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Original Article

(–)-Xanthatin induces the prolonged expression of c-Fos through an *N*-acetyl-L-cysteine (NAC)-sensitive mechanism in human breast cancer MDA-MB-231 cells

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ABSTRACT — We reported that (–)-xanthatin, a xanthanolide sesquiterpene lactone present in the Cocklebur plant, exhibited potent anti-proliferative effects on human breast cancer cells, in which *GADD45γ*, a novel tumor suppressor gene, was induced. Mechanistically, topoisomerase IIα (Topo IIα) inhibition by (–)-xanthatin was shown to be the upstream trigger that stimulated the expression of *GADD45γ* mRNA and concomitantly produced reactive oxygen species (ROS) to maintain this expression. Since the anti-cancer drug etoposide, a selective Topo IIα inhibitor, has also been shown to induce intracellular ROS, (–)-xanthatin may exert its anti-proliferative effects on cancer cells in a similar manner to those of etoposide. In the present study, to generalize its applicability to cancer therapy, we further investigated the biological activities of (–)-xanthatin by comparing its activities to those of the established anti-cancer drug etoposide. After the exposure of breast cancer cells to (–)-xanthatin or etoposide, a prolonged and marked up-regulation in the expression of *c-fos*, a proapoptotic molecule, was detected together with *GADD45γ*; and the expression of these molecules was stabilized by ROS and abrogated by the pretreatment with *N*-acetyl-L-cysteine (NAC), a potent ROS scavenger. (–)-Xanthatin in particular exhibited stronger anti-proliferative potential than that of etoposide, which underlies the marked induction of *c-fos*/*GADD45γ* and ROS production.

Key words: (–)-Xanthatin, c-Fos, GADD45γ, ROS, Breast cancer

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

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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 ^{1}H- and ^{13}C-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).

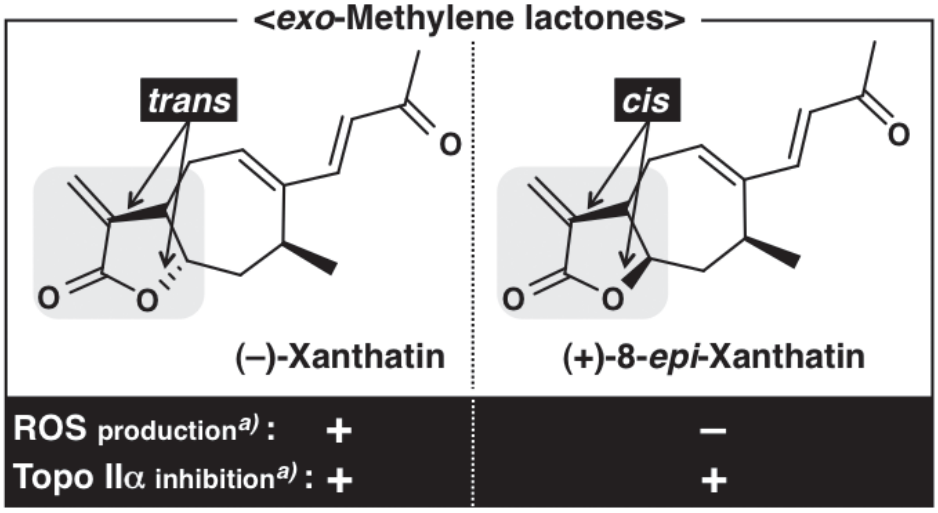


Fig. 1. The chemical structures of (–)-xanthatin (*trans*) and (+)-8-*epi*-Xanthatin (*cis*) are shown. [a)]Although the *exo*-methylene lactone moiety, indicated by a gray inclusion in the xanthanolides, has been suggested as an active center, (–)-xanthatin, but not (+)-8-*epi*-Xanthatin, exerts biological 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% CO_2, 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.). The specific gene expression pattern in MDA-MB-231 cells was examined by DNA microarray analysis in comparison with vehicle-controls. Total RNA was extracted from both cell types, and complementary DNA (cDNA) synthesis and cRNA labeling were conducted using a Low RNA Fluorescent Linear Amplification Kit (Agilent, Palo Alto, CA, USA). Overall changes in gene expression were evaluated using two-color microarray-based gene expression analysis (Hwang *et al.*, 2011; Takeda *et al*., 2011, 2013b; Toyama *et al.*, 2011). Labeled cRNA (Cy3 to control, Cy5 to (–)-xanthatin) was hybridized to human oligo DNA microarray slides (Agilent) that carried spots for human genes. Specific hybridization was analyzed using a Microarray scanner (Agilent) and evaluated as a scatter-plot graph for gene expression. Hokkaido System Science (Sapporo, Japan) provided assistance with the experiments.

