[11] from microorganisms and investigated their molecular mechanisms in *S. cerevisiae*.

EDBD (Fig. 1C) showed more potent activity against the mutant yeast than hydroxyurea and mycophenolic acid and the phenotype was also different (Fig. 1A–C). EDBD showed cytotoxicity against human chronic myelogenous leukemia K562 and human prostate carcinoma LNCaP cell lines with IC_{50} values of 9.1 and 23.4 μM, respectively.[6] DNA fragmentation and condensation of chromatin, the hallmarks of apoptosis, appeared in K562 cells after an 18 h treatment with EDBD.[6] α-Curcumene[12] (Fig. 1D), a bisabolane sesquiterpene that lacks the endoperoxide moiety of EDBD, also showed cytotoxicity against both K562 and LNCaP cell lines at over

10-times higher dose than that of EDBD. It indicated that the endoperoxide structure within EDBD is important for its anti-tumor activity in vitro.[6]

Artemisinin (ART) and its derivatives are sesquiterpene lactone endoperoxides found in the Chinese medicinal plant *Artemisia annua* and are widely used as anti-malarial drugs.[13,14] They have also been reported to inhibit the growth of a set of tumor cells such as Ehrlich ascites tumor cells,[15] 55 cancer cell lines[16] and at present are under clinical trials for several tumors.[17] Dihydroartemisinin (DHA), the main and potent semi-synthetic ART derivative, has been reported as having an endoperoxide bridge that was essential for the cytotoxicity, and that carbon-centered radicals were involved in apoptotic cell death by DHA.[18,19] DHA induces apoptosis through the dependence of iron and p38 MAPK in human promyelocytic leukemia HL-60 cells.[20] Although it has been reported that human fortilin is a molecular target of DHA,[21] the mechanism of action is ambiguous. Thus, EDBD with an endoperoxide is a unique sesquiterpene compound, because DHA has no activity against the mutant yeast (Fig. 1E).

We describe in this report the anti-tumor mechanism of EDBD against HL60 cells compared with DHA. We propose a reaction mechanism of EDBD against cancer cells which is different from that of DHA with respect to the structure of the radical intermediate, its conversion time and the COMPARE Analysis. Additionally, EDBD also had anti-tumor activity against the melanoma cell line, Lox-IMVI in nude mice.

2. Materials and methods

2.1. Strains, medium and reagents

The *S. cerevisiae* strain used was disrupted in the *RAD9* gene in *cdc2-1* mutant background by replacing the *RAD9* ORF with the *URA3* gene by PCR-directed one-step gene disruption (WCTR312A, *MATa ade2 his3 trp1 ura3 rad9D::URA3 cdc2-1*).[7] Hydroxyurea was purchased from MP Biomedicals LLC, Irvine, USA. Mycophenolic acid, RNase A and proteinase K were purchased from Wako Pure Chemical Co., Ltd, Osaka, Japan. α-Curcumene was isolated from the essential oil of ginger (Seikatsunoki Co., Tokyo, Japan).[6] DHA was purchased from LKT Lab. Inc., St. Paul, USA. YPD broth, YPD agar and MAPK activation sampler kit (antibodies of p38α and p38MAPK (pT180/pY182)) were purchased from Becton, Dickinson and Company, Sparks, USA. Hoechst 33342, agarose and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Dojindo Laboratories, Kumamoto, Japan. Buffer concentrate Titrisol (pH 3–10) were purchased from Merck (Calbiochem), Darmstadt, Germany. TBE (Tris-Borate-EDTA) was purchased from Takara Bio Inc., Shiga, Japan. Paper discs (8 mm) were purchased from Toyo Roshi Kaisya, Ltd, Tokyo, Japan. RPMI 1640 medium, penicillin and streptomycin were purchased from Gibco, Invitrogen Corp., Carlsbad, USA. Fetal bovine serum was purchased from Bio West Co. Ltd, Vancouver, Canada. MAPK inhibitors SB203580 was purchased from Enzo Life Sciences Inc., NY, USA, SP600125 from Jena Bioscience GmbH, Jena, Germany and PD98059 from Cayman Chemical Company, Ann Arbor, USA. DCFH-DA (2′,7′-dichlorofluorescin diacetate), HE (dihydroethidium) and radical scavengers (*N*-acetyl-L-cysteine, vitamin E (α-tocopherol) and allopurinol) were purchased from Sigma Aldrich Corp., St. Liouis, USA. Unless otherwise stated, chemicals used in this study were of the best grade commercially available.

2.2. Isolation of EDBD

EDBD was isolated from *Cacalia delphiniifolia* using a previously reported protocol.[6] EDBD (205.7 mg) was isolated from fresh *Cacalia delphiniifolia* (6,435 g). We also isolated EDBD from *Cacalia hastata* (the Japanese name is Yobusumasou), another edible wild plant in northern Japan, by the same method. EDBD (83.9 mg) was obtained as a colorless oil from 4,846 g of fresh *Cacalia hastata*.

2.3. Screening procedure

Screening was performed with a slight modification of the literature method.[7] The cultured broth of the mutant strain (1.75 ml of A_{590} = 0.8) was added to 48.25 ml YPD agar medium and it was suspended at 50 °C, followed by spreading on each 4 plate (8 cm diameter). Samples to be tested were applied to a 8 mm paper disc (40 μl/disc) and the disc was placed on the surface of the agar. The plates were incubated at 37 °C for 6 h and then at 28 °C for 2 days. The modulated activity of the DNA damage checkpoint pathways, including cell cycle blocker, was determined by the diameter of growth zone of the yeast compared to that of a clinical drug, mycophenolic acid (50 μg/disc).

2.4. Cell culture and cytotoxicity

HL60 cells (RCB0041, RIKEN BioResource Center, Tsukuba, Japan) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin (50 units/ml)-streptomycin (50 μg/ml) in a humidified atmosphere at 37 °C under 5% CO_2 for 48 h. The cytotoxicity of the compounds was examined by MTT assay, as described elsewhere.[22]

2.5. Microscopic observations

HL60 cells were seeded in a 60 mm dish at a density of 5×10^5 cells/ml. HL60 cells were treated with 10 μg/ml of Hoechst 33342 for 10 min at 37 °C. Nuclear morphology was observed under a fluorescence microscope (excitation/emission wavelength = 365/420 nm, BX51, Olympus Corp., Tokyo, Japan).[22]

2.6. Gel electrophoresis

HL60 cells were seeded in a 30 mm dish at a density of 5×10^5 cells/ml. The cells were harvested and washed with PBS. Cell pellets were resuspended in lysis buffer (100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 0.2% SDS and 0.1% RNase A), and incubated for 60 min at 50 °C. The lysates were then incubated for an additional 60 min at 50 °C with 1 mg/ml of proteinase K, and phenol:chloroform:isoamyl alcohol (25:24:1)-extracted DNA samples were then subjected to electrophoresis on a 2% agarose gel in TBE buffer for 2 h at 50 V. After electrophoresis, the gel was stained with 10 μg/ml of ethidium bromide and photographed.[22] MAPK inhibitors and radical scavengers were added to the cell culture at 1 h before exposure to EDBD.

