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Letter

(–)-Xanthatin-mediated marked up-regulation of RhoB, a sensor for damaged DNA

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ABSTRACT — Sesquiterpene lactones exhibit toxicity in humans and animals by non-selectively interacting with cellular macromolecules. Among the sesquiterpene lactones identified to date, (–)-xanthatin, which was obtained in an extract from *Xanthium strumarium* (the Cocklebur plant), is reportedly less toxic to animals. Although we have shown that (–)-xanthatin has anti-proliferative effects, coupled with the induction of DNA damage-inducible GADD45γ, on highly aggressive human MDA-MB-231 breast cancer cells, the molecular mechanisms of anti-proliferative activity have not yet been elucidated in detail. Furthermore, evidence for the involvement of DNA damage is currently not sufficient. In the present study, we chemically synthesized pure (–)-xanthatin, and attempted to obtain more concrete evidence for DNA damage caused by (–)-xanthatin, which leads to cell death. The results obtained revealed the marked up-regulation of RhoB, which is up-regulated by DNA damage. We summarized the anti-proliferative effects of (–)-xanthatin in combination with our previous findings.

Key words: (–)-Xanthatin, RhoB, GADD45γ, MDA-MB-231 cells, Topoisomerase IIα, ROS

INTRODUCTION

The human MDA-MB-231 cell line is a model of basal-like triple-negative [*i.e.*, estrogen receptor α (ERα), progesterone receptor, and HER2/ErbB2 negative] breast cancer (Rochefort *et al.*, 2003). If breast cancers express ERα, blockers for the receptor may be an effective treatment strategy. Thus, molecular targets need to be identified and chemical candidates developed in order to effectively treat triple-negative breast cancers. Sesquiterpene lactones (SLs) exhibit toxicity in animals and human by non-selectively interacting with cellular macromolecules (Piovano *et al.*, 2000); however, 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 LD_{50} value of ~800 mg/kg (Roussakis *et al*., 1994).

Although we and others (using extracts) demonstrated the anti-proliferative effects of (–)-xanthatin on human MDA-MB-231 breast cancer cells (Ramírez-Erosa *et al*., 2007; Takeda *et al*., 2011, 2013a, 2013b), the molecular mechanisms underlying (–)-xanthatin-induced anti-proliferative activity have not yet been elucidated in detail. We recently obtained evidence to show that i) (–)-xanthatin selectively induces growth arrest and the DNA damage-inducible gene 45 (GADD45) γ isoform, known as a stress sensor (Fornace *et al*., 1989), and ii) (–)-xanthatin inhibits DNA topoisomerase IIα (Topo IIα); inhibition of the Topo IIα enzyme, (possibly) accompanied by DNA damage, leads to deficient DNA replication (Mamouni *et al*., 2014). In addition, the up-regulated levels of DNA damage-inducible GADD45γ by (–)-xanthatin were

