BPB Reports

Report

Exposure to (–)-Xanthatin during the Haploid Formation of Mouse Spermatocyte GC-2spd(ts) Cells, an *in vitro* Male Germ Cell Model

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Received November 8, 2021; Accepted December 5, 2021

We and others have previously reported the possible usefulness of (–)-xanthatin, one of the naturally occurring xanthanolides present in the Cocklebur plant, as an anticancer cytotoxic agent in both *in vivo* and *in vitro* settings. Anticancer agents generally act as a double-edged sword during cancer chemoprevention, as these drugs can cause infertility in males and females. Although (–)-xanthatin is known to be an anti-proliferator against cancer cells, the adverse effect on spermatogenesis has not yet been investigated, even *in vitro*. In this study, we utilized chemically synthesized pure (–)-xanthatin to analyze whether this molecule affects the appearance of haploid cells in the population of a GC-2spd(ts) cell line, an *in vitro* model of male germ cells. We did not observe any remarkable effects on the haploid formation of GC-2spd(ts) at a (–)-xanthatin concentration below 0.5 μM.

Key words (–)-xanthatin, haploid, GC-2spd(ts) cells, spermatocyte

INTRODUCTION

The anti-proliferative effect of purified xanthatin from *Xanthium strumarium* L. (not crude extract) was first demonstrated on two murine lymphocytic leukemia cell lines, P388 and L1210 cells, as well as non-small lung carcinoma NSCLC-N6 cells, in 1994.[1)] However, to clarify the biological properties of xanthatin, it is crucial to apply chemically synthesized xanthatin to the experimental systems. Thus, we previously established a method for the total synthesis of (–)-xanthatin (Fig. 1A),[2,3)] and using this pure (–)-xanthatin, we generated substantial experimental evidence to demonstrate that (–)-xanthatin effectively kills cancer cells, especially breast cancer cells, such as human MCF-7 and MDA-MB-231 cells.[4–8)] In these reports, (–)-xanthatin was not only shown to be a catalytic inhibitor of topoisomerase IIα (topo IIα) but also an inducer of GADD45γ,[5)] which is one of the possible molecular targets of anticancer drugs, such as etoposide.[6,9)]

As mentioned above, the anti-proliferative properties of (–)-xanthatin against *in vivo* tumor cell growth as well as *in vitro* cancer cell growth have been repeatedly proven experimentally.[1,4–8,10,11)] Structurally, the (–)-xanthatin molecule contains an *exo*-methylene lactone moiety, which can act as a Michael-type acceptor toward nucleophiles (*i.e.*, Michael donor) in cells (Fig. 1A). Studies have also expressed concerns as to whether (–)-xanthatin could affect normal (non-neoplastic) cells, as observed in the suppression of cancer cell proliferation. According to Yu *et al*., when compared with normal (non-neoplastic) mammary gland epithelial cells (HCC1937), the anti-proliferative effects of (–)-xanthatin are preferably observed in several breast cancer cell lines, including MCF-7 and MDA-MB-231 cells.[10)] These selective anti-proliferative effects on cancer cells have also been documented in a trial between non-small cell lung cancer (A549, H1975, H1650, and HCC827) and normal bronchial epithelial cells, such as BEAS-2B.[11)] Thus, (–)-xanthatin might be considered a desirable candidate against many cancers in the future once the concerns regarding the effects on normal cells are resolved. In clinical settings, anticancer drugs can be used as a single treatment or in combination with other drugs to treat several cancers. However, this can also produce several undesired side effects, such as the disorder of spermatogenic function (spermatogenesis), thereby causing male infertility.

In 1994, Hofmann *et al*. established an *in vitro* model of male germ cells, GC-2spd(ts) cells.[12)] The GC-2spd(ts) cell line can produce postmeiotic cells with the appearance of "haploid cells." Although studies have reported some difficulties (*i.e.*, a failure in haploid cell formation) in handling GC-2spd(ts) cells,[13)] the cells are currently being used as a general model of male germ cells.[14,15)] In the present study, we re-assessed whether GC-2spd(ts) cells maintained in our laboratory could form haploid cells. Following this, the possible effects of (–)-xanthatin on the haploid cell formation were studied.