Analysis of *c-fos*, *GADD45γ*, and *β-actin* mRNAs by the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from MDA-MB-231 cells using the RNeasy kit (Qiagen, Inc., Hilden, Germany) and 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,

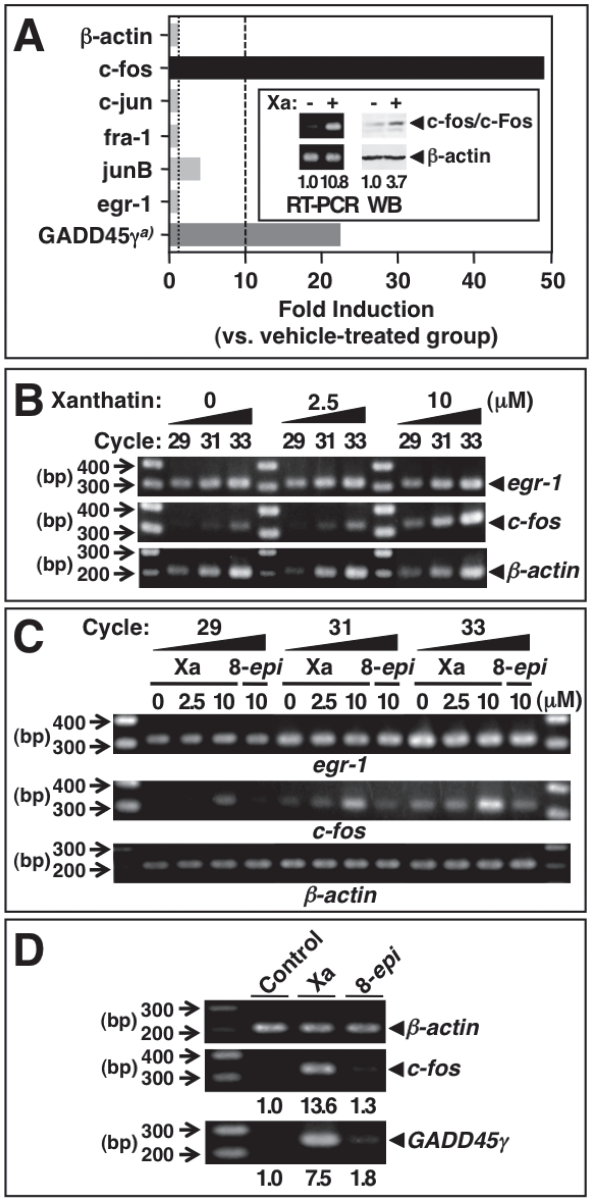


Fig. 2. Selective up-regulation of the *c-fos* gene by (–)-xanthatin. (A) Results of DNA microarray analysis. Data are expressed as fold induction vs. vehicle-treated groups. MDA-MB-231 cells were treated with vehicle or 10 μM (–)-xanthatin for 48 hr, followed by mRNA isolation. The details of microarray 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. [a)] 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.