2.7. Caspase activity

HL60 cells were seeded in a 96-well plate at a density of 2×10^5 cells/ml. The cells were treated with the compounds and the activity of caspase 3/7, 8 and 9 were detected using the Caspase-Glo® 3/7, 8 and 9 assay kits (Promega Corp., Madison, USA) according to the manufacturer's procedures. Caspase activities were determined using a Powerscan HT spectrophotometer (Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan).[22]

2.8. Western blotting

Compound-treated cells were harvested, washed with ice-cold PBS, and lysed by incubating at 4 °C for 30 min in lysis buffer (10 mM Tris–HCl (pH 7.5), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA) containing protease

inhibitor cocktail Complete Mini (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor cocktail (Wako Pure Chemical Industries, Osaka, Japan). After centrifugation (15,000×*g* for 30 min at 4 °C), supernatant protein concentrations were determined using a BCA kit (Pierce, Waltham, USA) and samples containing 20 μg total protein were loaded on 12% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred onto a PVDF membrane (Millipore, Billerica, USA) and blocking was performed in Tris-buffered saline (TBS) containing 1% polyvinylpyrrolidone and 0.1% Tween 20. Blots were then incubated with anti-mouse p38α (1:5,000) or phospho-specific anti-mouse p38 MAPK (1:2,000) for 1 h at room temperature. After washing membranes with 0.1% Tween 20 in TBS, each blot was incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:10,000) (Upstate and Cell Signaling Technology, Danvers, USA) for 1 h at room temperature. Peroxidase activity was detected by the ECL™ system (GE Healthcare, Little Chalfont, England).

2.9. Reaction of EDBD with $FeSO_4$

The effects of various pH and $FeSO_4$ concentrations on the reaction of Fe^{2+} and EDBD were examined. EDBD stock solution was prepared in MeOH (10 mg/ml) and stock solutions of $FeSO_4$ were freshly prepared in water (100 mM). The typical reaction consisted of $FeSO_4$ (3 mM), EDBD (500 μg) in each 0.1 M of pH buffer (final volume 0.1 ml) for pH dependence experiment and 0.1 M buffer (pH 8), EDBD (500 μg) in each $FeSO_4$ concentration (final volume 0.1 ml) for $FeSO_4$ dose-dependence experiment at 37 °C. New products produced by the reaction were analyzed using HPLC (Capcell pak (4.6 mm i.d. × 150 mm, Shiseido Co. Ltd, Tokyo, Japan), 75% MeOH for EDBD, 87.5% MeOH for α-curcumene or 60% MeOH for DHA, 1 ml/min, Photo Diode Array (PDA) system (JASCO 880-PU and MD-910, Shimadzu Co. Ltd, Kyoto, Japan)).

2.10. Isolation of a new stable rearrangement product (1) from EDBD reaction with Fe^{2+}

EDBD (10 mg) and 25 mM $FeSO_4$ were mixed in 2 ml of 0.1 M buffer (pH 8) and **1** was purified by reverse-phase HPLC (20 mm i.d. × 250 mm; Shiseido Co. Ltd, Tokyo, Japan, 70% MeOH, 10 ml/min). Extraction of **1** using hexane yielded a pure compound (3.4 mg).

2.11. Structure elucidation of a new stable rearrangement product (1)

Structure elucidation of **1** was performed by instrumental analyses using HR-EI-MS (JEOL JMS700) and NMR spectra (JEOL ECA600), including ^{1}H NMR, ^{13}C NMR, DEPT, DQF-COSY, HSQC-TOCSY and HMBC.

2.12. Cytotoxicity in the presence of DFOM

Cytotoxicity of EDBD, α-curcumene and DHA in the presence of DFOM against HL60 cells was measured with 5 μM DFOM as described in the literature.[23]

2.13. Cytotoxicity in the presence of Fe^{2+} or Fe^{3+}

HL60 cells (100 μl of 1×10^5 cells/ml) were cultured with various concentrations of $FeSO_4$ or $Fe_2(SO_4)_3$ for 3 h, following added EDBD (final concentration 2 μM in MeOH) and incubated for 2 days. The cytotoxicity of EDBD was examined by MTT assay, as described elsewhere.[22]

2.14. Measurement of ROS (an unstable carbon-centered radical intermediate) (DCFH-DA or HE assay)

HL60 cells (198 μl of 6.5×10^5 cells/ml) and EDBD (final concentration 10 μM in MeOH) were added to 96-well white plate following incubation for the indicated times. DCFH-DA or HE was dissolved in DMSO, and 10 μM DCFH-DA or 5 μM HE (final concentration) was added to wells 30 min prior to measurement (excitation/emission wavelength = 485/530 nm for DCF and excitation/emission wavelength = 530/590 nm for HE) using a Powerscan HT spectrophotometer (Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan).[24] DCFH (10 μM) was prepared from DCFH-DA and reacted with EDBD (125 μg/ml) and $FeSO_4$.[25]

2.15. COMPARE Analysis

A panel of 39 human cancer cell lines, known as JFCR39, was used as described previously.[26–29] All the cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in humidified air containing 5% CO_2.

2.16. Anti-tumor effect in vivo

Female nude mice, 6-week old, were purchased from Charles River Breeding Laboratories, Yokohama, Japan and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. Lox-IMVI cells (8×10^6 cells) were trypsinized and resuspended in 0.3 ml of 10% FBS-DMEM and then combined with 0.5 ml of growth-factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA). One hundred microliters of the cell suspension (1×10^6 cells) were injected subcutaneously in the left lateral flank of mice as described previously.[30] Five mice were used for each experiment set. EDBD and CDDP (cisplatin) were dissolved in saline and injected intravenously (iv) at 25 mg/kg on days 1, 6, 11, 16 or 5 mg/kg on days 1, 8, 15 respectively after tumor inoculation. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × width^2)/2. After the indicated times, tumors were surgically dissected.[30]

2.17. Statistical analysis

Data are means ± S.D. Differences in mean values among groups was assessed using the Student's *t*-test. The level of significance was set at P <0.05, 0.01 and 0.001 for Figures 5, 6B and S5.