MATERIALS AND METHODS

Reagents (–)-Xanthatin was chemically synthesized according to a published protocol,[2)] and purified as described previously (purity: > 95%).[4–8)] A commercially available (–)-xanthatin (purity: ≥ 98%) was also used (AdooQ BioScience,

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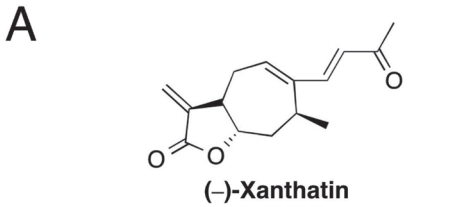

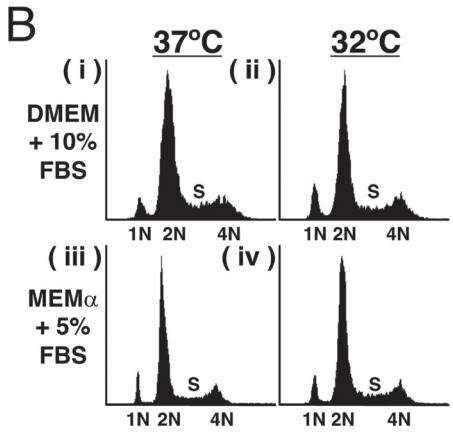

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	(%)	1N	2N	4N	S
(i)	DMEM (37°C)	5.6	58.3	5.6	30.5
(ii)	DMEM (32°C)	9.9	55.2	5.3	29.6
(iii)	MEMα (37°C)	6.3	60.1	6.9	26.6
(iv)	MEMα (32°C)	7.1	58.9	3.8	30.1

Fig. 1. Effects of Culture Conditions on the Haploid Population of GC-2spd(ts) Cells

(A) Structures of (–)-xanthatin. (B and C) GC-2spd(ts) cells were incubated for 6 d post-confluence under the following culture conditions: (i) 37°C with Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS), (ii) 32°C with DMEM/10% FBS, (iii) 37°C with minimum essential medium α (MEMα)/5% FBS, or (iv) 32°C with MEMα/5% FBS. (B) Representative histograms. (C) Percentage of a haploid population (1N), a diploid population (2N), a tetraploid population (4N), and an S phase population.

Irvine, CA, USA) for comparison. No significant differences were observed in the biological activity of either (–)-xanthatin sample. (–)-Xanthatin was dissolved in ethanol.

Cell Culture A mouse spermatocyte-derived GC-2spd(ts) cell line, human breast cancer cell lines, MCF-7 and MDA-MB-231 cells, and human promyelocytic leukemia cell line, HL-60 cells, were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The culture method for GC-2spd(ts) cells was based on previous report.[12] The MCF-7/MDA-MB-231/HL-60 cells were also cultured as described previously.[4,5,16,17]

Flow Cytometry Analysis of the Cell Cycle and Cell Viability Analysis Cell staining with propidium iodide and flow cytometry analysis were performed as described previously.[16] ModFit LT™ v3.0 (Verity Software House, Topsham, ME, USA) was used for data analysis. To determine cell viability, an MTS assay was performed as described previously.[4,5,16] For the determination of IC_{50} value, HL-60 cells were treated with 0.5, 2.5, 5, and 10 μM (–)-xanthatin at 37°C for 48 h. Details for the case of MCF-7 and MDA-MB-231 cells are indicated in the figure legend.