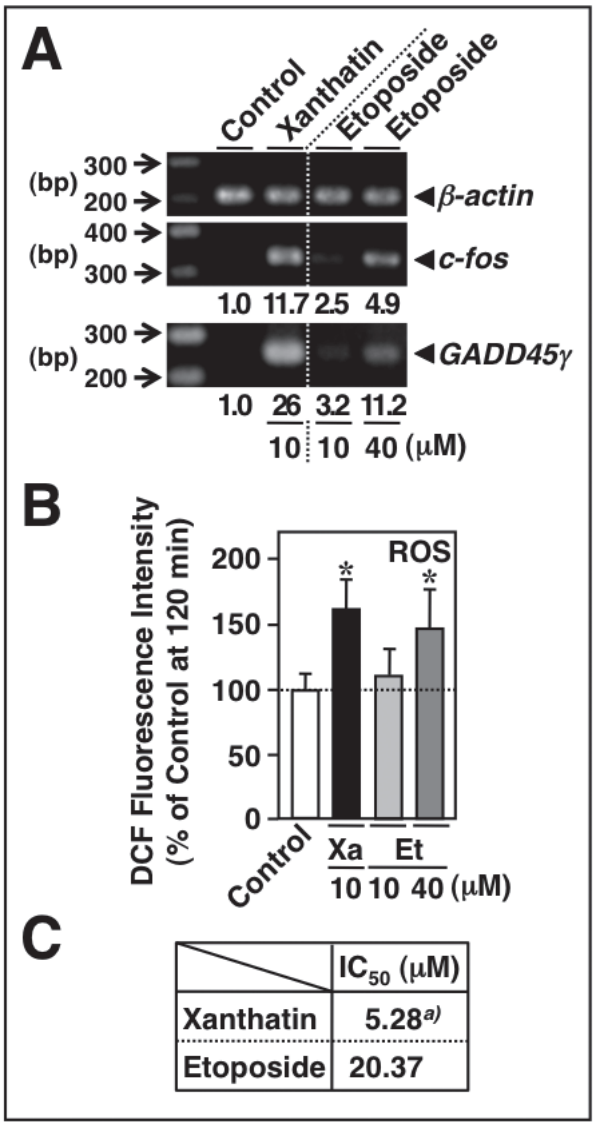
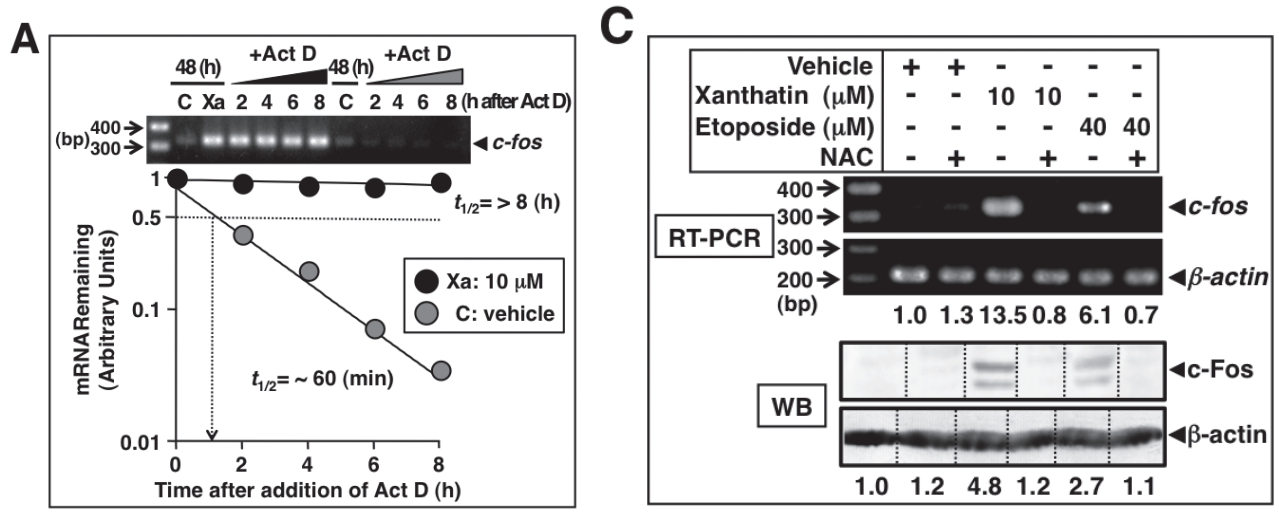


