6-Methylsulfinylhexyl Isothiocyanate Inhibits Cell Cycle Progression in Quiescent JB6 Cells Stimulated with Epidermal Growth Factor

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TH and MK contributed equally to this study. Author TH designed the study and wrote the first draft of the manuscript. Author MK managed the analyses of the study, performed the statistical analysis, and wrote the protocol. Author KK managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The effects of 6-MSITC on cell cycle progression were investigated in quiescent mouse epidermal JB6 cells.

Background: 6-Methylsulfinylhexyl isothiocyanate (6-MSITC) derived from wasabi (*Wasabia japonica*) has been reported to prevent tumor development *in vivo*.

Material and methods: Treatment with epidermal growth factor (EGF) to quiescent JB6 cells, which were serum-starved for 36 h, promoted cell cycle progression from the G₀/G₁ phase to the S phase. Effects of pretreatment with 6-MSITC on cell cycle progression were estimated by flowcytometry and real-time RT-PCR.

Results: Pretreatment with 6-MSITC at 0.25-1.0 µg/ml prior to the growth stimulation with EGF significantly inhibited cell cycle progression. Pretreatment with 6-MSITC inhibited the gene

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expression of DNA synthesis-related proteins cyclin A2, dumbbell former 4, and proliferating cell nuclear antigen.

Conclusion: These results showed that 6-MSITC inhibits cell cycle progression in quiescent cells, accompanied by the inhibition of gene expression of DNA synthesis proteins.

Keywords: 6-Methylsulfinylhexyl isothiocyanate; JB6 cells; Cyclin A; Cell cycle; Epidermal growth factor.

1. INTRODUCTION

Wasabi (*Wasabia japonica*) is a very popularly pungent spice in Japan. It has been reported to have many physiological functions, such as appetite enhancement [1], anti-bacterial activity [2-4] and inhibition of platelet aggregation [5]. The active ingredients of wasabi are considered to be isothiocyanates. Recently, 6-methylsulfinylhexyl isothiocyanate (6-MSITC) has been reported to possess potent anti-cancer effects. For example, oral administration of 6-MSITC reduced the development of mouse skin tumors *in vivo* [6] and suppressed tumor metastasis in mice implanted cancer cells [7]. 6-MSITC suppressed cell proliferation in human breast cancer MCF-7 and MDA-MB-231 cells and human melanoma LOX- IMVI cells [8] and induced apoptosis in human monoblastic leukemia U937 cells and human stomach cancer MKN45 cells [9]. It is well known that induction of apoptosis is associated with cell cycle arrest [10], e.g., benzyl isothiocyanate induces apoptosis mediated by G₂/M cell cycle arrest [11]. However, the effects of 6-MSITC on cell cycle progression are not well known.

Carcinogenesis is generally considered to consist of three distinct steps, carcinogenic initiation, promotion, and progression [12]. Carcinogenic promotion is a relatively lengthy process, in which the initiated cells *i.e.*, mutated cells are transformed into premalignant tumor cells by carcinogenic promoters such as growth factors and chemical agents and start abnormal proliferation. Mouse epidermal JB6 clone 41 cells (JB6 cells) have been used to identify the tumor-promoting activity of various compounds because they respond irreversibly to tumor promoter treatment with colony growth under anchorage-independent conditions and induced tumor formation [13]. Anchorage-independent transformation has been observed in JB6 cells treated with epidermal growth factor (EGF), tumor necrosis factor α [14], and 12-O-tetradecanoylphorbol-13-acetate [15]. Thus, JB6 cells are useful in studies of the molecular events specific to carcinogenic promotion [16]. In addition, quiescent JB6 cells that have had their

cell cycle synchronized into the G₀ phase were used for estimation of the inhibitory effects of chemicals on abnormal proliferation stimulated by growth factors as a tumor promotion model [17].

