Inhibition of ATR protein kinase activity by schisandrin B in DNA damage response

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ABSTRACT

ATM and ATR protein kinases play a crucial role in cellular DNA damage responses. The inhibition of ATM and ATR can lead to the abolition of the function of cell cycle checkpoints. In this regard, it is expected that checkpoint inhibitors can serve as sensitizing agents for anti-cancer chemo/radiotherapy. Although several ATM inhibitors have been reported, there are no ATR-specific inhibitors currently available. Here, we report the inhibitory effect of schisandrin B (SchB), an active ingredient of *Fructus schisandrae*, on ATR activity in DNA damage response. SchB treatment significantly decreased the viability of A549 adenocarcinoma cells after UV exposure. Importantly, SchB treatment inhibited both the phosphorylation levels of ATM and ATR substrates, as well as the activity of the G2/M checkpoint in UV-exposed cells. The protein kinase activity of immunoaffinity-purified ATR was dose-dependently decreased by SchB *in vitro* (IC$_{50}$: 7.25 μM), but the inhibitory effect was not observed in ATM, Chk1, PI3K, DNA-PK, and mTOR. The extent of UV-induced phosphorylation of p53 and Chk1 was markedly reduced by SchB in ATM-deficient but not siATR-treated cells. Taken together, our demonstration of the ability of SchB to inhibit ATR protein kinase activity following DNA damage in cells has clinical implications in anti-cancer therapy.

INTRODUCTION

Ultraviolet (UV), ionizing radiation (IR) as well as many anti-cancer drugs induce DNA damage and activate cellular responses, such as DNA damage checkpoint signaling cascades, resulting in cell cycle arrest at the G1/S, intra-S and G2/M phases. This allows time for damage repair, or leads to apoptosis when the extent of DNA damage is not compatible with cell survival (1,2). Thus, DNA damage checkpoints, which prevent the passage of damaged DNA to the next generation of cells, can hamper tumor progression by inducing tumor cell death in patients undergoing chemo/radiotherapies (3).

ATM [ataxia telangiectasia (AT) mutated] and ATR (ataxia telangiectasia and Rad-3-related) protein kinases act as master controllers in DNA damage checkpoint signaling (1,2,4,5). ATM-deficient AT cells, which are derived from human AT patients, were found to exhibit chromosomal instability, telomere shortening and defects in cellular responses to DNA double-strand breaks (DSBs) following exposure to IR and radiomimetic chemicals (1). On the other hand, *in vivo* functions of ATR are less understood than those of ATM because animals with homozygous ATR gene disruption, unlike ATM, are embryonic lethal (6,7). However, cellular functions of ATR have recently been investigated using small interfering RNA (siRNA) and the over-expression of kinase-deficient mutants in cultured cells (7–9). The transient knockdown of ATR by siRNA was found to cause prominent chromosome instability and mitotic catastrophe in cells (10,11).

Given that most cancer cells do not have functional p53 (12,13), a key molecule for G1/S checkpoint control, the use of inhibitors of the G2/M checkpoint can selectively sensitize p53-defective cancer cells to the cytotoxic effect of DNA-damaging anti-cancer drugs (14,15). Thus, the G2/M checkpoint may serve as a drug target, which is more useful than the G1/S checkpoint in anti-cancer therapy. The discovery of specific inhibitors of ATM and ATR is beneficial both for our understanding of the fundamental functions of these kinases and for potential clinical applications through checkpoint abrogation in anti-cancer therapy. Although several ATM/ATR inhibitors have been reported (14), a specific compound that inhibits ATR protein kinase has yet to be discovered.

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In the search for potential ATR inhibitors, we screened a number of herbal extracts and ingredients. Among them, schisandrin B (SchB) was the most active. SchB is a dibenzocyclooctadiene derivative isolated from *Fructus Schisandrae*, the fruit of *Schisandra chinensis*, which is commonly used in traditional Chinese medicine for the treatment of hepatitis and myocardial disorders (16,17). A recent study reported that SchB treatment enhances the effect of doxorubicin (DOX), a topoisomerase II inhibitor, which induces cell death via caspase-9 activation in SMMC7721 (human liver carcinoma) and MCF-7 (human breast adenocarcinoma) cells (18). Presumably, SchB augments the DOX-induced apoptotic pathway upstream of caspase-9. Alternatively, it is possible that SchB might produce an inhibitory effect on ATM and/or ATR, which are activated after DNA damage induced by DOX in cancer cells (19,20).

