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Differential Effect of Schisandrin B Stereoisomers on ATR-Mediated DNA Damage Checkpoint Signaling

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Abstract. We have previously reported that schisandrin B (SchB) is a specific inhibitor of ATR (ataxia telangiectasia and Rad-3-related) protein kinase. Since SchB consists of a mixture of its diastereomers gomisin N (GN) and γ-schisandrin (γ-Sch), the inhibitory action of SchB might result from a stereospecific interaction between one of the stereoisomers of SchB and ATR. Therefore, we investigated the effect of GN and γ-Sch on UV (UVC at 254 nm)-induced activation of DNA damage checkpoint signaling in A549 cells. UV-induced cell death (25 – 75 J/m2) was amplified by the presence of the diastereomers, especially GN. At the same time, GN, but not γ-Sch, inhibited the phosphorylation of checkpoint proteins such as p53, structural maintenance of chromosomes 1, and checkpoint kinase 1 in UV-irradiated cells. Moreover, GN inhibited the G2/M checkpoint during UV-induced DNA damage. The in vitro kinase activity of immuno-affinity-purified ATR was dose-dependently inhibited by GN (IC50: 7.28 μM) but not by γ-Sch. These results indicate that GN is the active component of SchB and suggest that GN inhibits the DNA damage checkpoint signaling by stereospecifically interacting with ATR.

Keywords: schisandrin B, gomisin N, ataxia telangiectasia and Rad-3-related (ATR), checkpoint, DNA damage

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Introduction

DNA provides the genetic information for a cell and damage to the DNA can have a major impact on cellular activities. DNA damage actually occurs routinely, caused by exogenous and endogenous factors such as radiation, chemicals, and reactive oxygen species (1), and cells have therefore developed DNA damage responses (DDR) for the protection and repair of such damage, in order to maintain genomic stability (2). The DNA damage checkpoint is one of the functions of DDR. When cells sense DNA damage, the cell cycle is arrested at the checkpoint in the G1, S, G2, or M phase for either the damage to be repaired or, when the damage is not repairable, for apoptosis to be induced. Two protein kinases, ATR (ataxia telangiectasia and Rad-3-related) and ATM (ataxia telangiectasia mutated), play a central role in activating DNA damage checkpoint signaling, that is, the phosphorylation of downstream effector proteins such as p53, structural maintenance of chromosomes 1 (SMC1), and checkpoint kinase 1 (Chk1) (3 – 5).

Although this checkpoint function is essential for cells to maintain their genomic stability (3, 4), it also works against cancer treatments such as radio- and chemotherapy: activation of the checkpoint facilitates the survival of cancer cells through the repair of DNA damage artificially produced by the treatments, thus contributing significantly to tolerance induction during cancer therapy. Abrogation of the checkpoint forces cells to cycle with damaged DNA and ultimately to mitotic catastrophe (6 – 8). Therefore, DNA damage checkpoint signals involving ATR and ATM are currently attracting
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Attention as potential new targets for sensitizing cancer cells to radio- and chemotherapy (9).

We previously reported schisandrin B (SchB) as the first example of a specific inhibitor of ATR in DNA damage checkpoint signaling (10). SchB is the major lignan isolated from Schisandra chinensis, one of the most frequently used herbs in traditional Chinese medicines (11, 12), and it has subsequently been revealed to be a mixture of gomisin N (GN) and γ-schisandrin (γ-Sch), which are the diastereomers of each other (Fig. 1). Therefore a question arose as to whether the inhibitory action of SchB involves a stereospecific interaction between one of the stereoisomers of SchB and ATR or ATR-related checkpoint signaling.

In this study, we investigated the individual effects of GN and γ-Sch on DNA damage checkpoint signaling, namely on ATR. The results reveal that the modulatory actions on checkpoint signaling by these stereoisomers are quite different and indicate that GN is the active isomer inhibiting ATR.

