

Letter

Possible involvement of FosB in (–)-xanthatin-mediated anti-proliferative effects in human cancer MDA-MB-231 cells

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ABSTRACT — Cancer cells can develop resistance to anti-cancer agents. Although some mechanisms have been suggested for this resistance to treatments, further detailed research is required. Historically, sesquiterpene lactones (SLs) have been shown to exhibit toxicity in humans and animals due to their chemical nature. Among the SLs identified to date, (–)-xanthatin, which was originally obtained in an extract from *Xanthium strumarium*, is reportedly less toxic to animals. Furthermore, accumulating evidence suggests that some SLs can kill cancer cells. Therefore, we have focused on (–)-xanthatin and established a method for the chemical synthesis of SLs in order to obtain a pure form. Although we showed that (–)-xanthatin exerts anti-proliferative effects on highly aggressive (poorly differentiated) human MDA-MB-231 breast cancer cells via a mechanism involving the induction of GADD45 γ , a tumor suppressor gene, other molecular target(s) of the molecule have not yet been identified. In the present study, we employed chemically synthesized pure (–)-xanthatin to investigate the targets involved in (–)-xanthatin-mediated cell death. The results obtained revealed marked increases in FosB, the expression of which is suggested to be down-regulated in poorly differentiated breast cancers, and the stimulated expression of FosB as well as cell death by (–)-xanthatin was abrogated by *N*-acetyl-L-cysteine (a ROS-scavenging agent). The possible participation of FosB in (–)-xanthatin-evoked cell death is discussed.

Key words: (–)-Xanthatin, FosB, GADD45 γ , MDA-MB-231 cells, *N*-acetyl-L-cysteine, ROS

INTRODUCTION

Sesquiterpene lactones (SLs) comprise a large group of natural products, and have been shown to exhibit toxicity in animals and humans by non-selectively interacting with cellular macromolecules due to the presence of an α -methylene- γ -lactone moiety (Piovano *et al.*, 2000). Among the SLs identified to date, (–)-xanthatin (Fig. 1A), which was obtained as an extract from *Xanthium strumarium* (the Cocklebur plant), is reportedly less toxic to animals, with an LD₅₀ value of \sim 800 mg/kg; however, this SL has a reactive moiety in its structure

(Roussakis *et al.*, 1994) (Fig. 1A, upper panel). To clarify the involvement of (–)-xanthatin in the extracts (Roussakis *et al.*, 1994; Ramírez-Erosa *et al.*, 2007) and to understand the SL's action mechanism, we have established a method for the complete synthesis of (–)-xanthatin in order to obtain “pure” (–)-xanthatin (Matsuo *et al.*, 2010; Matsumoto *et al.*, 2013) and applied this SL to biochemical analyses focusing on its anti-proliferative effects on the MDA-MB-231 cell line (Takeda *et al.*, 2011, 2013a, 2013b, 2015), a model of basal-like triple negative (*i.e.*, estrogen receptor α , progesterone receptor, and HER2/ ErbB2 negative) highly aggressive breast cancer (Roche-