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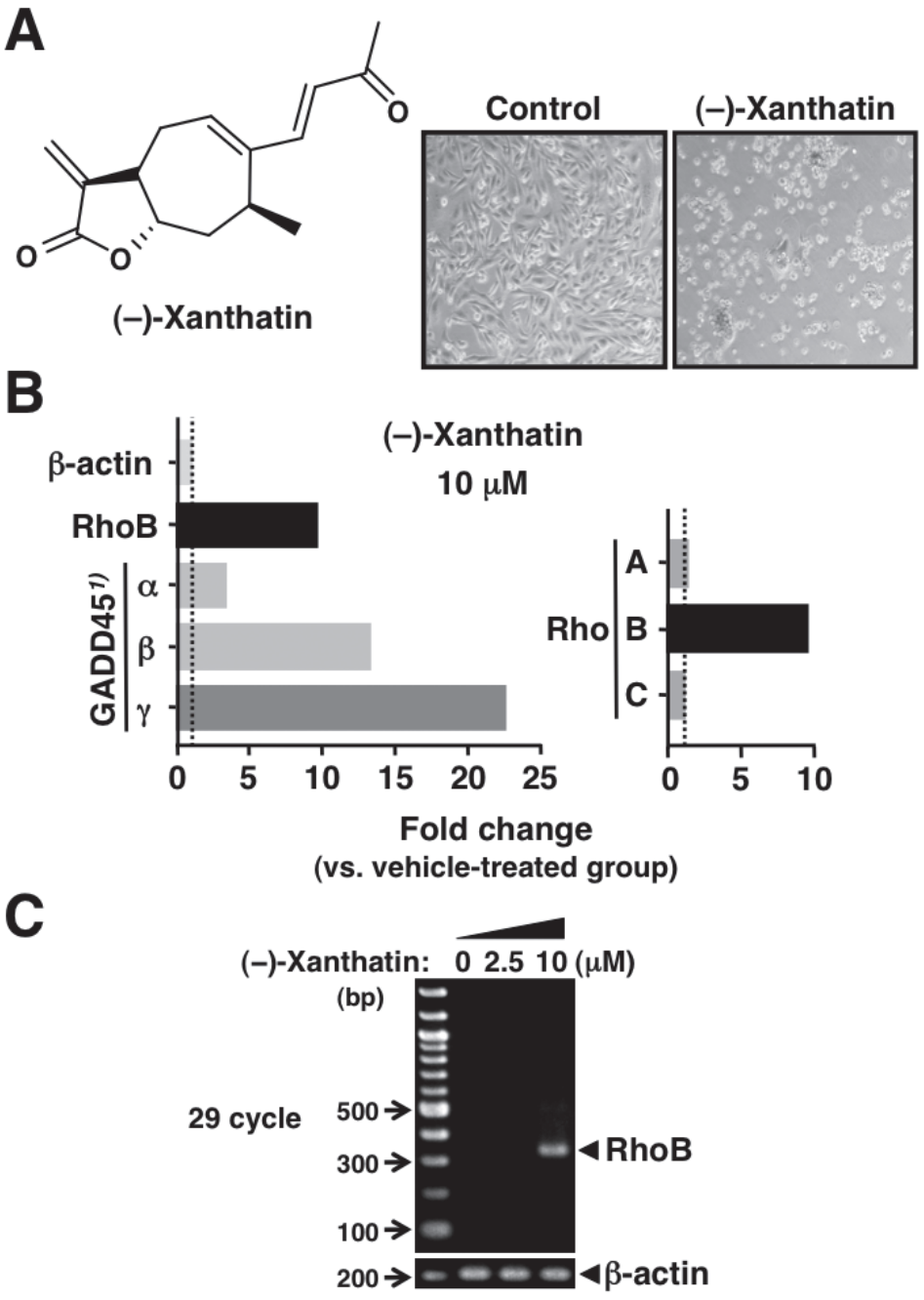

Fig. 1. (–)-Xanthatin markedly up-regulates the expression of RhoB. (A, left panel) The chemical structure of (–)-xanthatin is shown. (A, right panel) Effects of (–)-xanthatin on the morphology and viability of highly aggressive human MDA-MB-231 breast cancer cells. The morphology of MDA-MB-231 cells 48 hr after the (–)-xanthatin (10 μM) treatment. Control cells were treated with vehicle alone. (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 of GADD45α/β/γ in the Figure were taken from a previous study (Takeda *et al*., 2011) [1]. (C) RT-PCR analyses (29 cycles) of RhoB levels in MDA-MB-231 cells 48 hr after exposure to 2.5 μM (below IC_{50}) and 10 μM (beyond IC_{50}) (–)-xanthatin. β-Actin was used as the housekeeping gene for RT-PCR.

returned to control levels by the reactive oxygen species (ROS) scavenger, *N*-acetyl-L-cysteine (NAC). Furthermore, (–)-xanthatin exhibited markedly stronger anti-proliferative activity in MDA-MB-231 cells than etoposide, an established anti-cancer drug (*i.e.*, 5.28 μM vs. 20.37 μM for 48 hr), which utilizes mechanisms of both Topo IIα inhibition and ROS production similar to (–)-xanthatin (Takeda *et al*., 2011, 2013a, 2013b). These findings have been implicated in the (–)-xanthatin-mediated cell death response observed in MDA-MD-231 breast cancer cells (Takeda *et al*., 2011, 2013a, 2013b). However, further experimental evidence is needed for DNA damage primed by (–)-xanthatin.