Materials and Methods

Reagents

GN and γ-Sch were prepared and kindly provided by Kotaro Pharmaceutical Co., Ltd., Osaka. Briefly, they were purified by repeated preparative HPLC (ODS, φ 2.0 × 30 cm, MeOH:H2O = 85:15) from the SchB-rich fraction of Schisandra chinensis. The yield of GN and γ-Sch were 0.075% and 0.033%, respectively, from Schisandra chinensis. The purity of GN and γ-Sch were > 99%, as determined by HPLC analysis. The specimen numbers of SchB stereoisomers (GN: 20070719, γ-Sch: 20100625) for this study were recorded and stored for 10 years at Niigata University of Pharmacy & Applied Life Sciences. Stock solutions of GN and γ-Sch (6 mg/ml) were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Dojindo (Kumamoto). Stock solutions of MTT (0.05%) and PI (1 mg/ml) were prepared in phosphate-buffered saline (PBS) and stored at 4°C. A stock solution of nocodazole (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was prepared in DMSO and stored at −20°C.

Cell culture

Human adenocarcinoma A549 cells and human embryonic kidney (HEK) 293T cells (adenovirus-transformed HEK293 cell line, expressing SV40 large T antigen) (ATCC, Manassas, VA, USA) were maintained in Dulbecco’s modified eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1% penicillin/streptomycin.

Fig. 1. HPLC chromatogram of schisandrin B (SchB) (A) and chemical structures of gomisin N (GN) and γ-Schisandrin (γ-Sch) (B). The chemical structure of SchB is shown in A. The HPLC chromatogram of SchB was obtained with a monitor wavelength of 254 nm, under a linear gradient elution with CH3CN and its aqueous solution. The 2 peaks of SchB were assigned as GN (a) and γ-Sch (b), respectively, using authentic samples. Data analysis of the chromatogram was done with Chromato-PRO software.
100 μg/ml streptomycin, and 100 units/ml penicillin (all purchased from Invitrogen, Carlsbad, CA, USA). All cultures were kept in a humidified atmosphere with 5% CO₂ at 37°C. DNA damage was induced by UVC irradiation using a CL-1000 Ultraviolet Crosslinker, 8-watt UVC lamp with 254 nm emission peak (UVP, Inc., Upland, CA, USA). The strength of UV was continually monitored and automatically adjusted in the exposure chamber.

**HPLC analysis**

For the analysis of SchB, HPLC was carried out using a Hitachi HPLC system (Hitachi, Ltd., Tokyo) composed of an L-4200 UV-VIS detector, L-6200 intelligent pump, and L-6000 pump. The following conditions were used: Mightysil RP-18 column (250 × 4.6 mm i.d., 5-μm particle size) (Kanto Chemical Co., Inc., Tokyo), linear gradient elution with CH₃CN and its aqueous solution, and flow rate at 0.7 ml/min. The gradient program was from 60% to 100% CH₃CN over 20 min. UV detection was set at 254 nm and 20 μl of SchB (4 μg/ml) was injected to the column. GN and γ-Sch were separated as a and b in Fig. 1A, respectively, as shown in the typical HPLC chromatogram. The chromatogram was analyzed using Chromato-PRO software (RunTime Instruments Co., Ltd., Kanagawa).

**Determination of cell viability by MTT assay**

Cell viability was measured by MTT assay as reported previously (13). A549 cells were seeded at 1 × 10⁴ cells/cm² in 96-well plates and cultured for 24 h. One hour prior to UV exposure, cells were pre-incubated with GN or γ-Sch at a concentration of 0, 5, 10, or 30 μM. After removing growth medium, the cells were exposed to UV (25 and 50 J/m²) and then incubated for 4 h. After the cells were incubated with 100 μl of lysis buffer [20% SDS, 50% N,N-dimethyl formamide (DMF), pH 4.7], absorbance was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 595 nm. All incubations were carried out at 37°C in 5% CO₂.

**Preparation of cell lysates**

A549 cells were seeded at 2 × 10⁴ cells/cm² in 60-mm dishes for immunoblot analysis. The cells were harvested by a cell scraper in ice-cold PBS and then centrifuged at 830 × g for 5 min at 4°C to obtain pellets. For immunoblot analysis, cell pellets were dissolved in UTB buffer (8 mM urea, 150 mM 2-mercaptoethanol, 50 mM Tris, pH 7.5) on ice for 15 min. After centrifugation at 15,500 × g for 20 min at 4°C, the supernatant was mixed with 4 × SDS sample buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 3.4 M 2-mercaptoethanol and bromophenol blue) and stored at −30°C until use. For the ATR kinase assay, cell pellets were dissolved in IP buffer [10 mM Tris, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 1 mM dithiothreitol (DTT), pH 7.4] on ice for 15 min. Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad Laboratories).