Most somatic cells are considered to be in the quiescent phase, *i.e.*, G₀ phase of cell cycle and proliferate in the presence of extracellular stimuli including EGF [18]. Extracellular growth signals promote cell cycle progression mediated by their receptors. EGF activates cyclin-dependent kinase (CDK) 4/6, resulting in increased phosphorylation of retinoblastoma (Rb) protein [19]. Phosphorylated Rb protein then releases a transcription factor, E2Fs [20], resulting in the gene expression of DNA synthesis-related proteins such as cyclin A2 [21], dumbbell former 4 (Dbf4) [22], and proliferating cell nuclear antigen (PCNA) [23]. These proteins regulate DNA replication in the S phase [24].

In the present study, the effects of 6-MSITC on cell cycle progression from the G₀ phase, phosphorylation of Rb protein, and mRNA expression of cyclin A2, Dbf4, and PCNA were investigated in quiescent JB6 cells stimulated by EGF.

2. MATERIALS AND METHODS

2.1 Chemicals

Eagle's minimum essential medium (MEM), penicillin, streptomycin, ribonuclease A (RNase A), and propidium iodide (PI) were purchased from Sigma Chemicals (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Biowest (Nuaille, France). Phospho-Rb (Ser780) and phospho-Rb (Ser795) antibodies were from Cell Signaling Technology (Beverly, MA, USA), and Rb antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 6-MSITC was provided from Kinjirushi (Aichi, Japan). All other chemicals used were of the highest quality commercially available.

2.2 Cell Culture

The mouse epidermal JB6 clone 41 cells (JB6 cells) were provided by Dr. Zigang Dong of the

Hormel Institute, University of Minnesota (Austin, MN, USA) and maintained in MEM containing 5% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin in an atmosphere of 95% air-5% CO₂ at 37°C. Synchronization of the cell cycle into the G₀ phase was performed as described previously [17]. Briefly, JB6 cells were seeded at 5.0×10⁴ cells/ml in dishes and cultured for 24 h. The cell cycle was synchronized by serum-starvation with 0.1% FBS in MEM containing 50 units/ml penicillin and 50 µg/ml streptomycin for 36 h. The cells that were found to be more than 80% synchronized into the G₀/G₁ phase by cell cycle analysis were used in the experiments as quiescent JB6 cells. Quiescent JB6 cells were pretreated with 6-MSITC for 30 min and treated with EGF at 300 pg/ml to stimulate cell growth.

2.3 Cell Cycle Analysis

The cell cycle was analyzed by a published method [17] with a slight modification. Cells synchronized on a 60-mm dish were collected by trypsinization, washed twice with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ethanol at -20°C overnight. The cells were then washed twice with PBS to remove ethanol completely and incubated with 50 µg/ml PI and 20 µg/ml RNase A in PBS at room temperature for 30 min in the dark. The fluorescence was measured by a flowcytometer using on EPICS-XL equipped with EXPO32 flowcytometry software (Beckman coulter, Fullerton, CA, USA). The data were analyzed by using Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

2.4 Morphological Observation of Cells

Cells synchronized on a 60-mm dish were washed twice with ice-cold PBS and fixed with 70% ethanol at -20°C overnight. The cells were washed twice with PBS, and the nuclei were stained with 1 µg/ml of PI in PBS at room temperature for 10 min in the dark. The cells were observed by a phase-contrast and fluorescent microscopes.

2.5 Western Blotting Analysis

Western blotting analysis was performed according to the protocol provided by Cell Signaling Technology with slight modifications. In brief, cells synchronized on a 100-mm dish were washed twice with ice-cold PBS and harvested with 20 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5

mM sodium pyrophosphate decahydrate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysate was stood on ice for 1 h with occasional mixing and centrifuged at 15,000 × g for 10 min at 4°C. The supernatant was collected as a cell lysate and stored at -20°C until use. The protein concentration of the cell lysate was determined by the Lowry method [25]. Aliquots of 30 µg cell lysate protein were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a poly(vinylidene difluoride) membrane (GE Healthcare, Piscataway, NJ, USA). After blocking the membrane with Blocking One or Blocking One-P (Nacalai Tesque, Kyoto, Japan) and washing them thrice with TBST buffer composed of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20, they were incubated with a primary antibody in TBST buffer containing 5% bovine serum albumin. Then, the membranes were washed thrice with TBST buffer and incubated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare). The immunoblots were visualized by an enhanced chemiluminescence kit (ECL-plus; GE Healthcare) according to the manufacturer's protocol.