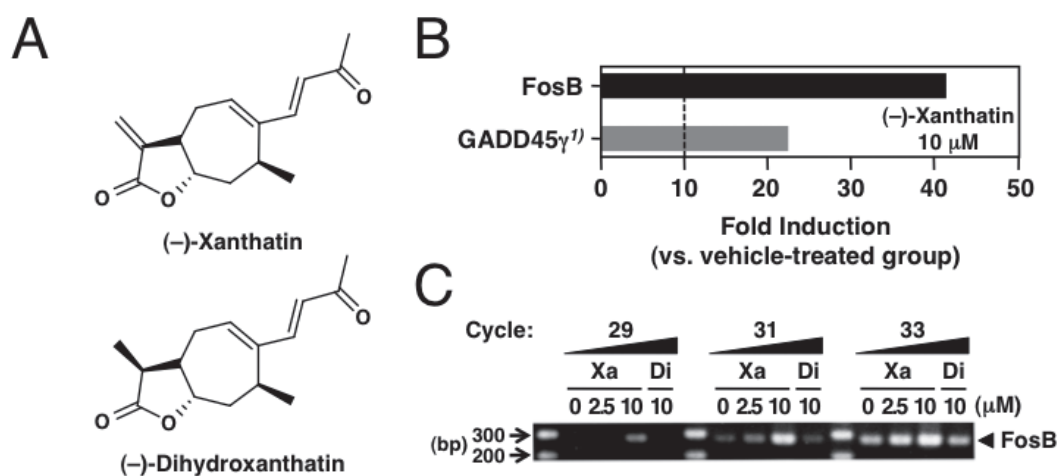


Fig. 1. (-)-Xanthatin-mediated up-regulation of FosB. (A) The chemical structures of (-)-xanthatin and its inactive congener (-)-dihydroxanthatin are shown. When compared to the former, the latter lacks the *exo*-methylene group in its structure. (B) Results of the DNA microarray analysis. Data are expressed as a fold change from the vehicle-treated group. MDA-MB-231 cells were treated with vehicle or 10 μ M (-)-xanthatin for 48 hr, followed by the isolation of total RNA. The results for GADD45 γ 1 in the Figure were taken from a previous study (Takeda *et al.*, 2011)¹⁾. (C) Semiquantitative RT-PCR analysis of the expression of FosB in MDA-MB-231 cells 48 hr after exposure to 2.5 μ M and 10 μ M (-)-xanthatin or (-)-dihydroxanthatin. RT-PCR was performed under constant PCR cycles (29, 31, and 33 cycles) as indicated in the Figure. β -Actin was used as a housekeeping gene for RT-PCR.

fort *et al.*, 2003): the findings of that study indicated that the inhibition of (-)-xanthatin-mediated DNA topoisomerase II α (Topo II α) and production of reactive oxygen species (ROS) are involved in its anti-proliferative effects through the up-regulation of GADD45 γ , an tumor suppressor gene (Ying *et al.*, 2005; Zerbini and Liebermann, 2005). Although we and others have demonstrated the anti-proliferative effects of (-)-xanthatin on MDA-MB-231 cells (Ramírez-Erosa *et al.*, 2007; Takeda *et al.*, 2011, 2013a, 2013b, 2015; Yu *et al.*, 2015), the molecular mechanisms underlying (-)-xanthatin-induced anti-proliferative activity have not yet been elucidated in detail.

FosB, a member of the AP-1 (activator protein-1) family of transcription factors involved in the regulation of cell proliferation/differentiation, has been suggested to play an important role in the normal proliferation/differentiation of mammary epithelial cells, and the down-regulation of FosB has been shown to participate in the dedifferentiation of breast tumorigenesis (Milde-Langosch *et al.*, 2003). Based on this phenomenon, one possibility may be that chemicals up-regulating the molecule of FosB effectively suppress the proliferation of poorly differentiated breast cancer cells, such as triple negative highly invasive MDA-MB-231 cells.

In the present study, we investigated whether (-)-xan-

thatin affects the expression status of FosB in MDA-MB-231 breast cancer cells using chemically synthesized pure (-)-xanthatin (Matsuo *et al.*, 2010; Matsumoto *et al.*, 2013), but not its extract form. The results obtained here showed that (-)-xanthatin stimulated FosB expression levels, and the up-regulated expression of FosB was affected by the application of *N*-acetyl-L-cysteine (NAC), a ROS-scavenging agent, and this was coupled with the abrogation of (-)-xanthatin cell death. We discussed the possible involvement of ROS-sensitive FosB in (-)-xanthatin-mediated cell death signaling.

MATERIALS AND METHODS

Materials and cell culture

(-)-Xanthatin and (-)-dihydroxanthatin were chemically synthesized based on previously reported protocols (Matsuo *et al.*, 2010; Matsumoto *et al.*, 2013), and were purified by HPLC (High-performance liquid chromatography) or column chromatography. Their purities (> 95%) were confirmed by ¹H- and ¹³C-NMR (Nuclear Magnetic Resonance) spectroscopy. Their ring-opened derivatives were not detected in these analyses (Takeda *et al.*, 2011). NAC was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents were of analytical grade, com-

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mercially available, and utilized without further purification. Cell culture conditions and methods were performed as described previously (Takeda *et al.*, 2011). Briefly, human breast cancer MDA-MB-231 cells (obtained from the American Type Culture Collection, Rockville, MD, USA) were routinely grown in phenol red-containing minimum essential medium α (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 5% fetal bovine serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Prior to the initiation of the two SL treatments, medium was changed to phenol red-free minimum essential medium α (Invitrogen) supplemented with 10 mM HEPES, 5% dextrin-coated charcoal-treated serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. Cultures of approximately 60% confluence in a 100-mm Petri dish were used to perform DNA microarray and RT-PCR analyses. (-)-Xanthatin and (-)-dihydroxanthatin were prepared in ethanol. Control incubations contained equivalent additions of ethanol.