**Immunoblot analysis**

For immunoblot analysis, a 20-μg protein sample was loaded and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by an electro blotting apparatus (Invitrogen). The membrane was blocked with 4% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. Immunoblotting was performed by incubating the membrane with the corresponding primary antibody for p53 (Cell Signaling Technologies, Danvers, NJ, USA), phospho-p53 Ser 15 (Cell Signaling Technologies), SMC1 (Bethyl Laboratories, Montgomery, TX, USA), phospho-SMC1 Ser 966 (Bethyl Laboratories), Chk1 (G-4) (Santa Cruz, Santa Cruz, CA, USA), phospho-Chk1 Ser 317 (Bethyl Laboratories), and tubulin (Cell Signaling Technologies) for 16 h at 4°C. Following incubation with secondary antibodies (anti-mouse or anti-rabbit IgG) conjugated with horseradish peroxidase (Cell Signaling Technologies) for 1 h at room temperature, the target proteins were visualized using an Immobilon Western Chemiluminescent HRP Substrate (Millipore) and X-ray film (Fujiﬁlm Co., Ltd., Tokyo). Densitometric analysis of the bands was done using Image Quant 5.2 software (GE Healthcare UK, Ltd., Amersham Place, Little Chalfont, UK).

**Cell cycle analysis by flow cytometry**

A549 cells were seeded at 2 × 10⁴ cells/cm² in 35 mm dishes and cultured for 24 h. The cells were incubated with 0.1 μg/ml nocodazole for 16 h to synchronize the cells in the G2/M phase (14). After the nocodazole treatment, FACS analysis indicated that approximately 90% of the cells were in the G2/M phase. GN or γ-Sch (5, 10 and 30 μM) was added to the medium 1 h prior to the washout of nocodazole. After removing the growth medium, the cells were exposed to UV (25 and 50 J/m²).
The cells were then cultured in fresh growth medium supplemented with γ-Sch or GN for 1 h, harvested by trypsinization, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol. Fixed cells were stored at −30°C until use. The cells were centrifuged at 2,300 × g for 5 min at 4°C, washed with 200 μl of ice-cold PBS, and incubated on ice for 30 min. Following centrifugation at 2,300 × g for 10 min at 4°C, the supernatant was removed and 100 μl of PI solution (50 μg/ml propidium iodide, 100 μg/ml RNase in PBS) was then added to each sample and incubated in the dark for 30 min. Cells were analyzed by FACS (COULTER EPICS® XL-MCL, Beckman Coulter, Brea, CA, USA). Cell populations in each cell cycle phase were analyzed using ModFit LT 3.2 software (Verity Software House, Inc., Topsham, ME, USA).

G2/M checkpoint analysis

Phosphorylation of histone H3 at Ser10 was used for monitoring mitosis. A549 cells (2 × 10⁴ cells/cm²) were seeded in 35 mm Ø dishes and cultured for 48 h before the experiments. After treating the cells with GN or γ-Sch (10 μM) for 1 h and removing the growth medium, the cells were exposed to UV irradiation (25 and 50 J/m²). The cells were cultured in fresh growth medium supplemented with GN or γ-Sch for 1 h, treated by trypsin, and harvested by centrifugation. The cells were fixed with ice-cold 70% ethanol, and the cell membrane was rendered permeable with 0.25% Triton X-100 in PBS on ice for 15 min. The cells were then incubated with a polyclonal rabbit phospho-histone H3 (Ser10) antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 4 h and an Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen) for 4 h and 100 μg/ml of PI solution was added to each sample and incubated in the dark for 30 min. The cells were then cultured in fresh growth medium supplemented with GN or γ-Sch for 1 h, treated by trypsin, and harvested by centrifugation. The cells were fixed with ice-cold 70% ethanol, and the cell membrane was rendered permeable with 0.25% Triton X-100 in PBS on ice for 15 min. The cells were then incubated with a polyclonal rabbit phospho-histone H3 (Ser10) antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 4 h and an Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen) for 1 h at room temperature. Cellular DNA was stained by 50 μg/ml of PI solution for 30 min at room temperature and then subjected to FACS analysis.