2.6 Real-time reverse transcription-polymerase chain reaction (RT-PCR)

The expression of mRNA was quantitatively measured by the real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) using the Applied Biosystems 7500 Fast (Applied Biosystems, Foster City, CA, USA) and related reagent kits according to the manufacturer's protocol. Total RNA was extracted from cells synchronized on a 60-mm dish with the RNeasy[®] Kit (Applied Biosystems). Complementary DNA (cDNA) was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit with an RNase Inhibitor (Applied Biosystems). TaqMan[®] Gene Expression Assays Inventoried; Mm00438064_ml for *Ccna2* (mRNA of cyclin A2), Mm00516462_ml for *Dbf4*, and Mm00448100_gi for *Pcna* (Applied Biosystems) was used as the PCR primer set for the real-time PCR. 18S ribosomal RNA was used as an endogenous control. Real-time PCR was performed with the TaqMan[®] Fast Universal PCR Master Mix (Applied Biosystems). The following protocol was run: denaturation at 90°C for 20 sec, amplification at 95°C for 3 sec, and quantification at 60°C for 30 sec were repeated 40 times. The mRNA of *Ccna 2*, *Dbf4*, and *Pcna* and 18S

ribosomal RNA were fluorescently determined by 5'-end labelling with FAM™ and VIC™, respectively. Data were normalized to 18S ribosomal RNA expression and presented as a ratio to the control cells, which were treated with neither EGF nor 6-MSITC.

2.7 Statistical Analysis

Data were expressed as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using the Turkey-Kramer test. The results were considered to be significant at $P < 0.05$.

3. RESULTS

3.1 6-MSITC Inhibited Cell Cycle Progression in Quiescent JB6 Cells

To investigate the effect of 6-MSITC on cell cycle progression stimulated by cell growth signal, quiescent JB6 cells (approximately 89% and 1% of which were in the G₀/G₁ and S phases, respectively) were pretreated with or without 6-MSITC for 30 min and treated with 300 pg/ml EGF as a stimulator of cell growth. After treatment with EGF, the cell population in the G₀/G₁ phase decreased, and that in the S phase increased time-dependently (Fig. 1). The G₀/G₁ phase population decreased to $5.9 \pm 1.2\%$, and the S phase population increased to $90.6 \pm 1.5\%$, 16 h after stimulation with EGF (Fig. 1). Thus, EGF stimulated cell cycle progression in quiescent JB6 cells time-dependently. On the other hand, pretreatment with 0.5 $\mu\text{g/ml}$ 6-MSITC significantly inhibited cell cycle progression, and $77.7 \pm 3.4\%$ of the cells remained in the G₀/G₁ phase 16 h after EGF-stimulation (Fig. 1). Additionally, pretreatment with 6-MSITC inhibited cell cycle progression in a dose-dependent manner in the range of 0.25-1.0 $\mu\text{g/ml}$, but not at 0.1 $\mu\text{g/ml}$ (Table 1). Morphological observation

also showed the appearances of mitotic cells 16 h after the EGF-stimulation, but not in 6-MSITC-pretreated cells as in the EGF-untreated cells (Fig. 2).

3.2 6-MSITC Did Not Change the Phosphorylation Level of Rb Protein

It is known that growth stimulation phosphorylates the serine 780 (Ser780) and serine 795 (Ser795) of Rb protein via the activation of CDK4/6 [26,27]. To elucidate the inhibitory mechanism of 6-MSITC on cell cycle progression, the effects of 6-MSITC on the phosphorylation levels of Rb protein were examined. Treatment with EGF increased the phosphorylation levels of Rb protein at Ser780 and Ser795 3 h after the stimulation, and the phosphorylated Rb protein remained at 9 h (Fig. 3). Pretreatment with 6-MSITC did not suppress the increased phosphorylation (Fig. 3). These results indicate that 6-MSITC did not affect the upstream intracellular cell growth signals related to the phosphorylation of Rb protein.

