INTRODUCTION

The transcription factor c-Fos protein hetero-dimerizes with members of the Jun family, such as c-Jun, and forms the activator protein-1 (AP-1) complex, which regulates the expression of AP-1 binding genes at the transcriptional level (Shaulian and Karin, 2002). c-Fos has been shown to exert its physiological activities including cell cycle modulation and apoptosis induction through the formation of the AP-1 complex (Shaulian and Karin, 2002); however, c-Fos mediates apoptosis in some settings ‘without’ the activation of transcription through the formation of AP-1 (Preston et al., 1996). Increased levels of c-Fos may also be responsible, at least in part, for sensitizing early preneoplastic cells to undergo apoptotic cell death and for inhibiting cancer cell proliferation (Preston et al., 1996), which suggests that c-Fos may be a negative regulator of cancer cell growth when its expression is continuously prolonged (Smeyne et al., 1993; Mikula et al., 2003). Although some stimulants, such as UV irradiation, have been shown to induce a transient up-regulation in c-fos expression (Dosch and Kaina, 1996; Blattner).
et al., 2000), other stimulants including chemicals that induce the stable and selective expression of c-fos are not fully known, except for the introduction of a plasmid carrying c-fos cDNA (Mikula et al., 2003).

We reported that (−)-xanthatin (Fig. 1), one of the major xanthanolides present in Xanthium strumarium (the Cocklebur plant), possessed potent anti-proliferative activity in breast cancer cells including the highly invasive human MDA-MB-231, which appeared to be mediated through the selective inhibition of Topoisomerase IIα (Topo IIα) and continuous production of reactive oxygen species (ROS), leading to the induction of GADD45γ tumor suppressor (Takeda et al., 2013a). Thus, ROS-mediated oxidative stress may participate in the biological activities of (−)-xanthatin. Since the anti-cancer drug etoposide, a selective Topo IIα inhibitor, has been shown to induce intracellular ROS (Kurosu et al., 2003), (−)-xanthatin may exert its anti-proliferative effects on cancer cells in a similar manner to those of etoposide. Although etoposide is one of the most active and useful anti-neoplastic agents, its cancer cell-killing mechanism(s) have not yet been fully understood. In the present study, to generalize the applicability of (−)-xanthatin to cancer therapy, we further analyzed the anti-proliferative effects of (−)-xanthatin on cancer cells by comparing to those of etoposide.

We showed here that i) in addition to GADD45γ, c-fos was revealed to be a (−)-xanthatin-regulated gene in MDA-MB-231 cells, and that ii) (−)-xanthatin was a more potent anti-proliferator than etoposide, as demonstrated by the more prolonged and stronger up-regulation of c-Fos via ROS, without influencing AP-1-regulated genes. The applicability of (−)-xanthatin as a phytomedicine for cancer therapy and possible interplay between c-Fos and GADD45γ in cancer cell-killing effects have also been discussed.

**MATERIALS AND METHODS**

**Reagents**

(−)-Xanthatin and (+)-8-epi-xanthatin were chemically synthesized according to a published protocol (Matsuo et al., 2010; Matsumoto et al., 2013). These synthesized compounds were purified by HPLC (High-performance liquid chromatography) or column chromatography, and their purity (> 98%) was confirmed by 1H- and 13C-NMR (Nuclear Magnetic Resonance) spectroscopy. No ring-opened derivatives of the xanthanolides’ lactones were detected in these analyses (Takeda et al., 2011, 2013a). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and etoposide were purchased from Sigma Co. (St. Louis, MO, USA). Actinomycin D (Act D) and NAC were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). L-Buthionine-sulfoximine (BSO) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

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**Fig. 1.** The chemical structures of (−)-xanthatin (trans) and (+)-8-epi-Xanthatin (cis) are shown. aAlthough the exo-methylene lactone moiety, indicated by a gray inclusion in the xanthanolides, has been suggested as an active center, (−)-xanthatin, but not (+)-8-epi-Xanthatin, exerts biological effects, including the suppression of MDA-MB-231 cell growth via both ROS production (indicated as +) and Topo IIα inhibition (indicated as +). (+)-8-epi-Xanthatin only exhibited Topo IIα inhibition (Takeda et al., 2011, 2013a).
All other reagents were of the highest grade commercially available.