Data Analysis The IC_{50} values were obtained using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA, USA). Differences were considered significant at *P*-values < 0.05. The statistical significance of the difference between multiple groups was determined using one-way ANOVA followed by Dunnett's post-hoc test. Calculations were performed using StatView 5.0J software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Although studies have suggested that the GC-2spd(ts) cell line shows a haploid peak in flow cytometry analysis at 32°C[12] and that GC-2spd(ts) is still being used as a model system for studying spermatogenesis,[14,15] some GC-2spd(ts) cells fail to differentiate into the spermatid state (*i.e.*, postmeiotic stage) with the haploid peak.[13] Thus, we first sought to refine the GC-2spd(ts) cells obtained from ATCC and maintained in our laboratory. Our study was based on the methodology described in the report that originally established the *in vitro* immortalized culture model of male germ cells, i.e., GC-2spd(ts).[12] The GC-2spd(ts) cells express a temperature-permissive p53 mutant protein, which is fully active at 32°C, and partially active at 37°C.[12] We performed flow cytometry analysis of GC-2spd(ts) cells that had been cultured for 6 d post-confluent under culturing at (i) 37°C with Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS), (ii) 32°C with DMEM/10% FBS, (iii) 37°C with minimum essential medium α (MEMα)/5% FBS, or (iv) 32°C with MEMα/5% FBS. As shown in Fig. 1, the clear appearance of haploid cells (indicated as 1N) was observed under all culture conditions, although the percentage of haploid cells in populations varied somewhat in the following order: (ii) 9.9% > (iv) 7.1% > (iii) 6.3% > (i) 5.6%. Among the focused parameters (temperature, medium, and FBS%), temperature was proven to be the most important for detecting a haploid peak in GC-2spd(ts) cells (Fig. 1B and C). Thus, we utilized 32°C with DMEM/10% FBS in the subsequent experiments as the culture condition.

We next studied whether (–)-xanthatin (Fig. 1A) can affect the viability of GC-2spd(ts) cells. The GC-2spd(ts) cells were treated with (–)-xanthatin at concentrations ranging from 0.5 nM to 25 μM at 32°C. No remarkable inhibitory effects were detected at (–)-xanthatin concentrations up to 500 nM. However, upon raising the (–)-xanthatin concentration to more than 1 μM, a striking decrease in the viability of GC-2spd(ts) cells was detected up to 25 μM. Furthermore, the IC_{50} value (48 h) was determined to be 1.45 ± 0.04 μM (Fig. 2A). To date, studies have only examined the anti-proliferative effects of (–)-xanthatin on immortalized cancer cell lines at 37°C. To directly compare the anti-proliferative effects of (–)-xanthatin on GC-2spd(ts) cells with immortalized cancer cell lines, such as breast cancer MCF-7 and MDA-MB-231 cells, we studied the effects of (–)-xanthatin on these cell lines at 32°C. As shown in Fig. 2B, when compared with GC-2spd(ts) cells, the two breast cancer MCF-7 and MDA-MB-231 cells exhibited slight resistance to (–)-xanthatin anti-proliferation, raising the IC_{50} values to 8.0 ± 0.4 and 3.61 ± 0.07 μM, respectively. Considering these findings, we focused on the (–)-xanthatin concentrations less than 1 μM (100, 250, and 500 nM), as these did not affect cell viability, for studying whether (–)-xanthatin