Preparation of total RNA and DNA microarray analyses

Total RNA was collected from 10 μ M (-)-xanthatin or vehicle-treated MDA-MB-231 cells (3×10^5 cells/well) 48 hr after exposure using the RNeasy kit (Qiagen, Inc., Hilden, Germany), and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). Specific gene expression patterns in MDA-MB-231 cells were examined by a DNA microarray analysis and compared with those in vehicle controls (Takeda *et al.*, 2011). Total RNA was extracted from both cell types, and the synthesis of complementary DNA (cDNA) and cRNA labeling were conducted using a Low RNA Fluorescent Linear Amplification kit (Agilent, Palo Alto, CA, USA). Hokkaido System Science (Sapporo, Japan) provided assistance with the experiments described above.

Analysis of FosB mRNAs by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from MDA-MB-231 cells using the RNeasy kit (Qiagen, Inc.) and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). The subsequent synthesis of cDNA (complementary DNA), RT, and PCR were performed using the SuperScript™ One-Step RT-PCR System with Platinum *Taq* polymerase (Invitrogen). The primers used for PCR were as follows: FosB (sense), 5'-TCC ATC GAC AGC CCT GAT AGT-3'; FosB (antisense), 5'-TTC CCA CGT CTA GCT TGC AGA-3'. The

PCR primers used for β -actin were taken from previous studies (Steuerwald *et al.*, 2000). 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 ethidium bromide. When the RT reaction was omitted, no signal was detected in any of the samples. β -Actin was used as a housekeeping gene for RT-PCR.

Determination of the FosB mRNA half-life

The FosB transcript half-life ($t_{1/2}$) was determined after a 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 the relative abundance of mRNA was determined by semiquantitative RT-PCR. Values were normalized relative to the value obtained 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 intensity was performed using ImageJ free software (ver. 1.46r, National Institutes of Health; Bethesda, MD, USA).

RESULTS AND DISCUSSION

After the treatment with 10 μ M (-)-xanthatin (Fig. 1A, upper panel) for 48 hr, the morphology of MDA-MB-231 cells became rounder than that of vehicle-treated control cells (data not shown) and cell death was more prominent (~10% viability) (Takeda *et al.*, 2011, 2015). Based on this result, we performed a DNA microarray analysis in order to obtain information on the genes responsible for (-)-xanthatin (10 μ M)-mediated anti-proliferative effects. As shown in Fig. 1B, among the genes up-regulated, the expression of FosB was positively stimulated more than 20-fold by (-)-xanthatin (41.3-fold), and this up-regulation of (-)-xanthatin was approximately two-fold higher than that of GADD45 γ (22.2-fold), an established gene sensitive to treatments with (-)-xanthatin; the result for GADD45 γ in Fig. 1B was taken from a previous study (Takeda *et al.*, 2011). The induction of FosB by (-)-xanthatin was evaluated using a semiquantitative RT-PCR analysis; the expression of RhoB was up-regulated by 10 μ M (-)-xanthatin in a concentration-dependent manner, but not by (-)-dihydroxanthatin (Fig. 1A, lower panel), an inactive congener of (-)-xanthatin, owing to the saturation of the *exo*-methylene group (*i.e.*, Topo II α inhibition/ROS production-negative) (Takeda *et al.*, 2011) (Fig. 1C). The following experiments focused on active (-)-xanthatin. As described in the Introduction section, (-)-xanthatin has the ability to

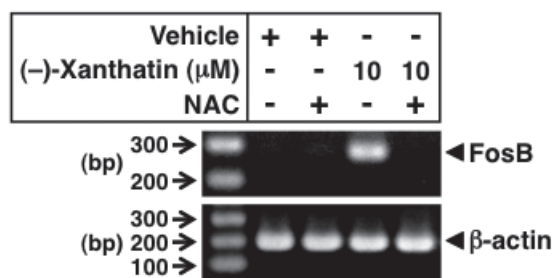


Fig. 2. *N*-Acetyl-L-cysteine-sensitive molecules are involved in the (-)-xanthatin-mediated up-regulation of FosB. RT-PCR analysis of FosB levels in MDA-MB-231 cells 48 hr after exposure to 10 μM (-)-xanthatin in the presence (+) or absence (-) of 1 mM NAC. NAC was added as a pretreatment 2 hr prior to the addition of (-)-xanthatin. β -Actin was used as a housekeeping gene for RT-PCR.

evoked the production of ROS, and this is coupled with cell death; however, rational evidence for ROS production mechanism(s) has not yet been obtained (Takeda *et al.*, 2013a). Although no observable effects of NAC on the expression of FosB were detected, its application clearly blocked the up-regulated expression of FosB as well as the decreases in cell viability induced by (-)-xanthatin to control levels (Fig. 2) (Takeda *et al.*, 2011, 2013a) (data not shown), suggesting that ROS induced by (-)-xanthatin are involved in the induction of FosB in MDA-MB-231 cells.