3. Results

3.1. Restored growth activity of EDBD, α-curcumene and DHA against the mutant yeast

Clinical drugs having anti-tumor activity, hydroxyurea and mycophenolic acid showed a growth zone in the temperature-sensitive mutant yeast strain (*cdc2-1 rad9*Δ) in a dose dependent manner under the conditions for the induction of DNA damage (37 °C incubation for 6 h)[7] (Fig. 1A and B). EDBD showed a different growth zone in the mutant yeast in a dose dependent manner (Fig. 1C). The diameter of the growth zones was 29.0, 24.1, 19.0 and 0 mm (at 4, 2, 1 and 0.5 μg/disc, respectively) compared to those of hydroxyurea and mycophenolic acid (14.3 and 18.5 mm at 10 and 0.05 mg/disc, respectively). This activity was about 50 times greater than that of mycophenolic acid and showed a fuzzy phenotype (diffusion) of the growth zone. On the other hand, the related sesquiterpenes, α-curcumene (without endoperoxide) and DHA (with endoperoxide) did not show a growth zone, even at

400 μg/disc (Fig. 1D and E). This indicates that EDBD is a potent compound, and that mechanism of EDBD is different from that of α-curcumene and DHA.

3.2. Cytotoxicity of EDBD, α-curcumene and DHA against HL60 cells

EDBD, α-curcumene and DHA showed cytotoxicity against HL60 cells (IC_{50} = 3.4, 108.6 and 0.26 μM) at 48 h and EDBD has comparable activity to DHA against cancer cells.[6] To characterize the cell death induced by EDBD, we examined the nuclear morphology of cells by using the fluorescent DNA-binding agent, Hoechst 33342. After 18 h of treatment with 2.5 μg/ml (10.6 μM) of EDBD, tumor cells clearly exhibited condensed and fragmented nuclei, indicative of apoptotic cell death (Fig. 2Ab). Altered nuclei morphology was not evident in control cells treated with MeOH (Fig. 2Aa).

The treatment of EDBD to HL60 cells caused DNA fragmentation in a dose and time dependent manner (Figs. 2Ba and Bb). EDBD, α-curcumene and DHA activated caspases 3/7, 8 and 9 after 3 h, 1 h and 12 h, respectively (Fig. 2C). EDBD and DHA also activated caspases 3/7, 8 and 9 in a dose dependent manner (Supplementary Fig. S1).

3.3. Effect of EDBD on the MAPK pathway

To explore whether the EDBD-induced DNA fragmentation was inhibited by MAPK inhibitors, three kinds of inhibitors were used. Of these, only SB203580 (p38 MAPK inhibitor) inhibited DNA fragmentation by EDBD against HL60 cells, and SP600125 (JNK inhibitor) and PD98059 (ERK inhibitor) had no effect (Fig. 3A). Thus, we examined the protein level of p38 and its phosphorylation (activation) in HL60 cells by Western blot, because it is involved in apoptosis. EDBD increased phospho-pT180/pY182 p38 from 5 μM (Fig. 3Ba) and after 3 h of treatment with 10 μM EDBD (Fig. 3Bb).

3.4. Dependence of pH and $FeSO_4$ to the conversion of EDBD to a new stable rearrangement product (1)

Ferrous ion is able to cleave the peroxide bond in DHA by reductive scission to form oxy-radicals.[19,31] The related compound of

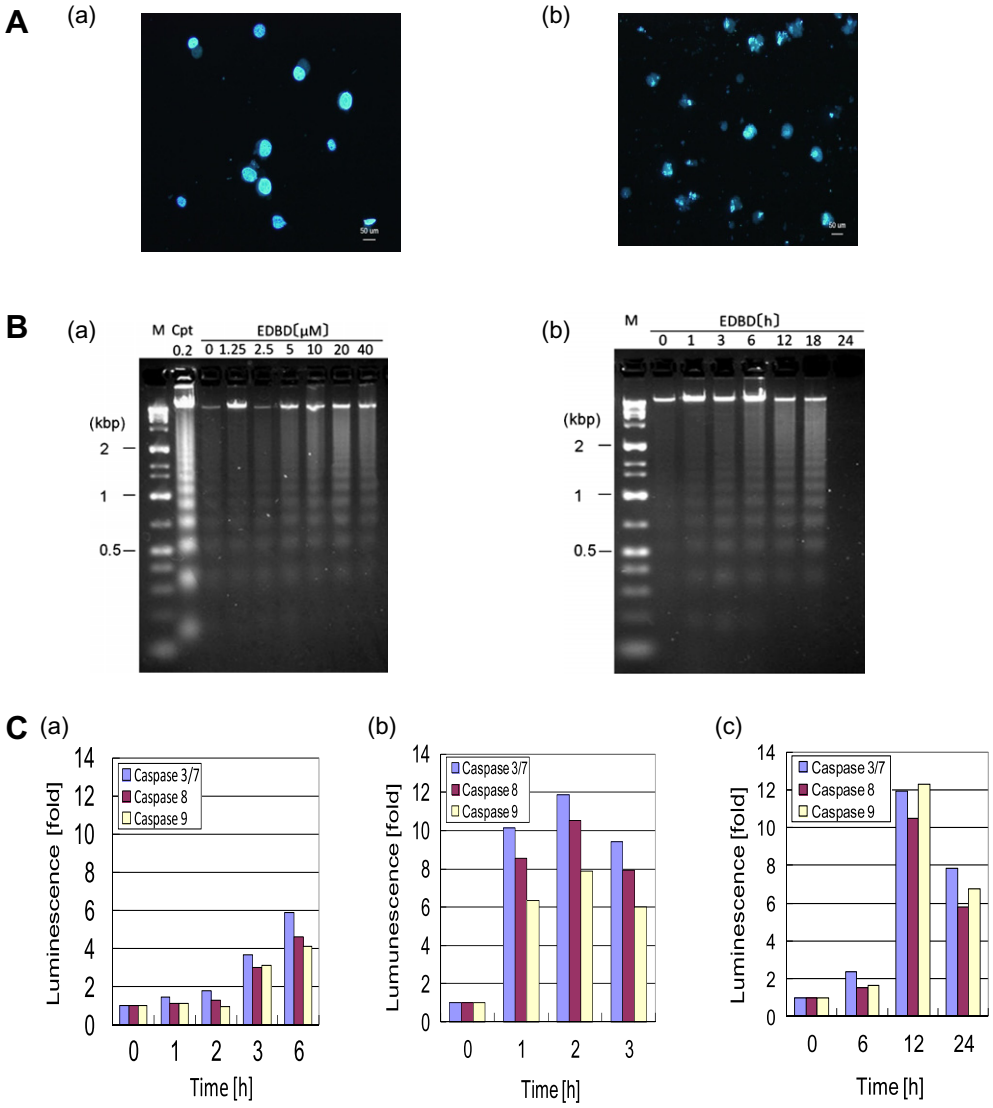

Figure 2. Nuclear morphology, DNA fragmentation and caspase activation of HL60 cells. (A) Microscopic observation of HL60 cells treated with MeOH (a) and EDBD (b). HL60 cells were incubated in the presence of methanol (1%), EDBD (10 μg/ml) as described in the methods. After incubating at 37 °C for 18 h, the cells were collected by centrifugation, washed with PBS, and stained with 10 μg/ml of Hoechst 33342 for 10 min at 37 °C. Bar, 50 μm. (B) Gel electrophoresis of the DNA fragmentation activity by EDBD showing the dependence on dose (a) and time (b). M: DNA marker, Cpt: 0.2 μM camptothecin. C: Time-dependent activation of caspases by 20 μM EDBD (a), 200 μM α-curcumene (b) and 1 μM DHA (c) in HL60 cells.