In the present study, we determined whether (–)-xanthatin affects the expression of RhoB (ras homolog gene family, member B), which is up-regulated by DNA damage (Fritz *et al*., 1995; Mamouni *et al*., 2014), using chemically synthesized pure (–)-xanthatin (Matsuo *et al*., 2010; Matsumoto *et al*., 2013), but not its extract form. The results obtained showed that among members of the Rho family comprising RhoA, RhoB, and RhoC (Wheeler and Ridley, 2004), (–)-xanthatin strongly up-regulated the expression of the RhoB isoform in a NAC-sensitive manner. We summarized the anti-proliferative effects of (–)-xanthatin in combination with our previous findings (Takeda *et al*., 2011, 2013a, 2013b) (*See* Fig. 3B).

MATERIALS AND METHODS

Materials and cell culture

(–)-Xanthatin was chemically synthesized according to a previously reported protocol (Matsuo *et al*., 2010; Matsumoto *et al*., 2013), and was purified by HPLC (High-performance liquid chromatography) or column chromatography. Its purity (> 95%) was confirmed by ^{1}H- and ^{13}C-NMR (Nuclear Magnetic Resonance) spectroscopy. Ring-opened derivatives of (–)-xanthatin 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, commercially available, and used without further purification. Cell culture conditions and methods were performed as described previously (Takeda *et al*., 2011, 2012, 2015) In brief, 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 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, within an atmosphere of 5% CO_2 at 37°C. Prior to the initiation of chemical treatments, the 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 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 was 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 (3×10^5 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). Overall changes in gene expression were evaluated using a two-color microarray-based gene expression analysis (Takeda *et al*., 2011, 2015). 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 RhoA, RhoB, RhoC, and β-actin 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 was performed using the SuperScript™ One-Step RT-PCR System with Platinum *Taq* polymerase (Invitrogen). The primers used for PCR were as follows: RhoA (sense), 5'-TCC ATC GAC AGC CCT GAT AGT-3'; RhoA (antisense), 5'-TTC CCA CGT CTA GCT TGC AGA-3'; RhoB (sense), 5'-TCT CAT GTG CTT CTC GGT GGA-3'; RhoB (antisense); 5'-AGC AGT TGA TGC AGC CGT TC-3'; RhoC (sense), 5'-TGC CTC CTC ATC

GTC TTC AGC-3'; RhoC (antisense), 5'-TCG TCT TGC CTC AGG TCC TTC-3'. 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. The quantification of band intensity was performed using ImageJ free software (ver. 1.46r, National Institutes of Health; Bethesda, MD, USA).

RESULTS AND DISCUSSION

MDA-MB-231 cells exhibited a mesenchymal-like morphology, a hallmark of tumor aggressiveness (Rochefort *et al.*, 2003; Takeda *et al.*, 2012) (Fig. 1A, right panel; Control). As shown in Fig. 1A (right panel), the morphology of most MDA-MB-231 cells became rounder, with marked cell death (~10% viability), compared with the vehicle-treated control cells after exposure to 10 μM (–)-xanthatin for 48 hr (data not shown) (Takeda *et al*., 2011). We performed a DNA microarray analysis in order to obtain information on the genes responsible for DNA damage, followed by a treatment with 10 μM (–)-xanthatin. RhoB was not only more strongly up-regulated than that in the control (9.33-fold), it was also selectively modulated by (–)-xanthatin among the three Rho isoforms (Fig. 1B). The result of GADD45α/β/γ in Fig. 1B (left panel) was taken from a previous study (Takeda *et al*., 2011). The induction of RhoB by (–)-xanthatin was evaluated using a RT-PCR analysis; the expression of RhoB was up-regulated by 10 μM (–)-xanthatin (Fig. 1C). We then performed a semiquantitative RT-PCR analysis on the expression of RhoA/B/C in MDA-MB-231 cells treated with 2.5 μM (below IC_{50}) and 10 μM (beyond IC_{50}) (–)-xanthatin. As expected, the RhoB isoform was strongly up-regulated at 10 μM (–)-xanthatin (Fig. 2). Furthermore, the results obtained showed that although RhoC was not affected, the expression of RhoA appeared to be positively stimulated by (–)-xanthatin (Fig. 2). RhoA inhibits, whereas RhoC enhances cancer cell invasion *in vitro* (Simpson *et al*., 2004; Bellovin *et al*., 2006), and the transcription of RhoB in most tumor-derived cancer cell lines is attenuated due to its potential anti-proliferative roles (Liu *et al*., 2001; Connolly *et al*., 2011). The phenomenon shown in Fig. 2 may be more clearly understood if these findings are applied to our (–)-xanthatin/MDA-MB-231 cells.