Fig. 3. (–)-Xanthatin and etoposide induced *c-fos/GADD45γ* and produced intracellular ROS. (A) RT-PCR analyses of *c-fos and GADD45γ* levels in MDA-MB-231 cells 48 hr after the treatment with (–)-xanthatin (10 μM), etoposide (10 μM and 40 μM), or vehicle (Control). *β-Actin* was used as an RNA normalization 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. (B) MDA-MB-231 cells were exposed for 120 min to (–)-xanthatin (Xa; 10 μM) or etoposide (Et; 10 μM and 40 μM). Intracellular ROS were measured as described in the *Materials and Methods* section. Data are expressed as a percentage of the vehicle-treated group (Control), as mean ± S.D. (n = 6). *Significantly different ($p < 0.05$) from the control. (C) IC_{50} values (μM) for MDA-MB-231 cell growth inhibition, obtained in the culture at 48 hr, were determined as described in the *Materials and Methods* section. The values indicate the means of three independent experiments performed with five technical replicates. [a)] The results for (–)-xanthatin presented in the Figure were taken from a reference (Takeda *et al*., 2011).

(–)-xanthatin was revealed to have superior anti-proliferative effects over those of etoposide on MDA-MB-231 cells *in vitro*.

(–)-Xanthatin and etoposide stabilized *c-fos* and *GADD45γ* mRNA

Because changes in *GADD45γ* mRNA and some mRNA stabilities have been modulated by treatments with



B

$t_{1/2}$	*c-fos*	*GADD45γ*	*β-actin*
Control	~ 60 (min)	~ 45 (min)	> 8 (h)
Xanthatin	> 8 (h)	> 8 (h)[a)]	> 8 (h)
8-*epi*-xanthatin	~ 2 (h)	~ 45 (min)[a)]	> 8 (h)
Etoposide (10)	~ 2 (h)	~ 45 (min)	> 8 (h)
Etoposide (40)	~ 6.5 (h)	~ 3 (h)	> 8 (h)

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. (B). 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). (C) 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 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.

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 incubated for 48 hr in the presence or absence of 10 μM (–)-xanthatin or 10 μM and 40 μM etoposide. Act D (4 μg/ml), an established transcriptional inhibitor that has no effect on cell growth (Zheng *et al*., 2005; Takeda *et al*., 2013a), was then added to the cell cultures. mRNA levels including *c-fos, GADD45γ*, and *β-actin* were determined by semi-quantitative RT-PCR every 2 hr up to 8 hr. *β-Actin* mRNA stability was not affected by the (–)-xanthatin, etoposide, or vehicle treatments (Fig. 4B). Previous studies showed the half-life ($t_{1/2}$) of *c-fos* and *GADD45γ* mRNA to be approximately ~60 min (Blattner *et al*., 2000; Bruemmer *et al*., 2003; Takeda *et al*., 2013a). In the present study, (–)-xanthatin markedly stabilized the *c-fos* and *GADD45γ* mRNA transcripts, > 11-fold (> 8 hr) (*see* also Takeda *et al*., 2013a) and > 8-fold (> 8 hr), respectively, relative to the vehicle-treated controls, and etoposide also stabilized both *c-fos* and *GADD45γ* mRNAs, although the degree of stabilization was weaker than that of (–)-xanthatin (Figs. 4A and 4B). As expected, 10 μM (+)-8-*epi*-xant-