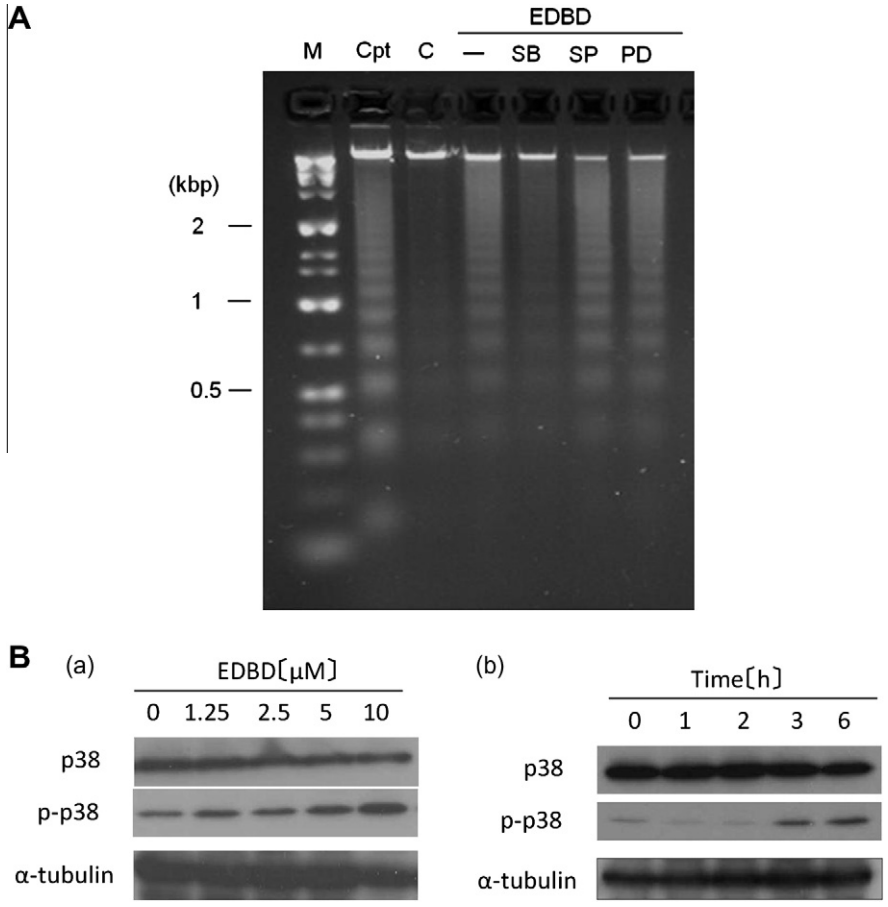

Figure 3. Effect of EDBD on the MAPK pathway in HL60 cells. (A) Effects of different MAPK inhibitors on EDBD-induced DNA fragmentation in HL60 cells. Cells were preincubated with 20 μM SB203580, 5 μM SP600125 or 10 μM PD98059 for 1 h prior to the addition of 10 μM EDBD and following incubated for 6 h. M: DNA marker, Cpt: 0.2 μM camptothecin, (C) control (MeOH and DMSO). (B) Western blot analyses for total protein of p38 and its phosphorylation (p-p38). Cells were treated with EDBD at the indicated concentrations for 6 h (a), and for indicated times with 10 μM EDBD (b). α-Tubulin was used as a loading standard.

EDBD, monoterpene endoperoxide ascaridole, which contains the peroxide bond, was also cleaved by Fe^{2+}.[32–34] There was a higher conversion rate of EDBD at pH 8 and 9 (Fig. 4Aa), and at $FeSO_4$ concentrations above 25 mM (Fig. 4Ab). EDBD was immediately converted to **1** (retention time: 7.1 min) in the presence of 25 mM $FeSO_4$ at pH 8 (Fig. 4Ba), but DHA was converted more slowly under the same conditions (Fig. 4Bc).[19] Both rates of conversion were parallel to the time course of caspase activation (Fig. 2C). α-Curcumene, without an endoperoxide moiety, could not be converted, but decreased under the same condition over 24 h (Fig. 4Bb).

3.5. Structure of a new stable rearrangement product (1)

A new HPLC peak (a new stable rearrangement product (**1**)) (retention time: 7.1 min, Fig. 4Ba) produced by the $FeSO_4$ reaction with EDBD was isolated as a colorless oil and was able to be dissolved in MeOH, ethyl acetate or $CHCl_3$. The R_f value on silica gel TLC (Wako, Silicagel $70F_{254}$, 0.25 mm) using the solvent system hexane–EtOAc (5:1) was 0.36, the $[\alpha]_D^{24}$ was +2.0° (*c* 0.1, MeOH) and the UV spectrum (MeOH) showed end absorption. Compound **1** was obtained as a 1:1 mixture of diastereomers with relative stereochemistry between *syn*-diepoxide portion and chiral center at C-7 with methyl group in side chain.[6] The relative stereochemistry on *syn*-diepoxide is speculated by mechanism of stereoselective rearrangement from EDBD.[34] ^{1}H NMR δ_H ($CDCl_3$, 600 MHz, JEOL JNM-ECA600): isomer 1; 5.06 (m, H-10), 3.12 (d, *J* = 3.0 Hz, H-2), 3.09 (d, *J* = 3.0 Hz, H-1), 2.00 (m, H-9a), 1.95 (m, H-9b), 1.82 (m, H-4a), 1.74 (m, H-5a), 1.69(br s, CH_3-12), 1.65 (m, H-4b) , 1.60 (m, H-5b), 1.60 (br s, CH_3-13), 1.39 (m, H-8a), 1.23 (m, H-8b), 1.35 (s, CH_3-15), 1.28 (m, H-7), 0.99 (d, *J* = 6.5 Hz, CH_3-14). Isomer 2; 5.08 (m, H-10), 3.13 (d, *J* = 3.0 Hz, H-2), 3.03 (d, *J* = 3.0 Hz, H-1), 2.00 (m, H-9a and H-9b), 1.83 (m, H-4a), 1.76 (m, H-5a), 1.67 (br s, CH_3-12), 1.63 (m, H-4b) , 1.61 (m, H-5b), 1.59 (br s, CH_3-13), 1.54 (m, H-8a), 1.24 (m, H-8b), 1.35 (s, CH_3-15), 1.31 (m, H-7), 0.93 (d, *J* = 6.5 Hz, CH_3-14).