3.3 6-MSITC Inhibited the mRNA Expression of DNA Synthesis-related Proteins

Hypo-phosphorylated Rb protein binds to transcription factors including E2Fs and inactivates them, but hyper-phosphorylated Rb protein releases E2Fs [20] and induces the gene expression of DNA synthesis-related proteins such as cyclin A2 [21], Dbf4 [22], and PCNA [23]. Treatment with EGF significantly increased the mRNA expression of *Ccna2*, *Dbf4*, and *Pcna* by 4.0-fold, 4.1-fold, and 2.7-fold, respectively, compared with untreated cells 16 h after the EGF-stimulation (Fig. 4). On the other hand, pretreatment with 6-MSITC at 0.5 $\mu\text{g/ml}$

Table 1. 6-MSITC inhibited cell cycle progression after quiescent JB6 cells were stimulated by EGF

6-MSITC ($\mu\text{g/ml}$)	EGF	G ₀ /G ₁ phase (%)	S phase (%)	G ₂ phase (%)
None	-	89.9 $\pm$ 7.6 ^a	5.7 $\pm$ 3.7 ^a	4.4 $\pm$ 4.1 ^{abd}
None	+	13.5 $\pm$ 1.3 ^b	82.7 $\pm$ 1.2 ^b	3.8 $\pm$ 0.2 ^{ab}
0.1	+	13.0 $\pm$ 0.7 ^b	84.1 $\pm$ 2.6 ^b	2.8 $\pm$ 2.5 ^{ab}
0.25	+	29.4 $\pm$ 5.2 ^c	56.6 $\pm$ 6.5 ^c	14.0 $\pm$ 1.3 ^{cd}
0.5	+	87.7 $\pm$ 5.5 ^a	0.8 $\pm$ 1.4 ^a	11.5 $\pm$ 4.2 ^{acd}
1.0	+	92.6 $\pm$ 0.3 ^a	0.3 $\pm$ 0.3 ^a	7.1 $\pm$ 0.4 ^{abcd}

Quiescent JB6 cells were pretreated for 30 min with the indicated concentration of 6-methylsulfinylhexyl isothiocyanate (6-MSITC) and treated with 300 pg/ml epidermal growth factor (EGF) for 16 h. Cell cycle analysis was performed by flowcytometry. The values are mean $\pm$ SD (n=3). Different letters in each phase indicate significant differences ($p < 0.05$) according to the Turkey-Kramer test ($p < 0.05$)

significantly inhibited the mRNA expression of *Ccna2* and *Dbf4*, and at 1.0 $\mu\text{g/ml}$ inhibited that of *Pcna*. Thus, the inhibitory effect of 6-MSITC

on cell cycle progression was accompanied by suppression of the EGF-increased gene expression of DNA synthesis-related proteins.

Fig. 1. 6-MSITC inhibited cell cycle progression after quiescent JB6 cells were stimulated by EGF

Quiescent JB6 cells were pretreated for 30 min with or without 0.5 $\mu\text{g/ml}$ 6-methylsulfinylhexyl isothiocyanate (6-MSITC) and treated with 300 pg/ml epidermal growth factor (EGF) for 12, 14, 16, and 18 h. Cell cycle analysis was performed by flowcytometry as described in Materials and methods. The values are mean $\pm$ SD ($n=3$). The right and left panels showed the representative data from the cells pretreated with and without 6-MSITC, respectively

Fig. 2. Morphological observation of 6-MSITC- and/or EGF -treated quiescent JB6 cells
Quiescent JB6 cells were pretreated for 30 min with 0.5 and 1.0 $\mu\text{g/ml}$ 6-MSITC and treated with 300 pg/ml EGF for 16 h. The cells were stained with propidium iodide as described in Materials and methods and observed by a phase-contrast and florescent microscopes. Arrows show mitotic cells