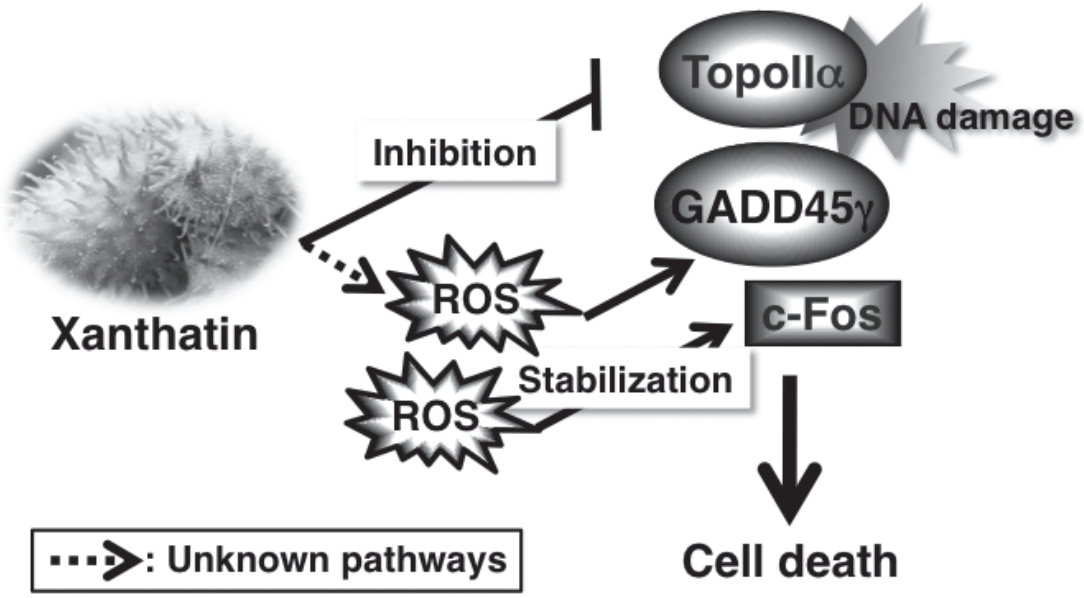


Fig. 5. A working model for the (–)-xanthatin-mediated up-regulation of c-Fos/GADD45γ coupled with cell death. In this study, it was suggested that (–)-xanthatin requires c-Fos in addition to GADD45γ to suppress human breast cancer MDA-MB-231 cell growth. (–)-Xanthatin inhibits Topo IIα (accompanied 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.

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*-Xantahtin, 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). Pre-treatment with NAC completely abolished the up-regula-

tion 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 IIα 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* (Figs. 4B and 4C). Accumulating experimental evidence suggests that the prolonged expression of c-Fos itself, without requiring AP-1 formation with c-Jun, is able to effectively induce cell death in different types of cancer (Preston *et al*., 1996), and etoposide-mediated cell death signaling has been shown to be independent of AP-1 complex formation (Jarvis *et al*., 1999). In the present study, we could not detect the up-regulation of known AP-1-regulated genes, including *collagenase IV*, *cyclin B1*, and *fra-1*, by (–)-xanthatin and etoposide (data not shown) (Fig. 2A). Although we could not address the possible interplay between c-Fos and GADD45γ in the (–)-xanthatin- or etoposide-mediated anti-proliferative effects on MDA-MB-231 cells, these anti-proliferative molecules may 'cooperate with each other' because i) GADD45γ is capable of modulating each of the stress-responsive MAPK (JNK and p38) signaling pathways, leading to the cell death (Takekawa and Saito, 1998; Schaeffer and Weber, 1999; Takeda *et al*., 2011), and ii) c-Fos-mediated cell death can be strongly 'enhanced' by a collaboration with MAPK signaling (Mikula *et al*., 2003). Furthermore, Gajate *et al*. showed that cells lacking c-Fos still underwent apoptotic cell death; therefore, c-Fos itself may not be essential for cell death (Gajate *et al*., 1996), which implies that GADD45γ is the primary molecule of the (–)-xanthatin/etoposide-mediated cell death pathways, and that c-Fos can possibly accelerate the potential of GADD45γ, or GADD45γ can compensate for the action of c-Fos when cancer cells lack the *c-fos* gene.

Here, we focused on the biological effects of (–)-xanthatin on human breast cancer (MDA-MB-231 cells); however, experimental evidence that (–)-xanthatin has broad anti-proliferative effects coupled with cell death for different kinds of cancer cells is accumulating, such as human gastric carcinoma (MKN-45 cells) (Zhang *et al*., 2012a), human non-small-cell lung cancer (A549 cells) (Zhang *et al*., 2012b), and human leukemia (HL-60 cells) (Nibret *et al*., 2011). Taken together with these findings, it is suggested that (–)-xanthatin may be an intriguing chemical entity for inclusion with the other sesquiterpene lactones in future cancer clinical trials (Ghantous *et al*., 2010). In support of this, several possibilities of (–)-xanthatin's potential for cancer prevention and therapy are being reported.

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 IIα, 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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REFERENCES

Adler, V., Yin, Z., Tew, K.D. and Ronai, Z. (1999): Role of redox potential and reactive oxygen species in stress signaling. Oncogene, **18**, 6104-6111.