^{13}C NMR δ_C ($CDCl_3$, 150 MHz): isomer 1; 131.82 (C-11), 124.13 (C-10), 59.39 (C-6), 55.87* (exchangeable, C-3), 55.45 (C-1), 54.60 (C-2), 39.72 (C-7), 33.15 (C-8), 27.51 (C-4), 25.91 (C-9), 25.69 (CH_3-12), 22.08** (exchangeable, CH_3-15), 20.46 (C-5), 17.69*** (exchangeable, CH_3-13), 15.20 (CH_3-14). Isomer 2; 131.57 (C-11), 124.37 (C-10), 59.55 (C-6), 55.83* (exchangeable, C-3), 54.60 (C-2), 53.93 (C-1), 39.66 (C-7), 32.49 (C-8), 27.81 (C-4), 25.77 (C-9), 25.69 (CH_3-12), 22.03** (exchangeable, CH_3-15), 20.85 (C-5), 17.65*** (exchangeable, CH_3-13), 16.02 (CH_3-14). Complete NMR assignments were performed by using 2D NMR spectral data including DQF-COSY, HSQC-TOCSY and HMBC (Supplementary Figs. S2–S4). HR-EI-MS *m/z* $(M)^+$: Calcd for $C_{15}H_{24}O_2$: 236.1777, Found: 236.1782 (JEOL JMS-700). All physico-chemical properties and NMR data indicated that compound **1** is a new compound with a structure as shown in Fig. 4C.

3.6. Biological activities of a new stable rearrangement product (1)

Compound **1** was over 300-times less effective than EDBD at inducing growth restored activity against mutant yeast (there

was no activity at 5 μg/spot), and was over 80-times less cytotoxic than EDBD to HL60 cells (there was no activity at 80 μM).

3.7. Cytotoxicity of EDBD, α-curcumene and DHA against HL60 cells in the presence of DFOM

As DFOM can chelate free iron ion in HL60 cells, the cytotoxocity of EDBD (bisabolane sesquiterpene with endoperoxide), α-curcumene (bisabolane sesquiterpene without endoperoxide) and DHA (sesquiterpene lactone with endoperoxide) was examined in the presence or absence of DFOM using a MTT assay. The IC_{50} value of EDBD and DHA increased approximately 10-fold and more than 100-fold in the presence of DFOM, respectively (Table 1). However, the cytotoxicity of α-curcumene (without an endoperoxide moiety) was not changed by the presence of DFOM (Table 1).

3.8. Cytotoxicity of EDBD against HL60 cells in the presence of Fe^{2+} or Fe^{3+}

HL60 cells were treated with EDBD in the presence of Fe^{2+} or Fe^{3+} and the cytotoxicity assessed using a MTT assay. The cell viability of HL60 cells decreased, depending on the concentration of Fe^{2+} or Fe^{3+}. Control cell viability treated by EDBD alone (77.8%) was decreased to 33.2% at 20 μM $FeSO_4$ and 34.9% at 20 μM $Fe_2(SO_4)_3$ (Supplementary Fig. S5).

3.9. Measurement of ROS (an unstable carbon-centered radical intermediate)

DCFH-DA (non-fluorescent) can penetrate through cell membranes and is cleaved to DCFH (non-fluorescent) by cellular