**Cell cultures and cytotoxicity assays**

Cell culture conditions and methods were performed as described previously (Takeda et al., 2011). Briefly, the human breast cancer cell line, MDA-MB-231 (obtained from the American Type Culture Collection, Rockville, MD, USA), was routinely grown in phenol red-containing minimum essential medium alpha (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator, within an atmosphere of 5% CO₂, at 37°C. Before the chemical treatments, the medium was changed to phenol red-free minimum essential medium alpha (Invitrogen) supplemented with 10 mM HEPES, 5% dextrin-coated charcoal-treated serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures of approximately 60% confluence in a 100-mm Petri dish were used to seed the proliferation experiments. In the cytotoxicity assays, the cells were seeded into 96-well plates at a density of approximately 5,000 cells/well, and test substances were introduced 4 hr after cell seeding. Cells were treated with increasing concentrations of (−)-xanthatin or etoposide for 48 hr. Cell viability was then analyzed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS reagent; Promega, Madison, WI, USA), according to the manufacturer’s instructions. Test chemicals were prepared in ethanol or dimethylsulfoxide (DMSO). Control incubations contained equivalent additions of ethanol or DMSO. No measurable influence of ethanol or DMSO at the final concentrations used was observed on cell viability.

**Preparation of total RNA and DNA microarray analyses**

Total RNA was collected from 10 μM (−)-xanthatin or vehicle-treated MDA-MB-231 cells 48 hr after exposure using the RNeasy kit (Qiagen, Inc., Hilden, Germany), and was purified using RNeasy/QiAamp columns (Qiagen, Inc.). cDNA synthesis, RT, and PCR were performed using the SuperScript™ one-step RT-PCR System with Platinum® Taq polymerase (Invitrogen). The primers used for c-fos were: c-fos (sense) 5’-CTG ACT GAT ACA CTC CAA GCG-3’ and c-fos (antisense) 5’-CAT CAA AGG GCT CGG TCT TCA-3’. PCR primers used for β-actin and GADD45γ were taken from previously published reports (Takeda et al., 2011). PCR was performed under conditions producing template quantity-dependent amplification. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-acetate EDTA (ethylenediamine-N,N,N’,N’-tetraacetic acid) buffer and stained with EtBr. When the RT reaction was omitted, no signal was detected in any of the samples. β-Actin was used as an internal control for RT-PCR. The quantification of band intensities was performed using NIH Image 1.61 software (http://rsb.info.nih.gov/nih-image/).

**Antibodies and Western blot analysis**

Antibodies specific for c-Fos (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (A5060; Sigma Co.) were used. Whole cell extracts were prepared as previously described (Takeda et al., 2013a). SDS–polyacrylamide gel electrophoresis/Western blot analysis was performed based on procedures described previously (Takeda et al., 2009, 2013a). Equal amounts of protein for each sample were confirmed by probing with β-actin. The quantification of band intensities was performed using NIH Image 1.61 software.

**ROS detection**

Cellular ROS levels were quantified according to reported methods using a DCFH-DA probe (LeBel et al., 1992; Takeda et al., 2013a).
Determination of c-fos and GADD45γ mRNA half-life

c-fos and GADD45γ transcript half-life ($t_{1/2}$) were determined after treatment with the transcription inhibitor, Act D. Samples were collected every 2 hr for 8 hr after the inhibition of transcription. Total RNA was extracted and mRNA relative abundance was determined by semiquantitative RT-PCR. The values were normalized relative to the value prior to the Act D treatment, plotted as a function of time, and subjected to a regression analysis according to our previous study (Takeda et al., 2013a). The quantification of band intensities was performed using NIH Image 1.61 software.

