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Mechanisms of chromosomal aberrations induced by sesamin metabolites in Chinese hamster lung cells

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ABSTRACT

Sesamin is a major lignan in sesame seeds and oil. We previously demonstrated that sesamin induces chromosomal aberrations (CA) in Chinese hamster lung (CHL/IU) cells in the presence of a metabolic activation system (S9 mix), although no genotoxicity was detected *in vivo*. To clarify the mechanism of CA induction by sesamin, we identified its principal active metabolite. A mono-catechol derivative, [2-(3,4-methylenedioxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7-dioxabi-cyclo[3.3.0]octane (SC-1)], was previously identified in culture medium when sesamin was incubated with S9 mix. In the present study, we show that SC-1 induces CA in CHL/IU cells but not in human hepatoblastoma (HepG2) cells. SC-1 was unstable in culture medium. Addition of glutathione (GSH) to the incubation mixture decreased the rate of decomposition and also suppressed induction of CA in CHL/IU cells. These results indicate that SC-1 itself may not contribute to the induction of CA. Two GSH adducts of SC-1 were identified when SC-1 was incubated with GSH, suggesting that SC-1 was converted to the semiquinone/quinone form and then conjugated with GSH in the culture medium. Sodium sulfite (a quinone-responsive compound) also suppressed CA induction by SC-1. These findings strongly suggest that SC-1 is oxidized to semiquinone/quinone derivatives extracellularly in culture medium, that these derivatives are responsible for the induction of CA in CHL/IU cells, and therefore that the positive results obtained with sesamin in *in vitro* CA tests using CHL/IU cells may not be relevant to the assessment of *in vivo* activity.

1. Introduction

Sesame (*Sesamum indicum*) seeds and oil have been recognized as traditional healthy foods since ancient times, particularly in Asia and Africa. During the refinement of non-roasted sesame seed oil, sesamin, a major lignan found in these foods, is partially epimerized to form episesamin [1]. Mixtures of sesamin and episesamin show multiple physiological effects, including antioxidant [2–5], hypocholesterolemic and hypolipidemic [6–10], antihypertensive [11–16], and hepatoprotective activities [17]. Therefore, sesamin and episesamin have attracted much attention as food constituents with potential health benefits. Nakai et al. [2] have shown that sesamin is metabolized by cytochrome P450-catalyzed oxidation to its mono-chatechol derivative [SC-1; 2-(3,4-methylenedioxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo [3.3.0]octane] and then to its di-chatechol derivative [SC-2; 2,6-bis (3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane]; both of these compounds are further metabolized by catechol-*O*-methyltransferase (COMT) catalyzed methylation to the methoxy metabolites SC-1m and SC-2m, respectively. We confirmed that sesamin is subject to extensive first-pass metabolism and that the plasma concentration of SC-1 is much higher than that of sesamin, after oral administration of sesamin in humans [39]; therefore, SC-1 might be responsible for physiological activities.

We previously reported that sesamin induces chromosomal aberrations (CA) in Chinese hamster lung cells (CHL/IU) in the presence of S9 mix (the supernatant from rat liver homogenate). However, no genotoxicity was detected using an *in vitro* bacterial reverse mutation assay (Ames test) with or without S9 mix, or *in vivo* genotoxic tests such as a bone marrow micronucleus (MN) test in mice and a liver cell comet assay in rats [18]. Sesamin only induced CA in the presence of S9 mix, suggesting that a metabolite of sesamin is responsible. No genotoxicity of sesamin has been detected in animals. One reason for this difference may be that the induction of CA in CHL/IU is accompanied by severe cytotoxicity (> 50%). This conclusion is consistent with the revised OECD test guideline (TG473), which notes that the effect of cell toxicity should be considered in the interpretation of CA results and that the CA test should be conducted within an appropriate concentration range [19]. In the present study, we have further examined the molecular

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mechanism underlying CA induction by sesamin in CHL/IU cells.