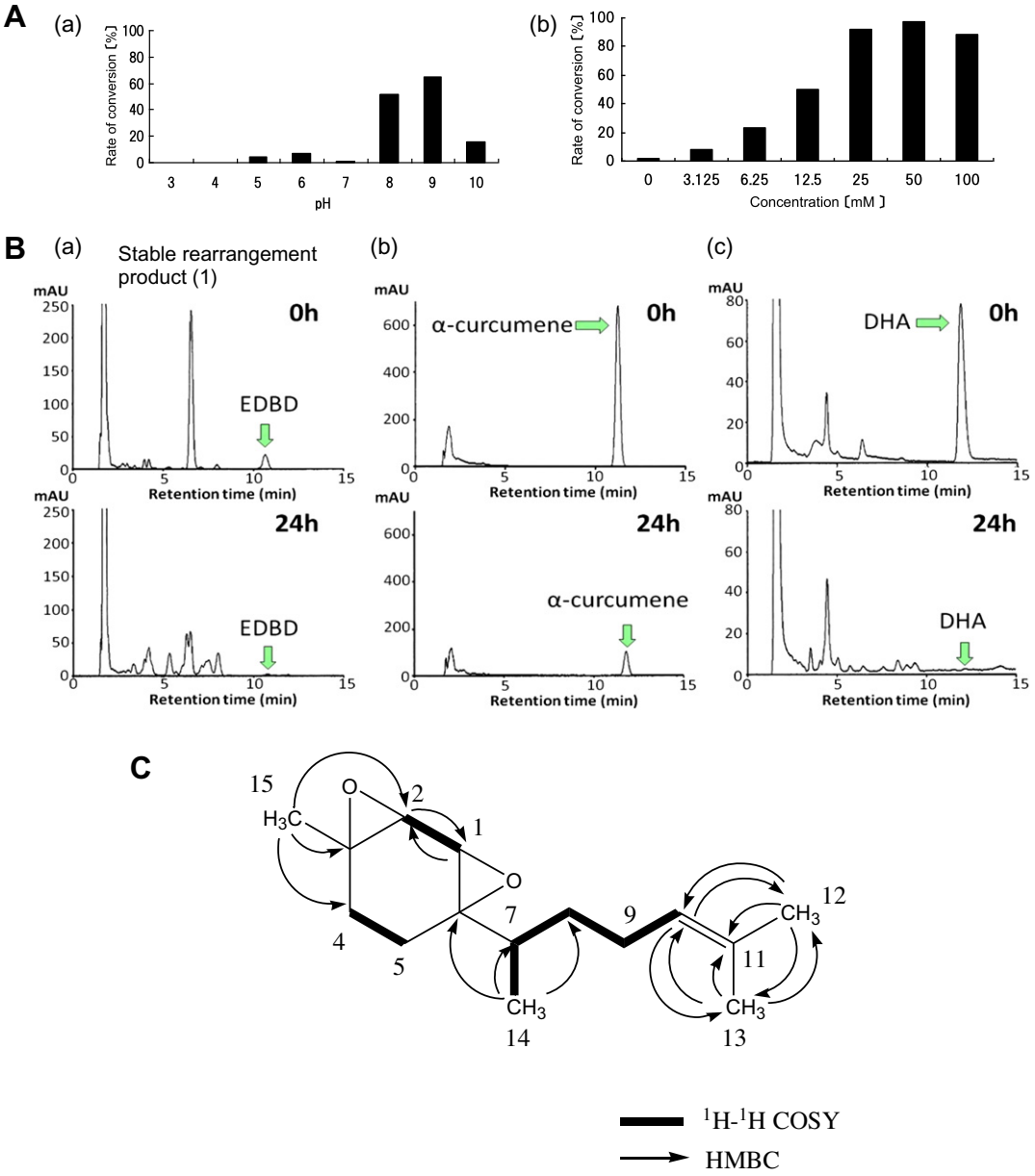

Figure 4. A new conversion product (**1**) from EDBD by $FeSO_4$. (A) Rate of EDBD conversion to **1** in the presence of Fe^{2+}. (a) pH dependency, (b) concentration of Fe^{2+} ion dependency. (B) HPLC profile of conversion of each compound by $FeSO_4$ (25 mM, pH 8). (a) EDBD, (b) α-curcumene, (c) DHA. Column: Capcell pak (ODS, 4.6 mm i.d. × 150 mm), Solvent: 75% MeOH for EDBD, 87.5% MeOH for α-curcumene, 60% MeOH for DHA, Flow rate: 1 ml/min, Detector: UV (205 nm for EDBD and α-curcumene, 198 nm for DHA). Profiles shown are the reaction start at 0 h and following 24 h treatment with Fe^{2+}. Arrows indicate the peak of the original compound. (C) Structure of compound **1**. Arrows indicate important HMBC signals. Bold lines show H–H coupling by ^1H–^1H COSY.

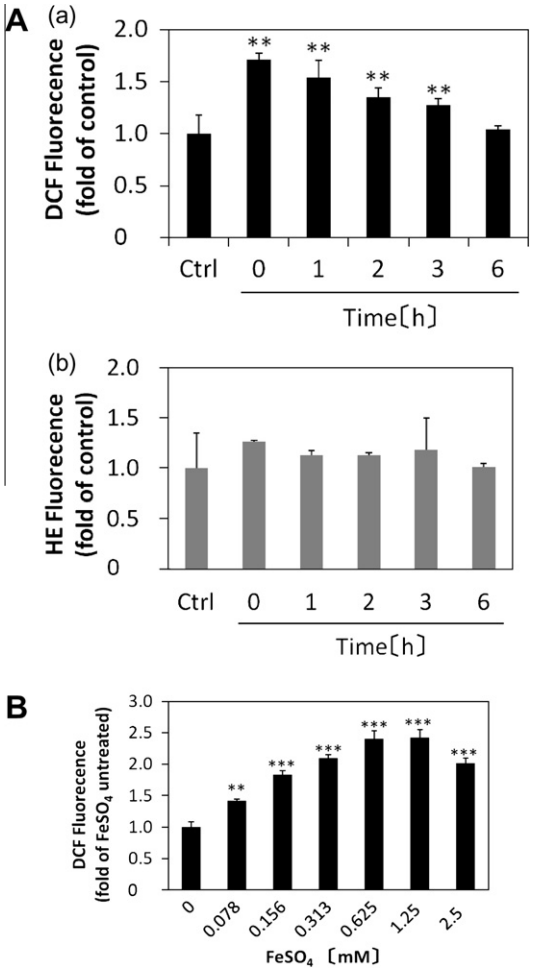

Figure 5. Measurement of ROS (a unstable carbon-centered radical intermediate) induced by EDBD (A) The production of ROS by EDBD in HL60 cells. The production of ROS was measured as fluorescence induced by DCF (a) or HE (b) at 0–6 h. $^{**}P$ <0.01 versus control. (B) The production of ROS by EDBD with Fe^{2+}. The production of ROS was measured as DCF on indicated concentration of $FeSO_4$ and EDBD. $^{**}P$ <0.01, $^{***}P$ <0.001 versus control.

Table 1
Cytotoxic activity of EDBD, α-curcumene and DHA against HL60 cells with or without DFOM

	−DFOM (IC_{50}: μM)	+DFOM (IC_{50}: μM)
EDBD	3.7	35.5
α-Curcumene	106.7	146.7
DHA	0.18	>20.0

esterases. If ROS (hydroxyl radical (HO·), peroxyl radical (ROO·) and hydrogen peroxide (H_2O_2)) were generated in the cells, the reaction with DCFH produce DCF which is fluorescent. HE also produces a red fluorescent product when it reacts with ROS (superoxide radical ($O_2^{\cdot-}$)).[24] When EDBD was added to HL60 cells, the intensity of fluorescence was increased immediately and gradually decreased over 3 h (Fig. 5Aa). This is comparable to the rate of conversion of EDBD to **1**. However, there was no increase of fluorescence from HE at these times (Fig. 5Ab).

When EDBD and $FeSO_4$ (without cells) were mixed with DCFH directly, the fluorescence intensity increased depending on the concentration of $FeSO_4$ (Fig. 5B). This also paralleled with the dose-dependent $FeSO_4$ conversion from EDBD to **1** (Fig. 4Ab).

3.10. Effect of radical scavengers to DNA fragmentation by EDBD

The free radical scavenger, vitamin E, inhibited DNA fragmentation by EDBD, but the superoxide scavenger, allopurinol and the hydrogen peroxide scavenger, *N*-acetyl-L-cysteine, failed to inhibit the fragmentation (Supplementary Fig. S6).

3.11. COMPARE Analysis

Four cell lines were identified for sensitivity to EDBD from a panel of 39 cancer cell lines, and these were HBC-5 (breast), HT-29 (colon), NCI-H522 (lung) and Lox-IMVI (melanoma) (Fig. 6Aa). Sensitive cell lines detected for DHA were HBC-4 (breast), HCT-15 (colon), HCT-116 (colon) and DMS114 (lung) (Fig. 6Ab). Although both EDBD and DHA are sesquiterpene compounds with an endoperoxide bridge, there was no similarity of sensitivity against the 39 cancer cell lines (correlation coefficient COMPARE Analysis $\gamma = 0.158$).

3.12. Anti-tumor activity

EDBD suppressed the tumor volume of the sensitive cell line Lox-IMVI bearing nude mice by 26.9% at 21 days after administration of EDBD (25 mg/kg, iv), without any decrease of body weight. By comparison, CDDP (5 mg/kg, iv) suppressed the tumor volume 69.6%, but this was accompanied by a decrease of body weight compared to un-treated mice (Figures 6B).