Data analysis

IC$_{50}$ values were determined using SigmaPlot 11® software (Systat Software, Inc., San Jose, CA, USA), according to analyses described previously (Takeda et al., 2011). Differences were considered significant when the $p$ value was calculated as less than 0.05. Significant differences between two groups were calculated by the Student’s $t$ test. Other statistical analyses were performed by Scheffe’s $F$ test, a post-hoc test for analyzing the results of ANOVA testing. Calculations were performed using Statview 5.0J software (SAS Institute Inc., Cary, NC, USA).

RESULTS

(−)-Xanthatin induced c-fos mRNA and c-Fos protein

We performed DNA microarray analysis to investigate the AP-1-related genes regulated by 10 μM (−)-xanthatin because (−)-xanthatin-mediated cell death was shown to be accompanied by the up-regulation of interleukin-1β (IL-1β) and hemeoxygenase-1 (HO-1) (Takeda et al., 2011). These are cellular stress-induced molecules and were shown to activate AP-1 and induce egr-1 (Oguro et al., 1998; Hungness et al., 2000). Although the marked up-regulation of c-fos was detected (49.1-fold), other transcription factors that composed a subunit of the AP-1 hetero-dimer were not markedly stimulated (Fig. 2A). The induction of c-fos by (−)-xanthatin was evaluated by RT-PCR analysis (indicated as ‘RT-PCR’), and further c-Fos protein expression was detected by Western blot analysis (indicated as ‘WB’) (Fig. 2A, inset). c-fos was up-regulated by (−)-xanthatin in a concentration-dependent manner relative to egr-1 (Fig. 2B).

We previously reported that GADD45γ is one of the (−)-xanthatin’s target genes (Takeda et al., 2011) (see Fig. 2A), and that two biological activities are involved; Topo IIα inhibition and ROS production (Takeda et al., 2013a). As is clearly shown in Figs. 2A and 2B, c-fos may also be a (−)-xanthatin-sensitive gene. Given that the c-fos gene is regulated by (−)-xanthatin underlying pathway(s) that are similar to those of GADD45γ gene induction, the c-fos gene would not be affected i) by a Topo IIα inhibitor without the potential to produce ROS, or ii) by an ROS producer without the ability to inhibit Topo IIα (the latter is discussed in the Discussion section). We thus compared the c-fos and GADD45γ induction potentials of (−)-xanthatin and (−)-8-epi-xanthatin because the latter Topo IIα inhibitory xanthanolide lacked the potential to produce ROS (Takeda et al., 2013a) (Fig. 1). Although egr-1 expression was not induced by either (−)-xanthatin or (−)-8-epi-xanthatin, (−)-xanthatin, but not (−)-8-epi-xanthatin, markedly stimulated the expression of both c-fos and GADD45γ genes (Figs. 2C and 2D). In addition, (−)-8-epi-xanthatin did not induce the expression of c-Fos and GADD45γ protein, which was different from (−)-xanthatin (Fig. 2A) (data not shown) (Takeda et al., 2013a).