4. DISCUSSION

In the present study, the effects of 6-MSITC on cell cycle progression were investigated using quiescent JB6 cells stimulated with EGF as a cellular model for studying carcinogenic promotion. 6-MSITC at 0.25 to 1.0 $\mu\text{g/ml}$ (1.2 to 4.8 μM) inhibited cell cycle progression in quiescent JB6 cells (Table 1) accompanied by inhibition of *Ccna2*, *Dbf4*, and *Pcna* mRNA expression (Fig. 4). Cyclin A2 and Dbf4 play important roles in DNA replication in the S phase of the cell cycle [24]. For the initiation of DNA replication, origin recognition complex proteins such as Cdc6 and minichromosome maintenance (MCM) proteins sequentially bind to chromatin and form the pre-initiation complex. After Cdc6 was phosphorylated by complexes of CDK2 and cyclin A2 and/or of Cdc7 and Dbf4 and then released from chromatin, MCM protein is loaded onto chromatin. MCM protein is further phosphorylated by complexes of CDK2 and cyclin A2 and/or of Cdc7 and Dbf4 and exerts a DNA helicase activity. Thus, the initiation of DNA replication in the S phase of the cell cycle depends on the complexes of CDK2 and cyclin A2 and/or of Cdc7 and Dbf4 [24]. Probably, 6-MSITC-suppression of *Ccna2* and *Dbf4* mRNA expression is implicated in the suppression of the activation of MCM helicase activity mediated by suppression of the phosphorylation of Cdc6 and MCM proteins. Additionally, p21 binds to PCNA [28] and inhibits PCNA-stimulated DNA polymerase activity [29]. Histone deacetylases (HDAC) negatively control the acetylation level of chromatin and its gene transcription [30]. Inhibition of HDAC promotes expression of specific genes including *p21* [31]. 6-MSITC is reported to inhibit HDAC activity at 15 μM in human colon cancer HCT116 cells [32]. Thus, 6-MSITC might increase p21 by inhibition of HDAC activity, leading to suppression of DNA polymerase activity by binding PCNA as in the present study.

Over-expression of cyclin A2, Dbf4, and PCNA has been reported in several cases of cancer. Volm et al. [33] indicated that out of the 187 non-small-cell lung carcinomas, 141 cases (75%) showed expression of cyclin A. Patients with cyclin A-positive carcinomas had significantly shorter median survival times than patients with cyclin A-negative carcinomas (79 vs. 129 weeks, $P = 0.045$). Over-expression of cyclin A has been shown to be a negative prognostic indicator of carcinomas in the kidneys [34] and colorectum [35]. Bonte et al. [36] indicated that Cdc7 protein

showed increased expression in approximately 50% of the 62 human tumor cell lines they examined, including colon, non-small cell lung, kidney, cancer, prostate, and breast cancers, and most cell lines with increased Cdc7 protein showed increased Dbf4 abundance. On the other hand, PCNA is used as a proliferative activity marker in gastric cancer [37], renal cell carcinoma [38] and colonic adenoma [39]. The present study demonstrated the cancer preventing activity of 6-MSITC using a cellular model to study carcinogenic promotion, and the cancer preventing activity was accompanied by inhibition of *Ccna2* and *Dbf4* expression (Fig. 4). If 6-MSITC inhibits the expression of cyclin A and Dbf4 in cancer cells, it may be able to be used as an anti-cancer drug for cancers associated with over-expression of these proteins.

Fig. 3. Pretreatment with 6-MSITC did not affect the phosphorylation levels of Rb protein in quiescent JB6 cells

Quiescent JB6 cells were pretreated for 30 min with or without 0.5 $\mu\text{g/ml}$ 6-MSITC and treated with 300 pg/ml EGF for 3, 6, and 9 h. The phosphorylation levels of Rb protein were examined by Western blotting analysis as described in Materials and methods