4. Discussion

We have isolated EDBD from *Cacalia delphiniifolia* and *Cacalia hastata* as a new type of anti-tumor substance for the first time using the unique screening system of a growth zone by the temperature sensitive mutant strain of *S. cerevisiae* (*cdc2-1 rad9*Δ). *Cacalia delphiniifolia* and *Cacalia hastata* are popular edible wild plants in the northern Tohoku district of Japan as Momijigasa (Shidoke) and Yobusumasou (Bouna), respectively, from spring to early summer. To date there has been little research about biologically active compounds contained in these edible wild plants. Until now, only the antioxidant compound cacalol has been isolated from *Cacalia delphiniifolia* and reported to have anti-tumor activity in vivo.[35,36] EDBD is now the second functional compound to be isolated from *Cacalia delphiniifolia* and would be a lead compound as a new type of dietary cancer-chemopreventive agent.

Although EDBD is sesquiterpene compound with an endoperoxide bridge that is the same as in the anti-malarial drug ART, the difference in structure and the activity against the mutant yeast suggests that its anti-tumor activity is different and that it would be expected to have anti-tumor activity against tumor-bearing mice (Fig. 1), because a similar but less potent compound, α-curcumene, has already been reported as an anti-tumor compound against tumor-bearing mice.[12]

We examined the anti-tumor mechanism of EDBD using well-characterized HL60 cancer cells that are p53 null.[37] Although the potency of EDBD is a little weaker than DHA in HL60 cells, the start time of caspase activation and the conversion time from Fe^{2+} catalyzed conversion of EDBD to **1** were different (Figs. 2C and 4B). It is also different from the DCFH-DA and HE assays compared with the literature.[20] The apoptosis mechanism of EDBD in HL60 cells that was involved in the increase of phospho-p38 MAPK resembles that of DHA,[20] but the sensitivity pattern against 39 cancer cell lines was very different for both compounds (Fig. 6A). The different cell specificity and activity appears to be due to the metabolism of EDBD to **1**, whereas this does not occur with DHA (Figs. 2C, 4B and 6A).

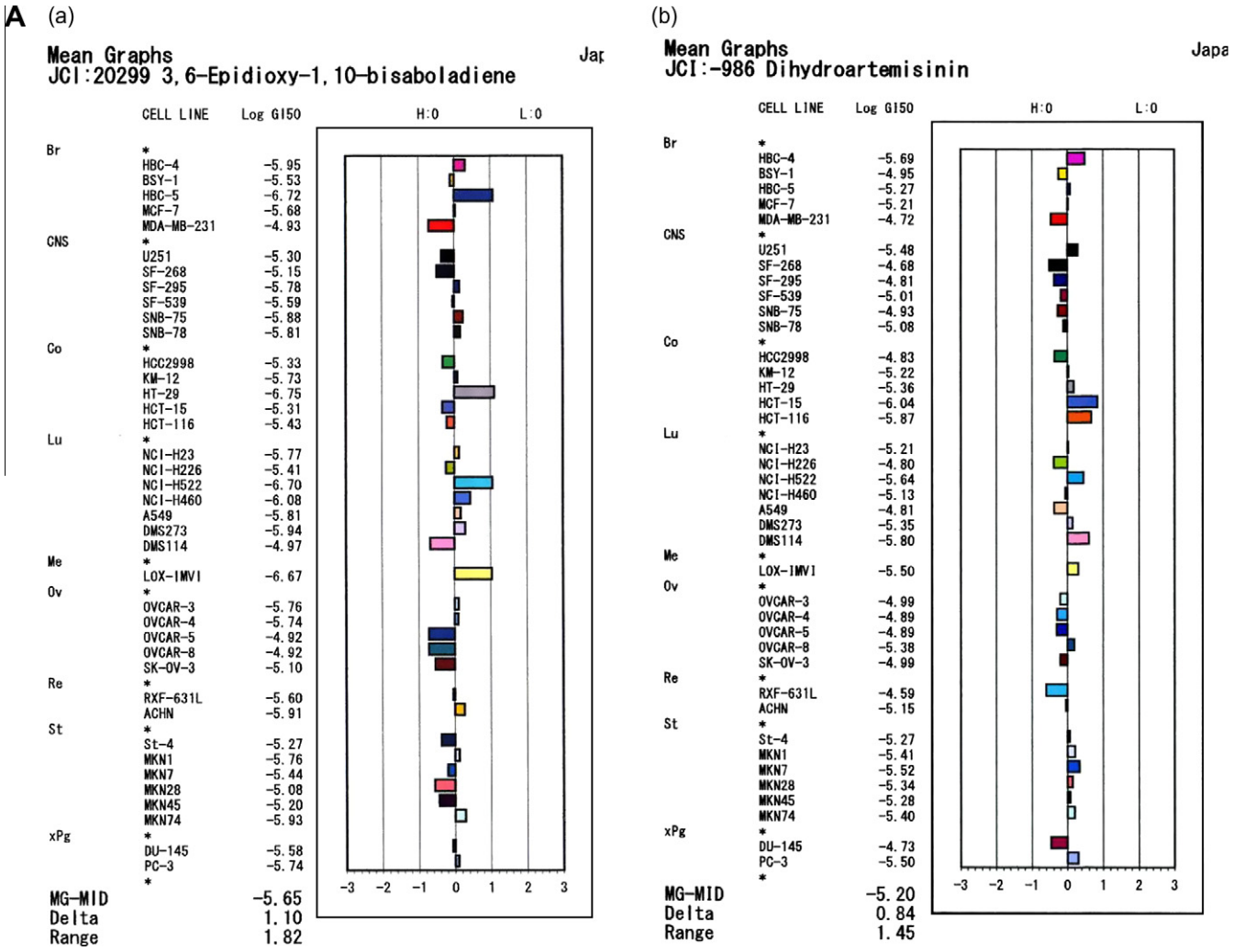

Figure 6. Anti-tumor activity of EDBD. (A) JFCR39 Fingerprints of EDBD (a) and DHA (b). The fingerprints indicate the differential growth inhibition pattern of the cells in the JFCR39 panel by EDBD (a) and DHA (b). The X-axis shows the difference in logarithmic scale between the mean of Log GI_{50} value for all 39 cell lines (MG-MID, expressed as 0 in the fingerprint) and the Log GI_{50} for each cell line in the JFCR39 panel. Columns to the right of 0 indicate the sensitivity of the cell lines to a given compound and columns to the left indicated the resistance. MG-MID = mean of Log GI_{50} values for all 39 cell lines; delta = difference between the MG-MID and Log GI_{50} value for the most sensitive cell line; range = reference between the Log GI_{50} values for the most resistant cell line and the most sensitive cell line. (B) Inhibitory effect of EDBD on mammary tumor growth in nude mice. EDBD and CDDP were dissolved in saline and injected iv at 25 mg/kg on days 1, 6, 11, 16 or 5 mg/kg on days 1, 8, 15 respectively after tumor innoculation. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × $width^2$)/2. (a) Tumor volume, (b) Tumor weight at 21 days, (c) Body weight. Bar = 2 cm. $^{**}P$ <0.01, $^{***}P$ <0.001 versus control.