Previous studies showed that compounds with a catechol structure induce DNA damage and CA in *in vitro* cell systems [20,21]. For example, tea catechins gave positive results in an *in vitro* mouse lymphoma test, CA test, and MN test, but were negative in *in vivo* tests (bone marrow MN test and transgenic rodent gene mutation test) [20,22,23]. Epigallocatechin gallate and dopamine have been reported to generate H_2O_2, semiquinones, and quinones *in vitro*, catalyzed by trace metals in the culture medium, inducing DNA damage and cytotoxicity [23–30,55,56]. Sesamin may act through a similar mechanism because its main metabolite, SC-1, has a catechol structure.

Several rodent and human cell lines, including CHL/IU, are commonly used to predict carcinogenicity. However, different results are observed, depending on the cell line used. HepG2 cells, which retain many of the functions of normal liver cells, are believed to be more useful for genotoxicity assessment of chemicals, compared to other mammalian cell lines which require an exogenous metabolizing system [57,58].

The aim of this study was to clarify the mechanism of CA-induction by sesamin in CHL/IU cells. We identified a major metabolite of sesamin produced by incubation with S9 mix, and evaluated the ability of this metabolite to induce CA in CHL/IU or HepG2 cells. The influence of reducing agents such as reduced glutathione (GSH) and sodium sulfite (Na_2SO_3) on the ability of sesamin and its metabolite to induce CA in CHL/IU cells was also examined.

2. Materials and methods

2.1. Preparation of reagents

Sesamin was purified as described previously [1,2]. Sesamin metabolites (SC-1 and SC-2) were prepared according to the method of Urata et al. [35]. Benzo[*a*]pyrene (B[*a*]P; CAS 50-32-8), dimethyl sulfoxide (DMSO; CAS 67-68-5) and sodium sulfite (Na_2SO_3; CAS 7757-83-7) were obtained from Wako Pure Chemical Industries (Japan). Mitomycin C (MMC; CAS 50-07-7) was purchased from Kyowa Hakko Kirin Co. Ltd. (Japan). Reduced glutathione (GSH; CAS 70-18-8) and silver oxide (Ag_2O; CAS 20667-12-3) were purchased from Nacalai Tesque, Inc. (Japan).

2.2. Cell culture

CHL/IU cells were obtained from Food and Drug Safety Center, Hatano Research Institute (Japan) and were grown in Eagle's minimal essential medium (MEM; GIBCO 11095-080, Japan) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, The Cell Culture Company, Canada). The doubling time was approximately 14.5 h and the modal chromosome number was 25. HepG2 cells were obtained from RIKEN Bioresource Center (Japan) and were grown in Eagle's minimal essential medium (MEM; GIBCO 10370-021, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B and 2 mM L-glutamine. The doubling time was around 39 h and the modal chromosome number was 52 (range 48–54 chromosomes per cell) [36]. The cells were maintained on culture plates at 37 °C in a humidified atmosphere with 5% CO_2 (BNA-111; Tabai Espec Corp., Japan).

2.3. Measurement of sesamin and its metabolites in culture medium

S9 (rat liver 9000 × *g* supernatant fractions, Oriental Yeast Co., Ltd., Japan) was used for metabolic activation. S9 was prepared from the livers of Sprague-Dawley rats pre-treated with phenobarbital and 5,6-benzoflavone. S9 mix had the following composition: S9, 30%; glucose-6-phosphate, 5 mM; $NADP^+$, 4 mM; KCl, 33 mM; $MgCl_2$, 5 mM; and HEPES, 4 mM. Sesamin (dissolved in DMSO) was incubated in the culture medium for CHL/IU cells with S9 mix at 37 °C, 5% CO_2 for 6 h. Before and at 0.5, 1, 4, and 6 h incubation, medium (100 μl) was removed and diluted methanol (200 μl). After centrifugation at 20,000 × *g* for 10 min, the supernatants were subjected to reversed-phase HPLC on a Develosil C30-UG-5 column (150 mm × 4.6 mm i.d.) with UV detection at 280 nm. A constant flow rate of 0.9 ml/min was used. The mobile phase was composed of solvent A (10 mM formic acid) and solvent B (10 mM formic acid in methanol). The following gradient was used: 0–10 min: 80% solvent A/20% solvent B – 45% solvent A/55% solvent B, 10–15 min: 45% solvent A/55% solvent B, 15–27 min: 45% solvent A/55% solvent B – 15% solvent A/85% solvent B, and 27–30 min: 15% solvent A/85% solvent B.