(−)-Xanthatin and etoposide induced c-fos/ GADD45γ mRNA and ROS

Based on the results displayed in Figs. 2C and 2D, we next performed RT-PCR analysis to compare the biological activities of (−)-xanthatin and etoposide because etoposide is known to be a selective Topo IIα inhibitor as well as a ROS producer (Kurosu et al., 2003). As is shown in Fig. 3A, c-fos and GADD45γ were induced by (−)-xanthatin and etoposide, although etoposide required much higher concentrations (i.e., 40 μM) to induce these at levels comparable to (−)-xanthatin. We next assessed the respective levels of intracellular ROS following the (−)-xanthatin and etoposide treatments using a DCFH-DA probe (LeBel et al., 1992; Takeda et al., 2013a). The results showed that the production of ROS with 40 μM, but not 10 μM etoposide was significantly higher than that with the vehicle-treated control ($p < 0.05$), which was almost comparable to the level of ROS produced by 10 μM (−)-xanthatin (Fig. 3B). In addition, the ROS produced by (−)-xanthatin or etoposide were blocked by NAC, a potent ROS scavenger (data not shown) (Takeda et al., 2013a). By comparing the IC$_{50}$ values of (−)-xanthatin and etoposide treatments for 48 hr, (−)-xanthatin’s anti-proliferative potential was found to be approximately four times stronger than that of etoposide (Fig. 3C; 5.28 μM vs. 20.37 μM), and this concentration requirement seems to be consistent with the efficacies of c-fos/GADD45γ induction and ROS production between (−)-xanthatin and etoposide (Figs. 3A and 3B). Thus,
Fig. 2. Selective up-regulation of the c-fos gene by (−)-xanthatin. (A) Results of DNA microarray analysis. Data are expressed as fold induction vs. vehicle-treated groups. MDA-MB-231 cells were treated with vehicle or 10 μM (−)-xanthatin for 48 hr, followed by mRNA isolation. The details of microarray conditions are described in the Materials and Methods section. Figure inset, cells were treated with vehicle (-) or 10 μM (−)-xanthatin (+) for 48 hr, followed by an investigation of c-fos mRNA/c-Fos protein using RT-PCR and Western blot analysis (indicated as WB), respectively. β-Actin was used as an internal loading control. The results of GADD45γ presented in the Figure were taken from a reference (Takeda et al., 2011). (B) RT-PCR analysis of egr-1 and c-fos transcript levels after treatment with (2.5 μM or 10 μM) or without (indicated as 0) (−)-xanthatin. A 100-bp DNA ladder marker was also loaded. (C) RT-PCR analysis of egr-1 and c-fos transcript levels after treatment with (−)-xanthatin (Xa; 2.5 μM or 10 μM), (+)-8-epi-xanthatin (8-epi; 10 μM), or without (indicated as 0) xanthanolide treatments. A 100-bp DNA ladder marker was also loaded. (D) RT-PCR analyses of c-fos and GADD45γ levels in MDA-MB-231 cells 48 hr after treatment with 10 μM (−)-xanthatin (Xa), 10 μM (+)-8-epi-Xanthatin (8-epi), or vehicle (Control). β-Actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded. Band intensities were quantified by densitometry (NIH Image 1.61 software) and normalized to β-actin levels. The normalized values were converted to fold change relative to vehicle-treated control.
(–)-Xanthatin was revealed to have superior anti-proliferative effects over those of etoposide on MDA-MB-231 cells in vitro.

Because changes in GADD45γ mRNA and some mRNA stabilities have been modulated by treatments with
ROS-producing agents including (–)-xanthatin (Adler et al., 1999; Zheng et al., 2005; Takeda et al., 2013a), we investigated whether (–)-xanthatin and etoposide affected the stability of c-fos mRNA in a manner similar to that of GADD45γ. MDA-MB-231 cells were treated for 48 hr with (–)-xanthatin (Xa; 10 μM), etoposide (10 μM and 40 μM), or vehicle (C), and the cells were subsequently exposed to the transcriptional inhibitor, actinomycin D (Act D, 4 μg/ml) for 2, 4, 6, or 8 hr. The Act D concentration used was determined based on both the efficacy and lack of toxicity following dose-response experiments. After the respective Act D exposures, total cellular RNA was isolated and RT-PCR analyses were performed as described in the Materials and Methods section. β-Actin was also used as an RNA internal control. (A) A representative semi-logarithmic plot of the decay of c-fos mRNA is shown. A 100-bp DNA ladder marker was also loaded. Based on each mRNA decay plot, the mRNA half-life \( t_{1/2} \) was determined and listed. a) The results for (–)-xanthatin and (+)-8-epi-xanthatin presented in the Figure were taken from a reference (Takeda et al., 2013a). (B) RT-PCR (upper panel) and Western blot (WB, lower panel) analyses of c-fos/c-Fos levels in MDA-MB-231 cells 48 hr after the treatment with 10 μM (–)-xanthatin, 40 μM etoposide, or vehicle in the presence or absence of 1 mM NAC. NAC was added as a pretreatment 2 hr prior to the (–)-xanthatin additions. β-Actin was used as an internal loading control. A 100-bp DNA ladder marker was also loaded. Band intensities were quantified by densitometry (NIH Image 1.61 software) and normalized to β-actin levels. The normalized values were converted to fold change relative to vehicle-treated control.