It is well known that isothiocyanates are rapidly absorbed in the stomach and intestines and conjugated to glutathione (GSH) in the liver [40]. ω -MSITC is also conjugated with GSH non-enzymatically to form dithiocarbamate (6-MSITC-GSH adducts) [5]. 6-MSITC is also absorbed and rapidly enters the circulatory system as 6-MSITC or its GSH conjugate [41]. After oral administration of 6-MSITC in rats, the plasma concentration of 6-MSITC and 6-MSITC-GSH adducts reached its maximum within 30 min and began to decrease within 1 h. However, this decrease was relatively slow, and it was detected in the plasma even at 4 h. Although the metabolism of 6-MSITC in humans is not well understood, the metabolism of its analogue 4-methylsulfinylbutyl isothiocyanate (4-MSITC) has been studied and reported [42,43]. A human perfusion experiment showed that about 75 % of the dosed 4-MSITC was absorbed in the jejunum, and a portion of the absorbed 4-MSITC returned

to the lumen of the jejunum as complexes of 4-MSITC and GSH [42]. In human, an oral dose of 200 μ mol broccoli sprouts isothiocyanates, in which the major isothiocyanate is 4-MSITC, produced a plasma concentration of isothiocyanate of 1.6 μ M after 1 h [43]. In the present study, 6-MSITC inhibited cell cycle progression at 0.25 to 1.0 μ g/ml (1.2 to 4.8 μ M).

If the pharmacokinetics of 6-MSITC resemble those of 4-MSITC, the effective concentration in the present study might be bioavailable for humans. Because 100 g fresh weight of wasabi rhizome contains 55.0-55.6 mg of 6-MSITC [41], 75 g of wasabi are needed to provide 200 μ mol 6-MSITC orally.

Fig. 4. Pretreatment with 6-MSITC inhibited the mRNA expression of *Ccna2*, *Dbf4*, and *Pcna*
Quiescent JB6 cells were pretreated for 30 min with 0.1, 0.25, 0.5, and 1.0 μ g/ml 6-MSITC and treated with 300 μ g/ml EGF for 16 h. The mRNA expression of *Ccna2* (A), *Dbf4* (B), and *Pcna* (C) were quantitatively measured by real-time RT-PCR as described in Materials and methods. Data were normalized to 18S ribosomal RNA expression and presented as a ratio to the cells treated with neither 6-MSITC nor EGF. The values are mean $\pm$ SD ($n=3$). Different letters indicate significant differences ($p < 0.05$) according to the Turkey-Kramer test ($p < 0.05$)

The present study demonstrated that 6-MSITC has inhibitory effects on cell cycle progression. Wasabi contains other kinds of isothiocyanates, such as allyl isothiocyanate, *n*-butyl isothiocyanate, 3-butenyl isothiocyanate, 4-pentenyl isothiocyanate, 5-hexysenyl isothiocyanate, 5-methylsulfinylpentyl isothiocyanate, 6-MSITC, 7-methylsulfinylheptyl isothiocyanate, 5-methylthiopentyl isothiocyanate, 6-methylthiohexyl isothiocyanate (6-MTITC), and 7-methylthioheptyl isothiocyanate [44]. It is reported that allyl isothiocyanate, which is contained 118 mg in 100 g fresh weight of wasabi rhizome [2], inhibited the proliferation of human prostate cancer PC-3 and LNCaP cells by causing G₂/M arrest at 5-20 μ M and induced apoptosis [45]. It is also reported that pretreatment with 6-MTITC for 4 consecutive days at 5 μ mol/day suppresses 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J strain female mice [46]. Thus, consumption of dietary wasabi is expected to provide the additive anti-carcinogenic activity of several isothiocyanates. To clarify such a hypothesis, it is necessary to study the inhibitory effects of dietary wasabi and / or 6-MSITC *in vivo* on gene expression of DNA synthesis proteins; Ccna2, Dbf4 and Pcn. On the other hand, it is also necessary to investigate a comprehensive biological molecular mechanism in the inhibitory effect of 6-MSITC on EGF-induced cell cycle progression in quiescent JB6 cells.

5. CONCLUSION

6-MSITC characteristic in wasabi (*Wasabia japonica*) has been reported to prevent tumor development *in vivo*. The present study indicated that 6-MSITC inhibited cell cycle progression in quiescent JB6 cells. The inhibitory effect was accompanied by the inhibition of gene expression of DNA synthesis proteins, Ccna2, Dbf4 and Pcn.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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