2.4. Chromosomal aberration (CA) assay with CHL/IU and HepG2 cells

The CA assay was carried out as described previously [37,38]. Briefly, CHL/IU cells (5 ml) were seeded at a density of 2.0×10^4 cells/60 mm plate and cultured in a 5% CO_2 incubator at 37 °C for 3 d. HepG2 cells were seeded at a density of 1.0×10^6 cells/60 mm plate and cultured for 24 h. Sesamin or SC-1 was dissolved in DMSO. The cells were treated with sesamin (100 μg/ml; 282 μM) in the presence of S9 mix or with SC-1 at doses of 12.5–50 μg/ml (37–146 μM) for 6 h; B[*a*]P and MMC (final concentrations, 20 and 0.1 μg/ml, respectively) were used as positive controls. GSH and Na_2SO_3 were separately dissolved in distilled water and their final concentrations are indicated in each experiment.

After 6 h treatment, the cells were washed and then grown in fresh medium for another 18 h (CHL/IU cells) or 41 h (HepG2 cells). At 2 h before the end of the incubation, colcemid (final concentration: 0.1 μg/ml, GIBCO) was added to arrest cell mitosis. The culture medium was removed and the cells were trypsinized and suspended in phosphate-buffered saline (PBS (−), Nissui Pharmaceutical Co., Ltd., Japan). The suspension was centrifuged at 1600 × *g* for 5 min and the CHL/IU pellet was resuspended in 0.075 M hypotonic KCl, 6 ml, whereas the HepG2 pellet was resuspended in Optimal Hypotonic Solution, ProCell (GGS-JL005/b; Funakoshi Co., Ltd., Japan), in order to spread the chromosomes. All cells were then fixed in a 3:1 (v/v) fixative solution of methanol and acetic acid. A drop of each suspension was placed on a glass slide, air dried, and stained with 3% Giemsa solution for 8 min. Two or three independent experiments per treatment group were carried out and two specimens were prepared from each plate.

The number of viable cells was counted for each plate and relative cell growth is presented as a percentage of cell growth on the negative control plate, which was considered to be 100%.

Specimens were coded with randomly assigned numbers. Using a microscope, a total of 200 cells/concentration (two specimens) for the CHL/IU cells or 150 cells/concentration (three specimens) for HepG2 cells were evaluated. CAs were classified as structural aberrations. No statistical analyses were conducted.

2.5. Identification of glutathione adducts of SC-1 in culture medium

We evaluated the stability of SC-1 in PBS or culture medium with or without GSH. SC-1 was incubated in PBS and in culture medium with or without GSH at 37 °C for 6 h (cell-free conditions). The concentration of SC-1 was measured by HPLC before and 1, 3, and 6 h after incubation. Medium containing SC-1 and GSH was mixed with an equal volume of methanol. The mixture was centrifuged and the supernatant was analyzed using an ACQUITY UPLC system (Waters Corp., U.S.A.) coupled to a Quattro micro MS System. LC conditions were described previously [39]. Detection was by absorbance at 280 nm.

The electrospray mass spectrometer was operated in positive ion mode. The cone voltage and capillary voltage were 25 V and 3.0 kV, and the source and desolvation temperatures were set at 80 °C and 250 °C, respectively. Full scans and product ion scans with a collision energy of 20 eV were performed.