Fig. 4. (–)-Xanthatin and etoposide stabilized c-fos/GADD45γ mRNA. (A and B) The effects of (–)-xanthatin, etoposide, or vehicle on the mRNA stability of c-fos, GADD45γ, and β-actin in MDA-MB-231 cells. MDA-MB-231 cells were treated for 48 hr with (–)-xanthatin (Xa; 10 μM), etoposide (10 μM and 40 μM), or vehicle (C), and the cells were subsequently exposed to the transcriptional inhibitor, actinomycin D (Act D, 4 μg/ml) for 2, 4, 6, or 8 hr. The Act D concentration used was determined based on both the efficacy and lack of toxicity following dose-response experiments. After the respective Act D exposures, total cellular RNA was isolated and RT-PCR analyses were performed as described in the Materials and Methods section. β-Actin was also used as an RNA internal control. (A) A representative semi-logarithmic plot of the decay of c-fos mRNA is shown. A 100-bp DNA ladder marker was also loaded. Based on each mRNA decay plot, the mRNA half-life \( t_{1/2} \) was determined and listed. a) The results for (–)-xanthatin and (+)-8-epi-xanthatin presented in the Figure were taken from a reference (Takeda et al., 2013a). (B) RT-PCR (upper panel) and Western blot (WB, lower panel) analyses of c-fos/c-Fos levels in MDA-MB-231 cells 48 hr after the treatment with 10 μM (–)-xanthatin, 40 μM etoposide, or vehicle in the presence or absence of 1 mM NAC. NAC was added as a pretreatment 2 hr prior to the (–)-xanthatin additions. β-Actin was used as an internal loading control. A 100-bp DNA ladder marker was also loaded. Band intensities were quantified by densitometry (NIH Image 1.61 software) and normalized to β-actin levels. The normalized values were converted to fold change relative to vehicle-treated control.
hatin and 10 μM etoposide did not exhibit any stabilizing effects on the c-fos and GADD45γ mRNA transcripts (Fig. 4B). Furthermore, we tested independent RT-PCR primer sets in parallel experiments and yielded the same quantitative conclusions (data not shown). Together, these results indicate that (−)-xanthatin and etoposide stabilize c-fos as well as GADD45γ mRNA in MDA-MB-231 cells, possibly through a ROS generation mechanism.

We reasoned that if ROS generation was involved as a mediator of the (−)-xanthatin and etoposide-mediated up-regulation of c-fos, as observed in the case of GADD45γ (Takeda et al., 2013a), then NAC, an effective ROS scavenger (Zhang et al., 2011), should interfere with this pathway. As is clearly shown in Fig. 4C, pre-treatment with 1 mM NAC largely blocked the induction of c-fos mRNA (indicated as RT-PCR) and c-Fos protein (indicated as WB) that resulted from exposure to 10 μM (−)-xanthatin (upper and lower panels, respectively). Treatment with NAC alone had no marked effect on the c-fos mRNA/c-Fos protein expression status. The same phenomenon was also detected in the case of GADD45γ (data not shown) (Takeda et al., 2013a). These results support the concept that NAC-sensitive pathways underlie (−)-xanthatin’s up-regulation of c-fos as well as GADD45γ, and suggest that (−)-xanthatin possesses, at least in part, common pathway(s) with etoposide in the induction of c-fos and GADD45γ, which may be coupled with these chemicals’ anti-proliferative effects on breast cancer cells (see Fig. 5).

DISCUSSION

In the current study, c-fos was shown to be a (−)-xanthatin-regulated gene, and c-fos mRNA could be stabilized by (−)-xanthatin-produced ROS in a similar manner to that of GADD45γ (Takeda et al., 2013a). Previous studies showed that c-fos and GADD45γ were activated in response to DNA damaging stimuli such as DNA alkylation and UV irradiation; however, these genes tended to be transiently activated, followed by ‘quickly’ decreased to basal levels (Dosch and Kaina, 1996; Blattner et al., 2000). (+)-8-epi-Xanthatin, a cis-isomer of (−)-xanthatin (Fig. 1), did not induce significant higher levels of c-fos and GADD45γ over those of (−)-xanthatin (Figs. 2C and 2D); however, the 8-epi form was shown to have Topo IIα inhibitory potential similar to that of (−)-xanthatin, which has been coupled with DNA damage as assessed by the formation of γH2AX (Takeda et al., 2013a). Pretreatment with NAC completely abolished the up-regula-