Since our previous experiments demonstrated that (-)-xanthatin abrogates the decay in GADD45 γ transcripts more than that in vehicle-treated control groups (Takeda *et al.*, 2013a), this phenomenon allowed us to investigate whether FosB mRNAs are also stabilized by this SL. The degree of the decay in FosB mRNA in the presence of (-)-xanthatin was more gradual than that in the vehicle-treated control (*i.e.*, $t_{1/2}$: ~ 3 hr vs. ~ 8 hr) (Fig. 3). Since the decay of FosB mRNA did not depend on the initial amount of the transcripts (data not shown), this difference in the decay of FosB mRNA may be attributed to (-)-xanthatin-mediated biological events, such as ROS production. However, further investigations are needed in order to validate this possibility.

In the present study, FosB, which may be involved in changing mammary epithelial cells into poorly differentiated breast cancer cells (Milde-Langosch *et al.*, 2003), has been suggested to be one of the molecular targets of cell death signaling stimulated by (-)-xanthatin (Fig. 4), and (-)-xanthatin may also have multiple action points to attack cancer cells, indicating its potential as a lead com-

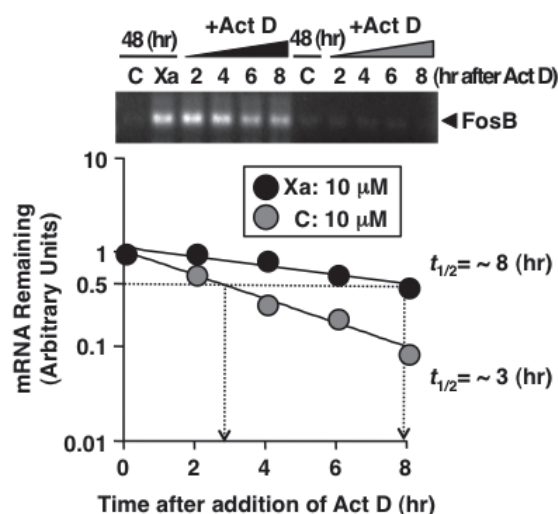


Fig. 3. (-)-Xanthatin abrogation of FosB mRNAs. A representative semi-logarithmic plot of the decay of FosB mRNA is shown. Based on the mRNA decay plot, the mRNA half-life ($t_{1/2}$) was determined for vehicle- and 10 μM (-)-xanthatin-treated samples.

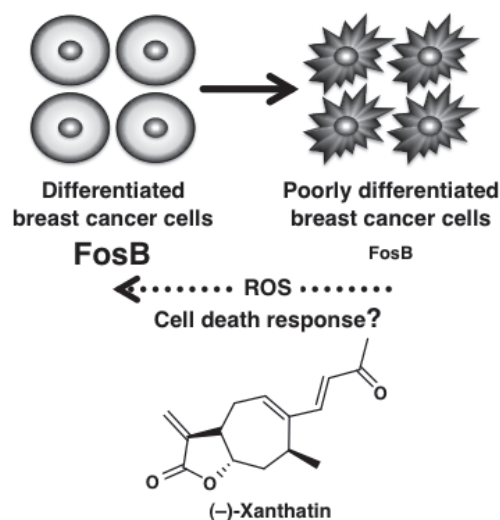


Fig. 4. A working model of (-)-xanthatin-mediated cell death via FosB. A working model of (-)-xanthatin-mediated cell death is described in combination with a previous study (Milde-Langosch *et al.*, 2003). As shown in the Figure, (-)-xanthatin may evoke cell death through the up-regulation of FosB, which is down-regulated in poorly differentiated breast cancer cells (Milde-Langosch *et al.*, 2003). In the Figure, ROS produced by (-)-xanthatin may be involved in the modulation of FosB; however, ROS production mechanism(s) by (-)-xanthatin currently remain unclear (Takeda *et al.*, 2013a).

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pound in the production of cancer chemotherapeutic agents. An analysis of the relationship between Topo II α inhibition/ROS production and FosB induction is ongoing.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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