As shown in Figs. 1 and 2, RhoB may be one of the molecular targets of (–)-xanthatin in MDA-MB-231 cells. DNA damage-inducible chemicals, such as farnesyl transferase inhibitors (FTIs), have been shown to elevate RhoB levels (Fritz *et al*., 1995; Liu *et al*., 2001). The quenching of ROS prevented the induction of RhoB by FTI, indicating that RhoB is induced as a result of ROS-mediated DNA damage. (–)-Xanthatin utilizes "two biological events" to exert its anti-proliferative effects on MDA-MB-231 cells, namely, i) Topo IIα inhibition (as a catalytic inhibitor) and ii) ROS generation (*See* Takeda *et al*., 2011, 2013a). These activities of (–)-xanthatin mutually evoke DNA damage responses. Thus, since (–)-xanthatin induces the expression of RhoB based on the oxidative DNA damage via the two points, (–)-xanthatin-induced elevations in RhoB may be reduced to control levels by the addition of the ROS scavenger, NAC. An RT-PCR analysis was performed under 32 constant cycles in order to compare control and (–)-xanthatin-treated groups in combination with NAC (*See* Fig. 2). As shown in Fig. 3A, in support of the hypothesis described above, the pre-treatment with NAC completely blocked the up-regulated expression of RhoB induced by (–)-xanthatin. This modulation appears to be specific to the RhoB isoform, suggesting that (–)-xanthatin induces oxidative DNA damage in addition to its catalytic inhibition of Topo IIα (Takeda *et al*., 2013a).

Collectively, the results of the present study suggest that (–)-xanthatin up-regulates the expression of RhoB, which is silenced/maintained at very low levels in MDA-MB-231 cells, and this may result in oxidative DNA dam-

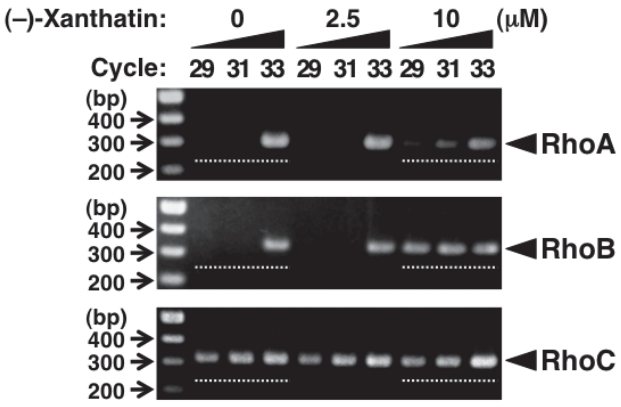

Fig. 2. (–)-Xanthatin up-regulates the expression of RhoB. RT-PCR analyses of the three Rho isoforms A/B/C in MDA-MB-231 cells 48 hr after exposure to 2.5 μM (below IC_{50}) and 10 μM (beyond IC_{50}) (–)-xanthatin. RT-PCR was performed under constant PCR cycles (29, 31, and 33 cycles) as indicated in the Figure.