Kinase assay

The effects of GN and γ-Sch on ATR kinase activity were determined by kinase assay. Flag-tagged ATR was introduced into HEK293T cells using calcium phosphate transfection with a flag-tagged ATR vector. The HEK293T cell line was derived from the human embryonic kidney 293 cell line by the addition of the SV40 large T antigen. The expressed flag-tagged ATR proteins from 6 mg of cell lysates were immuno-precipitated for 2 h at 4°C with 27.6 μg (6 μl) of anti-Flag® M2 monoclonal antibody (Sigma-Aldrich). Cell lysates with antibodies were subsequently incubated with protein-G sepharose (GE Healthcare UK, Ltd.) for 1 h at 4°C. Immunocomplexes were washed twice with TGN buffer (50 mM Tris, pH 7.4, 50 mM glycerophosphate, 150 mM NaCl, 1% Tween 20, and 10% glycerol) and once with kinase buffer containing 10 mM HEPES (pH 7.5), 50 mM glycerophosphate, 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, and 5 mM DTT. The phosphorylation reaction was performed by mixing 0.6 μg of recombinant GST-p53, a substrate for ATR kinase, with 10 μM 32P-ATP (50 Ci/mmoll; MP Biomedicals, Inc., Santa Ana, CA, USA), as previously described (10, 15). After incubation for 20 min at 37°C, the reaction was terminated by adding 4 × SDS sample buffer. The denatured samples were separated on 7% SDS-PAGE and the gel was dried using a Bio-rad Gel Dryer (Bio-Rad Laboratories). The dried gels were exposed to a Storage Phosphor Screen (Amersham Biosciences, Piscataway, NJ, USA). The radioactivity incorporated in the substrate was visualized by a Typhoon 9210 PhosphorImager (GE Healthcare UK, Ltd.) and quantified using Image Quant 5.2 software (GE Healthcare UK, Ltd.). A linear relationship between ATR activity and the level of GST-p53 phosphorylation was verified under the above assay conditions.

Statistical analyses

All data are expressed as the mean ± S.D. Statistical analysis of the data was carried out by one-way ANOVA using the Tukey–Kramer test. Differences were considered significant at P < 0.05.

Results

Effects of the SchB components GN and γ-Sch on the viability of A549 cells after UVC irradiation

First, the cytotoxicities of GN and γ-Sch (structures in Fig. 1B) were examined in A549 cells. Up to 50 μM, both GN and γ-Sch showed no significant effect on cell viability as determined by MTT assay. However, at concentrations higher than 70 μM, cell viability was significantly decreased (Fig. 2A, B). In the high concentration range, GN showed stronger cytotoxicity than γ-Sch (Fig. 2A).

Next, the effects of GN and γ-Sch were examined on UV-induced cell death in their non-toxic concentration range. The viability of A549 cells was determined 3 days after UV exposure in the presence or absence of GN and γ-Sch. Control cells without GN or γ-Sch treatment showed a moderate decrease in viability following UV irradiation, whereas UV toxicity was significantly enhanced in both GN- and γ-Sch-treated cells (Fig. 2; C, D). It was notable that the sensitizing effect of GN on UV-induced cell death was more marked than that of γ-Sch, especially in the cells after higher-dose UV irradiation (Fig. 2C).

Effects of GN and γ-Sch on checkpoint signal proteins

We further examined whether GN and γ-Sch modulate...
the DNA damage checkpoint signaling pathways. UV irradiation increased the phosphorylation levels of checkpoint proteins p53 at Ser 15, SMC1 at Ser 966, and Chk1 at Ser 317 (Fig. 3: A, B, lane 2). GN dose-dependently prevented the UV-induced phosphorylation of SMC1 and Chk1 (Fig. 3A: third and fifth panels; Fig. 3C, second and third graphs). GN also tended to prevent the UV-induced phosphorylation level of p53 (Fig. 3A, top panel; Fig. 3C, top graph). On the contrary, γ-Sch rather enhanced the UV-induced phosphorylation of checkpoint proteins, especially that of Chk1 (Fig. 3B, fifth panel; Fig. 3D, third graph). Also, the expression of p53 protein itself was enhanced by γ-Sch, whereas GN did not influence the p53 protein level (Fig. 3: A, B, second panel). Together, this strongly indicates that GN is the active isomer of SchB responsible for inhibiting ATR-related checkpoint signals.