Blattner, C., Kannouche, P., Litfin, M., Bender, K., Rahmsdorf, H.J., Angulo, J.F. and Herrlich, P. (2000): UV-Induced stabilization of *c-fos* and other short-lived mRNAs. Mol. Cell. Biol., **20**, 3616-3625.

Bruemmer, D., Yin, F., Liu, J., Berger, J.P., Sakai, T., Blaschke, F., Fleck, E., Van Herle, A.J., Forman, B.M. and Law, R.E. (2003): Regulation of the growth arrest and DNA damage-inducible gene 45 (GADD45) by peroxisome proliferator-activated receptor gamma in vascular smooth muscle cells. Cirs. Res., **93**, e38-47.

Dosch, J. and Kaina, B. (1996): Induction of *c-fos, c-jun*, *junB* and *junD* mRNA and AP-1 by alkylating mutagens in cells deficient and proficient for the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) and its relationship to cell death, mutation induction and chromosomal instability. Oncogene, **13**, 1927-1935.

Gajate, C., Alonso, M.T., Schimmang, T. and Mollinedo, F. (1996): C-Fos is not essential for apoptosis. Biochem. Biophys. Res. Commun., **218**, 267-272.

Ghantous, A., Gali-Muhtasib, H., Vuorela, H., Saliba, N.A. and Darwiche, N. (2010): What made sesquiterpene lactones reach cancer clinical trials? Drug Discov. Today, **15**, 668-678.

Hwang, G.W., Lee, J.Y., Ryoke, K., Matsuyama, F., Kim, J.M., Takahashi, T. and Naganuma, A. (2011): Gene expression profiling using DNA microarray analysis of the cerebellum of mice treated with methylmercury. J. Toxicol. Sci., **36**, 389-391.

Hungness, E.S., Pritts, T.A., Luo, G.J., Sun, X., Penner, C.G. and Hasselgren, P.O. (2000): The transcription factor activator protein-1 is activated and interleukin-6 production is increased in interleukin-1β-stimulated human enterocytes. Shock, **14**, 386-391.

Jarvis, W.D., Johnson, C.R., Fornari, F.A., Park, J.S., Dent, P. and Grant, S. (1999): Evidence that the apoptotic actions of etoposide are independent of c-Jun/activating protein-1-mediated transregulation. J. Pharmacol. Exp. Ther., **290**, 1384-1392.

Kurosu, T., Fukuda, T., Miki, T. and Miura, O. (2003): BCL6 overexpression prevents increase in reactive oxygen species and inhibits apoptosis induced by chemotherapeutic reagents in B-cell lymphoma cells. Oncogene, **22**, 4459-4468.

LeBel, C.P., Ischiropoulos, H. and Bondy, S.C. (1992): Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol., **5**, 227-231.

Matsumoto, K., Koyachi, K. and Shindo, M. (2013): Asymmetric total syntheses of xanthatin and 11,13-dihydroxanthatin using a stereocontrolled conjugate allylation to γ-butenolide. Tetrahedron, **69**, 1043-1049.

Matsuo, K., Ohtsuki, T., Yoshikawa, K., Shishido, K., Yokotani-Tomita, M. and Shindo, M. (2010): Total synthesis of xanthanolides. Tetrahedron, **66**, 8407-8419.

Mikula, M., Gotzmann, J., Fischer, A.N., Wolschek, M.F., Thallinger, C., Schulte-Hermann, R., Beug, H. and Mikulits, W. (2003): The proto-oncoprotein c-Fos negatively regulates hepatocellular tumorigenesis. Oncogene, **22**, 6725-6738.

Nibret, E., Youns, M., Krauth-Siegel, R.L. and Wink, M. (2011): Biological activities of xanthatin from *Xanthium strumarium* leaves. Phytother. Res., **25**, 1883-1890.

Oguro, T., Hayashi, M., Nakajo, S., Numazawa, S. and Yoshida, T. (1998): The expression of heme oxygenase-1 gene responded to oxidative stress produced by phorone, a glutathione depletor, in the rat liver; the relevance to activation of c-jun N-terminal kinase. J. Pharmacol. Exp. Ther., **287**, 773-778.