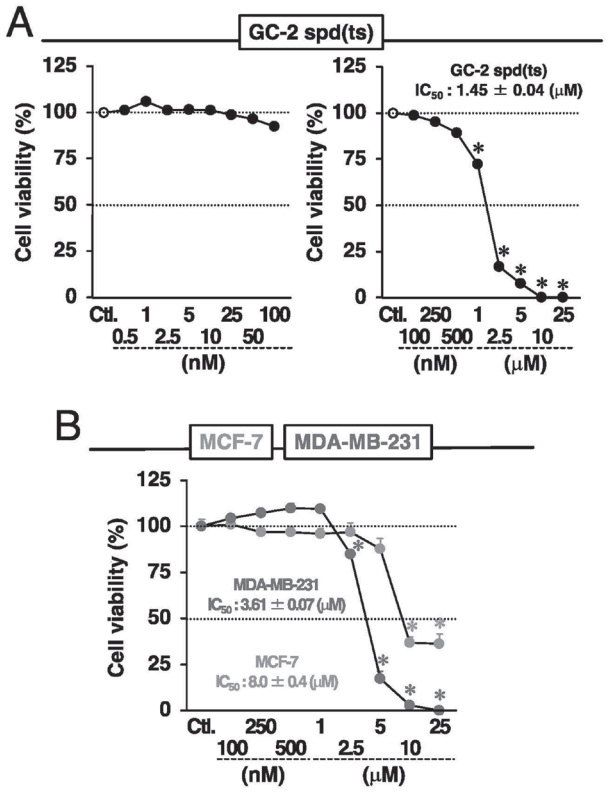

Fig. 2. Effects of (–)-Xanthatin on the Viability of GC-2spd(ts), MCF-7, and MDA-MB-231 Cells

(A) GC-2spd(ts) cells were treated with (–)-xanthatin (0.5 nM–25 μM) at 32°C for 48 h. (B) MCF-7 and MDA-MB-231 cells were treated with (–)-xanthatin (100 nM–25 μM) at 32°C for 48 h. Data are presented as mean ± standard error ($n = 6$), percentage of the vehicle-treated control (Ctl.). *, $P < 0.05$ *versus* the vehicle-treated control.

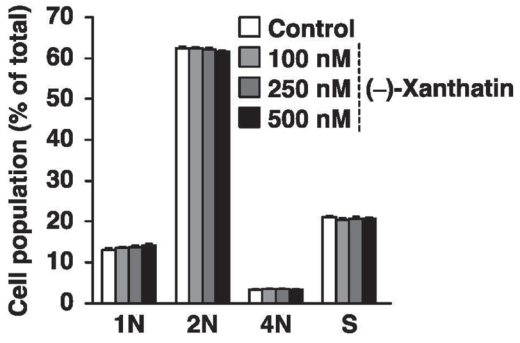

Fig. 3. Effects of (–)-Xanthatin on the Haploid Population of GC-2spd(ts) Cells

GC-2spd(ts) cells were treated with (–)-xanthatin (100–500 nM) at 32°C for 96 h. The percentages of a haploid population (1N), a diploid population (2N), a tetraploid population (4N), and an S phase population are shown. The control group was treated with the vehicle alone. Data are presented as mean ± standard error ($n = 3$).

could affect the haploid formation in GC-2spd(ts) cells. As shown in Fig. 3, although it was not a statistically significant difference ($P < 0.05$) when the control and (–)-xanthatin-treated groups were compared, the cell population after exposure to (–)-xanthatin was somewhat affected; a haploid population (1N) tended to be stimulated in concert with a decrease in a diploid population (2N), implicating possible progress of spermatogenesis. Although (–)-xanthatin exhibited relatively strong ablation in the viability of GC-2spd(ts) cells (*versus* MCF-7/MDA-MB-231 cells) (Fig. 2A and B), when focusing on xanthatin interaction with the mouse leukemic P388 cells, the IC_{50} value has been reported to be 73 nM.[1] Furthermore, in the human leukemic HL-60 cells (–)-xanthatin displayed an IC_{50} value of 1.27 ± 0.21 μM, and at 0.5 μM the residual cell viability was 72.2 ± 4.13% ($P < 0.05$) compared with control cells. Thus, it is suggested that (–)-xanthatin may preferably target leukemia cells as an anti-proliferator. In this study, it was also revealed that the xanthanolide does not exert inhibitory effects on the haploid cell formation at concentrations below 0.5 μM (Fig. 3). Clearly, further studies are required to assess whether (–)-xanthatin can induce a testis-specific marker for acrosome biogenesis in GC-2spd(ts) cells, similar to SP-10.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research [JP21K12261 (to S.T.)] and [JP20H04780 (to M.S.)] from the Japan Society for the Promotion of Science (JSPS) KAKENHI. This study was also supported in part by a Setouchi Satoyama-Satoumi Research Project Grant of FUKUYAMA UNIVERSITY (to S.T.) and in part by the Cooperative Research Program of the Network Joint Research Center for Materials and Devices [Research No. 20211327 (to S.T.)].

Conflict of interest The authors declare no conflict of interest.

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