GN inhibits the UVC-activated G2/M checkpoint

We next examined the effect of GN and γ-Sch on the cell cycle. The cell cycle transition from G2/M to G1 phase was determined by flow cytometric analysis using cells synchronized with nocodazole (Fig. 4). After 16-h incubation with nocodazole as a G2/M blocker, approximately 90% of A549 cells were concentrated in the G2/M phase. One hour after removing the nocodazole, G1 phase cells were markedly increased coincident with a decrease in G2/M phase cells, indicating that the cell cycle was released to progress. However, UV exposure inhibited the cell cycle transition from the G2/M to G1 phase in a UV dose-dependent manner (Fig. 4A) because of checkpoint activation. Consequently, the cell population in the G2/M phase remained high, especially in the cells irradiated with higher-dose UV (39% in control cells without UV irradiation vs. 41% and 50% in the cells irradiated at 25 and 50 J/m² UV, respectively). The cell population in the G2/M phase was significantly smaller in cells treated with GN compared to control cells without GN treatment, indicating that the cell cycle progressed and thus the checkpoint did not properly operate (Fig. 4B). In contrast to the effect of GN, more γ-Sch-treated cells were in the G2/M phase than the control cells, indicating that the checkpoint functioned more strictly in the presence of γ-Sch (Fig. 4C). This contrasting effect of GN and γ-Sch on cell cycle progression is more clearly illustrated by the bar graph of the G2/M cell populations in the cells that were irradiated by higher-dose UV, as determined by FACS (Fig. 4D).

For further confirmation of the differential effect of both isomers on checkpoint function, we examined the effects of GN and γ-Sch on mitosis in the UV-irradiated cells (Fig. 5). The mitotic cells were identified by histone H3 phosphorylation at Ser 10. Treatment for 1 h with GN
Fig. 3. Effect of SchB stereoisomers on checkpoint proteins. A549 adenocarcinoma cells were pre-incubated with or without the SchB stereoisomers (10, 30, and 100 μM) for 1 h followed by irradiation with UV (25 J/m²) and then incubated at 37°C for 3 h. A and B: Dose-dependency of GN or γ-Sch on p53, SMC1, and Chk1 activations were examined by western blotting using increasing concentrations of GN or γ-Sch added before and after DNA damage induction by UV irradiation (25 J/m²). Tubulin was used as a loading control. C and D: Densitometric analysis of the bands (phosphorylation of p53, SMC1, and Chk1) using Image Quant 5.2 software. Each value represents the mean ± S.D. of three independent experiments (*P < 0.05).
or γ-Sch did not give rise to any change in the mitotic cell population in the non-irradiated cells. After UV irradiation, the percentage of phospho-histone H3–positive cells (indicative of cells in mitosis) in the control cells was clearly decreased (Fig. 5A), indicating that the checkpoint was operative. However, treatment of cells with GN significantly increased the percentage of mitotic cells in the UV-irradiated cells, compared to the irradiation-only control cells. GN at 30 μM also showed a similar effect on the percentage of mitotic cells (data not shown). On the other hand, γ-Sch showed no effect on the percentage of mitotic cells in the UV-irradiated cells (Fig. 5: A, B). These results indicate that GN inhibited the G2/M checkpoint that is activated by UV-induced DNA damage.

**GN inhibits ATR kinase activity**

Finally, we examined whether the checkpoint inhibition by GN is due to a direct inhibitory effect on ATR. ATR kinase activity was assessed using Flag-tagged ATR expressed in HEK293T cells. After purification of Flag-tagged ATR protein by immunoprecipitation with Flag-M2 antibody, we measured in vitro the ATR kinase activity toward its specific substrate GST-p53. The ATR kinase activity was dose-dependently inhibited by GN, with an IC50 of 7.28 μM (Fig. 6A). In contrast, the inhibition of ATR kinase activity by γ-Sch was fairly low compared to that of GN, with an IC50 of > 30 μM (Fig. 6B).