Preston, G.A., Lyon, T.T., Yin, Y., Lang, J.E., Solomon, G., Annab, L., Srinivasan, D.G., Alcorta, D.A. and Barrett, J.C. (1996): Induction of apoptosis by c-Fos protein. Mol. Cell. Biol., **16**, 211-218.

Schaeffer, H.J. and Weber, M.J. (1999): Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol., **19**, 2435-2444.

Shaulian, E. and Karin, M. (2002): AP-1 as a regulator of cell life and death. Nat. Cell Biol., **4**, E131-E136.

Smeyne, R.J., Vendrell, M., Hayward, M., Baker, S.J., Miao, G.G., Schilling, K., Robertson, L.M., Curran, T. and Morgan, J.I. (1993): Continuous *c-fos* expression precedes programmed cell death *in vivo*. Nature, **363**, 166-169. [Erratum in Nature **365**, 279, 1993]

Takeda, S., Ishii, Y., Iwanaga, M., Nurrochmad, A., Ito, Y., Mackenzie, P.I., Nagata, K., Yamazoe, Y., Oguri, K. and Yamada, H. (2009): Interaction of cytochrome P450 3A4 and UDP-glucuronosyltransferase 2B7: evidence for protein-protein association and possible involvement of CYP3A4 J-helix in the interaction. Mol. Pharmacol., **75**, 956-964.

Takeda, S., Matsuo, K., Yaji, K., Okajima-Miyazaki, S., Harada, M., Miyoshi, H., Okamoto, Y., Amamoto, T., Shindo, M., Omiecinski, C.J. and Aramaki, H. (2011): (–)-Xanthatin selectively induces GADD45γ and stimulates caspase-independent cell death in human breast cancer MDA-MB-231 cells. Chem. Res. Toxicol., **24**, 855-865.

Takeda, S., Noguchi, M., Matsuo, K., Yamaguchi, Y., Kudo, T., Nishimura, H., Okamoto, Y., Amamoto, T., Shindo, M., Omiecinski, C.J. and Aramaki, H. (2013a): (–)-Xanthatin up-regulation of the *GADD45γ* tumor suppressor gene in MDA-MB-231 breast cancer cells: Role of topoisomerase IIα inhibition and reactive oxygen species. Toxicology, **305**, 1-9.

Takeda, S., Harada, M., Su, S., Okajima, S., Miyoshi, H., Yoshida, K., Nishimura, H., Okamoto, Y., Amamoto, T., Watanabe, K., Omiecinski, C.J. and Aramaki, H. (2013b): Induction of the *fatty acid 2-hydroxylase (FA2H)* gene by Δ^9-tetrahydrocannabinol in human breast cancer cells. J. Toxicol. Sci., **38**, 305-308.

Takekawa, M. and Saito, H. (1998): A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell, **95**, 521-530.

Toyama, T., Yoshida, E., Shinkai, Y. and Kumagai, Y. (2011): DNA microarray analysis of human neuroblastoma SH-SY5Y cells exposed to methylmercury. J. Toxicol. Sci., **36**, 843-845.

Zhang, F., Lau, S.S. and Monks, T.J. (2011): The cytoprotective effect of *N*-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glutathione synthesis. Toxicol. Sci., **120**, 87-97.

Zhang, L., Tao, L., Ruan, J., Li, W., Wu, Y., Yan, L., Zhang, F., Fan, F., Zheng, S., Wang, A. and Lu, Y. (2012a): Xanthatin induces G2/M cell cycle arrest and apoptosis in human gastric carcinoma MKN-45 cells. Planta. Med., **78**, 890-895.

Zhang, L., Ruan, J., Yan, L., Li, W., Wu, Y., Tao, L., Zhang, F., Zheng, S., Wang, A. and Lu, Y. (2012b): Xanthatin induces cell cycle arrest at G2/M checkpoint and apoptosis via disrupting NF-κB pathway in A549 non-small-cell lung cancer cells. Molecules, **17**, 3736-3750.

Zheng, X., Zhang, Y., Chen, Y.Q., Castranova, V., Shi, X. and Chen, F. (2005): Inhibition of NF-κB stabilizes *gadd45α* mRNA. Biochem. Biophys. Res. Commun., **329**, 95-99.