Fig. 5. A working model for the (−)-xanthatin-mediated up-regulation of c-Fos/GADD45γ coupled with cell death. In this study, it was suggested that (−)-xanthatin requires c-Fos in addition to GADD45γ to suppress human breast cancer MDA-MB-231 cell growth. (−)-Xanthatin inhibits Topo IIα (accompanied by DNA damage) (see Takeda et al., 2013a), followed by c-fos/ GADD45γ induction, and the up-regulated c-fos/GADD45γ mRNA transcripts are stabilized by concomitantly generated ROS. (−)-Xanthatin-mediated ROS production pathways are not resolved.
(-)-Xanthatin induces the prolonged expression of c-Fos

dition of c-fos/GADD45γ mRNA and protein expression by (-)-xanthatin/etoposide in MDA-MB-231 cells (Fig. 4C) as well as the cell-killing effect, and, as expected, blocked increased ROS production (data not shown). These phenomena suggest that an increased oxidative state is one of the critical factors of the (-)-xanthatin- and etoposide-mediated cell death mechanism; however, the viability of breast cancer cells treated with L-buthionine-sulfoximine (BSO) was not affected (data not shown), suggesting that ROS alone are not sufficient to induce c-fos and GADD45γ, leading to the cell death response. When these results and our previous findings are taken into consideration, they suggest that ROS may assist in further enhancing the activity of (-)-xanthatin and etoposide.

As shown in Figs. 3B and 3C, four times higher concentrations of etoposide than those of (-)-xanthatin exhibited biological effects almost comparable to (-)-xanthatin (i.e., anti-proliferative effect and ROS production potential); however, there is a discrepancy between (-)-xanthatin and etoposide in induction potential of c-fos/GADD45γ at the respective concentrations (40 μM vs. 10 μM) (Fig. 3A). It is likely that 40 μM etoposide induces 'sufficient' level of c-fos/GADD45γ to cause anti-proliferative effects as observed in the case of 10 μM (-)-xanthatin. It is noteworthy that (-)-xanthatin in particular exhibited much stronger anti-proliferative potential than that of etoposide, underlying marked c-fos/GADD45γ induction and ROS production in vitro. Further studies are needed to establish the biological effects of (-)-xanthatin in vivo.

Although etoposide is one of the most active and useful anti-neoplastic agents, the exact mechanism(s) of the agent-mediated anti-neoplastic effect has remained unknown. In the present study, we compared the biological activities of (-)-xanthatin and etoposide because etoposide was previously shown to be a Topo IIa inhibitor as well as a ROS producer (Kurosu et al., 2003). In support of this, etoposide induced both c-fos and GADD45γ in a NAC-sensitive manner. In addition, etoposide exhibited mRNA stabilization effects on c-fos/GADD45γ to cause anti-proliferative effects as observed in the case of 10 μM (-)-xanthatin. It is noteworthy that (-)-xanthatin in particular exhibited much stronger anti-proliferative potential than that of etoposide, underlying marked c-fos/GADD45γ induction and ROS production in vitro. Further studies are needed to establish the biological effects of (-)-xanthatin in vivo.

Collectively, based on the results obtained here, a working model for the (-)-xanthatin-mediated up-regulation of c-Fos/GADD45γ coupled with cell death has been suggested (see Fig. 5). It is proposed that c-Fos/GADD45γ activation results from (-)-xanthatin’s ability to inhibit Topo IIa, thereby enhancing cellular DNA damage, and that these activities are stabilized by concomitantly generated ROS. However, we could not address the ROS production machinery in the present study (see Fig. 5). By focusing on the difference in the biological activities of (-)-xanthatin and (+)-8-epi-xanthatin (Fig. 1), we are currently investigating this machinery, and any information obtained will allow us to clarify the ROS-assisted stabilization mechanism(s) of c-fos and GADD45γ.

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