**Discussion**

ATR and ATM are members of the phosphoinositide-3-kinase–like protein kinase (PIKK) family and function as key regulators of DNA damage response signaling, together with other kinases such as DNA-PK (16). Both ATR and ATM are activated by DNA damage, but their functional modes are not identical as they respond to different types of DNA damage. ATM is mainly activated by DNA double-strand breaks (DSBs) such as those caused by γ-radiation, whereas ATR responds to a broad spectrum of DNA damage leading to DNA single-strand breaks (SSBs), such as arrested replication forks, or damage caused by UV irradiation or hydroxyurea (17 – 19). Dysfunctions of these kinases are closely related to the onset of cancer and other diseases (20 – 26). Specific inhibition of DNA damage checkpoint mole-

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**Fig. 4.** Effect of the SchB stereoisomers on the G2/M checkpoint activated by UVC in A549 adenocarcinoma cells synchronized to G2/M with nocodazole. Cell cycle progression was analyzed by FACS after 1 h of UV exposure. Data were analyzed using ModFit LT 3.2 software. Histogram on the left represents the cells synchronized at the G2/M phase by nocodazole. A, B, and C: Cells were treated with control (DMSO), GN, or γ-Sch (0, 5, 10, and 30 μM) 1 h before UV irradiation (0, 25, and 50 J/m²). Arrow shows the cells distributed in the G2/M phase. D: Data are expressed as the percentage of G2/M cells in total cells. Each value represents the mean ± S.D. of three independent experiments (*P < 0.05 vs. UV-untreated cells).
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cules, including ATR and ATM, are currently attracting attention as novel targets of anti-cancer therapies such as radiotherapy and chemotherapy (9) because the DNA damage checkpoint function is one of the reasons that cancer cells are able to acquire tolerance against such therapies. Indeed, this approach is promising as an effective reduction in sarcoma size has been reported when the tumor was treated with caffeine, an inhibitor of ATR and ATM, during chemotherapy (27 – 31).

Many natural products in foods show high potentiality in protecting against the tissue damage associated with cancer radio- and chemotherapy not only through their antioxidant and anti-inflammatory functions but also through the modulation of genes of, for example, antioxidant enzymes (32, 33). They might be able to play a more beneficial role in complimentary cancer treatment as an adjuvant or sensitizer when they have additional pharmacological activity such as checkpoint modulating function. We previously reported that SchB, isolated from Schisnadra chinensis, is a specific inhibitor of ATR and enhanced the cytotoxic effects of UV (10) and anticancer drugs such as doxorubicin (DOX) on cancer cells in vitro, as well as in xenograft and allograft animal experimental systems (unpublished data). Later, SchB was found to consist of the two stereoisomers (diastereomers) GN and γ-Sch (34) (Fig. 1), which led us to examine whether a stereospecific interaction occurs between one of them and ATR. Determining if such an interaction occurs would help elucidate the underlying mechanisms.

Fig. 5. GN inhibits the cell cycle checkpoints induced by UVC irradiation. A: Dot plot analysis was performed by FACS. The percentage of mitotic cells was determined by the number of phosphorylated histone H3 (Ser10)-positive cells in the whole cell population. B: Data are expressed as the percentage of mitotic cells with respect to the total cells. Each value represents the mean ± S.D. of three independent experiments (*P < 0.05 vs. untreated control).

Fig. 6. Inhibition of ATR kinase activity by GN. A and B: ATR kinase activity was measured in vitro using GST-p53 as the substrate, in the presence of GN or γ-Sch (0.3, 1, 3, 10, and 30 μM). A flag-tagged ATR-wt plasmid was transfected into HEK293T cells. ATR protein kinases were purified by immunoprecipitation using anti-Flag® M2 antibody and protein-G sepharose. Kinase activity was monitored for 20 min at 37°C. Each value represents the mean ± S.D. of three independent experiments.
molecular mechanism of action of SchB on checkpoint signaling.

The present study clearly demonstrated the diverse effects of these two isomers on DNA damage checkpoint signaling. Although both GN and γ-Sch significantly enhanced the cytotoxicity of UV in A549 cells, especially at high doses of UV (Fig. 2: C, D), the enhancing effect of GN was stronger than that of γ-Sch. At the same time, GN inhibited the UV-dependent activation of checkpoint molecules related to G1, S, and G2/M checkpoint functions, whereas γ-Sch enhanced it. In particular, the activation profile of Chk1 was clearly dependent on the γ-Sch dose. The phosphorylation levels of p53 and SMC1 were also enhanced slightly by γ-Sch, but only at low concentrations; at high concentration, phosphorylated p53 was even inhibited by γ-Sch (Fig. 3). These observations are consistent with the effects of GN and γ-Sch on the cell cycle (Fig. 4) and on mitosis (Fig. 5). Together, these results clearly indicate that GN inhibits G2/M checkpoint function but γ-Sch does not. Since GN inhibited UV-dependent activation of all the downstream signaling molecules related to each checkpoint, it is suggested that the site of GN action is located upstream of the checkpoint signal cascade. ATR was expected to be the target molecule of GN, because SchB, a mixture of GN and γ-Sch, inhibited ATR (10). This was confirmed by examining the direct inhibitory action of GN and γ-Sch on ATR kinase activity. GN, but not γ-Sch, showed a specific inhibitory action on ATR kinase activity with an IC50 value comparable with the previously reported value for SchB (Fig. 6). We thus conclude that the previously observed ATR inhibition by SchB was the result of a stereospecific reaction of GN with ATR.