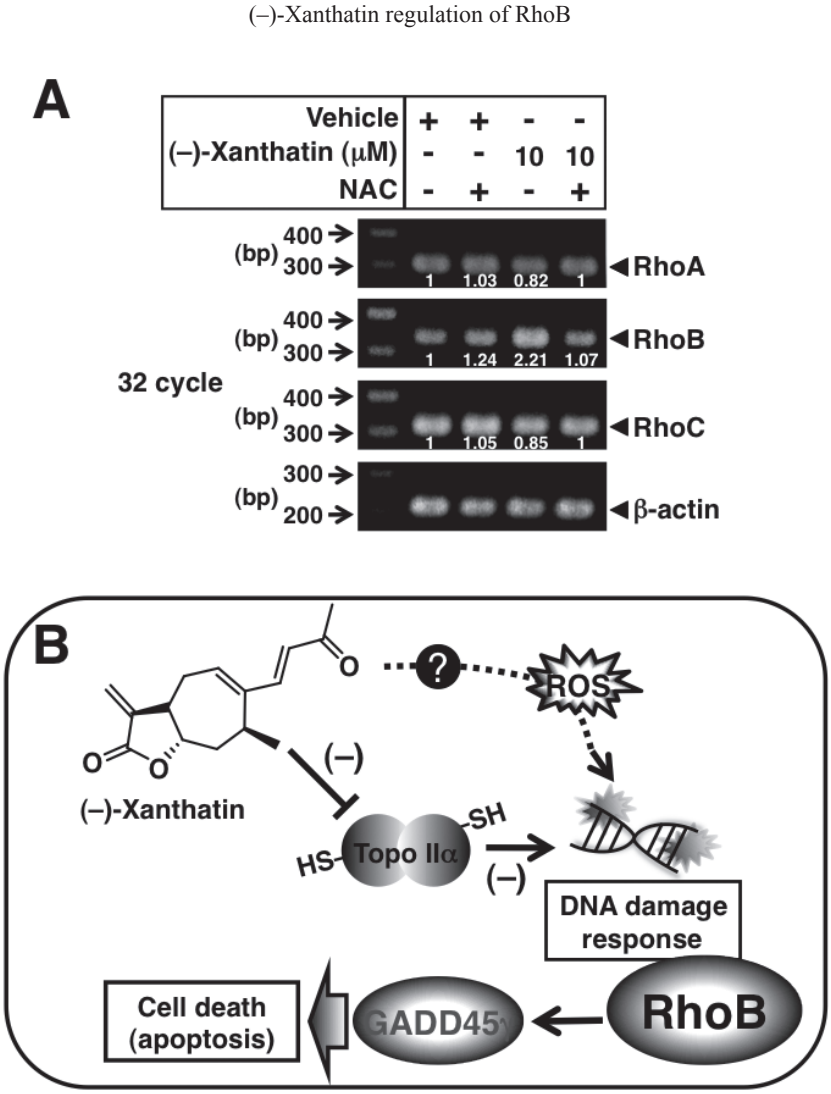

Fig. 3. (–)-Xanthatin up-regulates the expression of RhoB in a NAC-sensitive manner. (A) RT-PCR analyses of RhoB levels in MDA-MB-231 cells 48 hr after exposure to 10 μM (–)-xanthatin in the presence (+) or absence (–) of 1 mM NAC. NAC was added as a pretreatment 2 hr prior to the addition of (–)-xanthatin. β-Actin was used as a housekeeping gene for RT-PCR. (B) A working model of (–)-xanthatin-produced cell death signaling was described in combination with previous findings (Takeda *et al*., 2011, 2013a, 2013b). As shown in the Figure, (–)-xanthatin inhibits the catalytic activity of Topo IIα, which is coupled with the concomitant production of ROS; GADD45γ, which is up-regulated by DNA damage, may be an execution factor in cell death. Thus, evidence for DNA damage by (–)-xanthatin has not yet been obtained. In the present study, (–)-xanthatin markedly up-regulated the expression of RhoB, a hallmark of DNA damage, in MDA-MB-231 cells, in which its expression was maintained at very low levels, and (–)-xanthatin was shown to evoke oxidative DNA damage in collaboration with Topo IIα inhibition and ROS production because the levels of RhoB induced were affected by NAC, a radical scavenger. "?" in the Figure indicates that the ROS production mechanism(s) by (–)-xanthatin remain unclear.

age in concert with the catalytic inhibition of Topo IIα. Since RhoB itself does not evoke cell death responses, other factor(s) may be responsible (Liu *et al*., 2001). Previous studies on (–)-xanthatin-mediated cell death identified the GADD45γ gene, an established tumor suppressor up-regulated by DNA damage (Ying *et al*., 2005; Zerbini and Liebermann, 2005), as a critical execution factor in cell death (Takeda *et al*., 2011). Thus, (–)-xanthatin may induce RhoB as a hallmark of DNA damage, coupled with GADD45γ (an execution factor) in order to induce anti-proliferative effects, resulting from Topo IIα inhibition and ROS production. We herein "(–)-xanthatin-medi-

ated cell death signaling" together with previously reported findings (Fig. 3B).

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

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