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 GADD45γ, 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, GADD45γ, 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 ~ 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-
fort et al., 2003): the findings of that study indicated that the inhibition of (–)-xanthatin-mediated DNA topoisomerase IIα (Topo IIα) and production of reactive oxygen species (ROS) are involved in its anti-proliferative effects through the up-regulation of GADD45γ, a 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). 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.

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In the present study, we investigated whether (–)-xanthatin 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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**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 μM (–)-xanthatin for 48 hr, followed by the isolation of total RNA. The results for GADD45γ in the Figure were taken from a previous study (Takeda et al., 2011). (C) Semiquantitative RT-PCR analysis of the expression of FosB in MDA-MB-231 cells 48 hr after exposure to 2.5 μM and 10 μM (–)-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.
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 μg/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 medium was changed to phenol red-free containing minimum essential medium α (Invitrogen, Carlsbad, CA, USA) supplemented with 10 μM HEPES, 5% dextrin-coated charcoal-treated serum, 100 U/mL of penicillin, and 100 μg/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 μM (−)-xanthatin or vehicle-treated MDA-MB-231 cells (3 × 105 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 (anti-sense), 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 (t1/2) 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 μM (−)-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 μM)-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 GADD45γ (22.2-fold), an established gene sensitive to treatments with (−)-xanthatin; the result for GADD45γ 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 μM (−)-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 IIα 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
evoke 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 GADD45γ 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., \( t_{1/2} \approx 3 \) hr vs. \( \sim 8 \) 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-
pound in the production of cancer chemotherapeutic agents. An analysis of the relationship between Topo IIα 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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