It is important to note that GN inhibits not only G1/S but also G2/M checkpoint activity through its inhibitory action on ATR. In as many as 50% of various human cancer cells, the G1/S checkpoint function is defective because the tumor suppressor gene p53 is frequently mutated (35–38) and DNA damage repair is therefore dependent on other checkpoints such as the S and G2/M checkpoints (9).

Accordingly, inhibitors of ATR/Chk1 are attracting attention in the fields of radio- and chemotherapy. Recently developed compounds such as VE-821, ETP-46464 (ATR inhibitor), and UCN-01 (Chk1 inhibitor) are expected to enhance mitotic catastrophe (6–8, 39–41). Indeed, the inhibition of Chk1 by UCN-01 inhibits the G2/M checkpoint to cause premature mitotic entry leading to cell death (42–44). Thus the fact that GN can inhibit the G2/M checkpoint makes it an even more interesting anti-cancer drug candidate. Given the inhibitory profile of GN, which targets upstream of the checkpoint signal cascade, GN might be a promising sensitizer applicable to all types of cancer cells in chemotherapy and radiotherapy (9, 10).

The inhibitory activity of γ-Sch on ATR kinase activity was quite low compared to that of GN. Moreover, γ-Sch enhanced the phosphorylation of downstream checkpoint molecules, especially Chk1, in a dose-dependent manner, indicating that γ-Sch enhances the checkpoint function rather than inhibiting it (Fig. 4). On the other hand, it enhanced the sensitivity of A549 cells to UV, especially at a high UV dose. It has been suggested that the enhanced checkpoint function may facilitate apoptosis of cells carrying damaged DNA; however, the increase in apoptosis by γ-Sch was not significantly different from that by GN on FACS analysis (data not shown). Therefore, it remains unclear how γ-Sch was able to sensitize cancer cells to UV as GN did.

The differential effects of GN and γ-Sch may be partly explained by the structural differences between these isomers. SchB has two chiral centers in the eight-membered rings and one axis-chirality in the molecule. GN and γ-Sch are the diastereomers of each other but each of them has two enantiomers, the P- and M-isomers, due to the configurations of the methyl group on the chiral carbon centers. GN was identified as a P-isomer, while γ-Sch is a racemic mixture of P- and M-enantiomers, indicating that the P-isomer of γ-Sch has the same bulky structure as GN, although the configuration on the chiral carbons is different. Therefore, it might be possible that the P-isomer of γ-Sch has a certain degree of ATR inhibitory action as GN does, which would explain the data in Fig. 3 showing that γ-Sch inhibits checkpoint signals only at a high concentration.

Since γ-Sch showed enhancement of UV-activated checkpoint signals, especially Chk1 (Fig. 3), and since the checkpoint signals play a crucial role in the routine repair of damaged DNA, it is reasonable to assume that γ-Sch may contribute in part to the chemopreventive function of Schisandraceae or SchB. However, another possibility needs to be considered that enhanced phosphorylation of checkpoint molecules resulted from inhibitory action of γ-Sch on certain phosphatases involved in dephosphorylation of activated checkpoint proteins. Further studies are needed to clarify the function of the γ-Sch M-isomer that has a different bulky structure from GN. It might react with other target molecules related to Chk1 activation besides ATR and ATM, or it may be responsible for other functions of γ-Sch such as potentiation of the cytotoxic effect of DOX in DOX-resistant cancer cells by increasing the accumulation of DOX inside the cells (45).

In conclusion, the present study revealed that GN is the active component of SchB that inhibited DNA
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