We therefore examined the in vivo anti-tumor activity of EDBD against one of the sensitive cell lines, Lox-IMVI (melanoma). As expected, the increase of tumor volume of Lox-IMVI in nude mice was suppressed at 25 mg/kg EDBD after 21 days without loss of body weight (Fig. 6B), indicating that EDBD was both effective in vitro and in vivo.

The structure of **1** suggested that a reactive compound of EDBD against HL60 cells might be an unstable carbon-centered radical intermediate.[32–34] In fact, the loss of biological activity of **1** also indicated that **1** is not the actual active compound. Moreover, the cytotoxicities of EDBD and DHA against HL60 cells were both decreased by DFOM (Table 1). On the contrary, the cytotoxicity of EDBD was increased depending on the concentration of Fe^{2+} or Fe^{3+} (Supplementary Fig. S5). When EDBD was added to HL60 cells, ROS was generated immediately in the cells comparable to the conversion rate of EDBD to **1** in the presence of $FeSO_4$ (Figs. 4Ab and Ba, and 5B). Additionally, EDBD produces ROS directly depending on the concentration of $FeSO_4$ in the absence of HL60 cells (Fig. 5B). These observation, together with the fact that vitamin E (scavenger for lipophilic free radicals) only inhibited DNA fragmentation induced by EDBD, suggested that an unstable carbon-centered radical intermediate of EDBD is the actual active compound in HL60 cells (Supplementary Fig. S6). Because an unstable carbon-centered radical intermediate of EDBD is a lipophilic compound, it can react with DCFH in the cells and enhance the fluorescence of DCF. The cleavage and reaction mechanism of EDBD against HL60 cells is summarized in Figure 7. But α-curcumene without the endoperoxide of EDBD has anti-tumor activity (Table 1)[12] and the Fe^{2+} concentration of HL60 cells may be lower than in this experiment (Figs. 4Ab and 5B). In addition of activation of intrinsic pathway (caspase 9), the extrinsic pathway of apoptosis (caspase 8) was activated by EDBD (Fig. 2C). These results suggested that intact EDBD is also involved in its anti-tumor activity.

Thus, collectively, these results indicate that EDBD appears to have a unique mechanism and/or molecular target. More detailed studies of the mechanism on apoptosis signal transduction are therefore justified, together with additional in vivo assessment of biological responses. EDBD is an unique anti-tumor compound

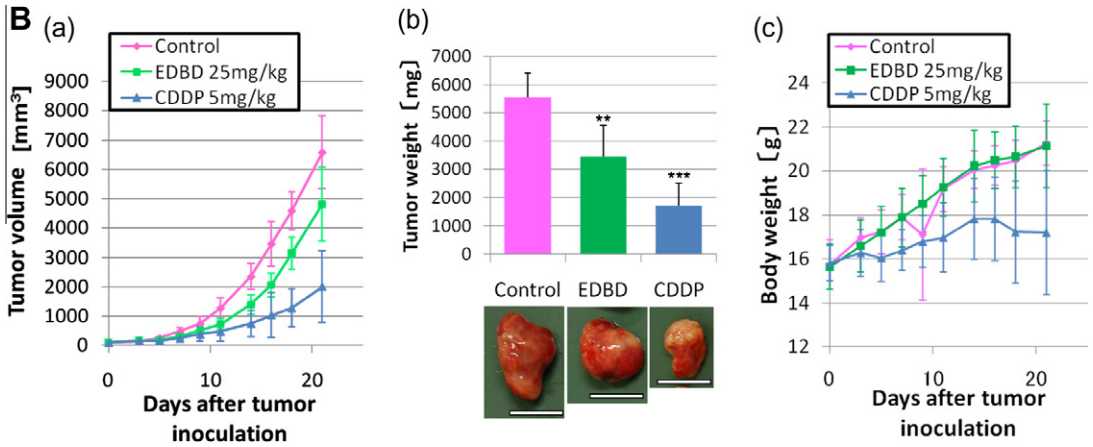

Fig. 6 (*continued*)

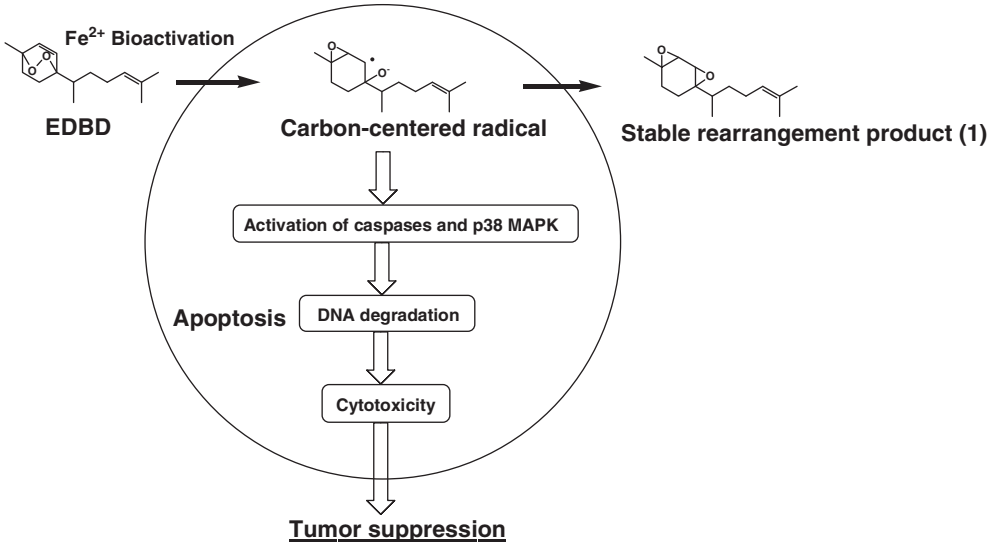

Figure 7. Proposed chemical and biological pathway of EDBD induced HL60 cell death.

different from DHA, and the edible wild plant sources of *Cacalia delphiniifolia* and *Cacalia hastata*, which include EDBD, have considerable potential as useful cancer-preventing food sources.[38,39]

5. Conclusion

3,6-Epidioxy-1,10-bisaboladiene (EDBD) from regional vegetables in Japan showed a unique phenotype of the mutant yeast (*cdc2-1 rad9*Δ). Conversion from EDBD to a new stable rearrangement product (**1**) in the presence of $FeSO_4$, together with ROS assay (DCFH-DA assay) suggested the role of an unstable carbon-centered radical compound in cells. The anti-tumor mechanism is different from that of another sesquiterpene endoperoxide compound, dihydroartemisinin (DHA), as indicated by the rate of conversion time to an active compound and also by the different response in the COMPARE Analysis. EDBD suppressed the tumor growth of Lox-IMVI cells (melanoma) in mice without compromising body weight. This is the first report of the anti-tumor mechanism and activity of EDBD.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.025.

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