Here, we demonstrate that SchB specifically inhibits ATR kinase activity *in vitro*, and that treating cells with SchB reduces the extent of *in vivo* phosphorylation of ATR substrates as well as the abrogation of G2/M checkpoints following UV (but not IR) irradiation. Consistent with the inhibition of ATR by SchB, our studies with siRNA and AT cells show that the cytotoxic effect of SchB on cancer cells is dependent on the presence of ATR but not ATM kinase.

MATERIALS AND METHODS

Cell culture

Human adenocarcinoma A549 cells (ATCC: American Tissue Type Collection, VA, USA) and GM18366 Seckel syndrome cells (Coriell Cell Repositories, NJ, USA) were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/ml streptomycin and 100 units/ml penicillin), all were purchased from Invitrogen (CA, USA). AT2KY cells were obtained from Health Science Research Resources Bank (Osaka, Japan), and cultured in RPMI-1640 medium containing 15% FBS and antibiotics. DNA damage of cells was induced by UV irradiation (Stratagene, Stratalinker model 2400, CA, USA) or γIR irradiation (^{137}Cs, 2 Gy/min, PS-3000SB, Pony Industry Co., Osaka, Japan).

Chemicals and reagents

SchB was isolated from the petroleum extract of *Fructus schisandrae*, and had greater than 95% purity, as assessed by HPLC and described by Ip *et al*. (20). The drug was dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml. For cell treatments, the drug solution was further diluted in culture medium with a final DMSO concentration <5 µg/ml. The proteasome inhibitor (*N*-acetyl-L-Leucyl-L-Leucyl-L-norleucinal: LLnL) for the immunoblot assay of p53 was purchased from Sigma (MO, USA). MEKs inhibitor (PD098059) and PMA were obtained from Sigma and Tocris (MO, USA), respectively. Caffeine was purchased from Sigma.

Protein preparation

Cells were harvested by scraping into ice-cold phosphate-buffered saline (PBS), and proteins were extracted from cell pellets into UTB buffer (8 mM urea, 150 mM 2-mercaptoethanol, 50 mM Tris, pH 7.5) for immunoblot analysis, or 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 dithiothreitol (DDT), pH 7.4] for the ATR kinase assay. Protein concentrations were determined using a protein assay kit (Bio-Rad, CA, USA).

Clonogenic assay

A549 or GM18366 cells were seeded at 500 cells/60-mm dish and grown overnight. One hour prior to UV exposure, cells were pre-incubated with SchB (0, 1, 10 and 30 µM). After the removal of growth medium, cells were sham treated or exposed to UV irradiation (20–75 J/m^2). The cells were cultured in a fresh growth medium supplemented with SchB. After incubating for 14 days, cells were stained with 2% (w/v) methylene blue (dissolved in 50% ethanol). Colonies were counted and the value was normalized to that of the sham-treated group with or without SchB treatment.

Immunoblot analysis

For immunoblot analysis, 50 µg of proteins was loaded and separated by SDS–PAGE, and the protein bands were transferred to a nitrocellulose membrane by electron blotting. The membrane was blocked by 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 for 1 h at room temperature. Immunoblotting was performed using primary antibodies for p53 (Calbiochem, Darmstadt, Germany), phospho-p53 (Cell Signaling Technologies, NJ, USA), phospho-ATM (Rockland Immunochemicals, PA, USA), phospho-Chk1 (Cell Signaling Technologies), ATR (Bethyl Laboratories, TX, USA), tubulin (Sigma) and GAPDH (Santa Cruz, CA, USA), and these antibodies were incubated with the membrane for 16 h at 4°C. Secondary antibodies conjugated with horse radish peroxidase (Zymed laboratories, CA, USA) were incubated for 1 h at room temperature. Target proteins were visualized using an ECL reaction kit (Amersham, NJ, USA) and a chemiluminescence film (Amersham).

siRNA experiments

siRNA oligonucleotides were synthesized by Qiagen. The target sequences of the siRNA duplex for ATM and ATR were 5′-AAG CGC CTG ATT CGA GAT CCT-3′ and 5′-AAG ACG GTG TGC TCA TGC GGC-3', respectively. A siRNA for GFP with a target sequence of 5′-CGGCAAGCTGACCCTGAAGTTCAT-3′ was used for the negative control. The siRNA duplexes were transfected into A549 adenocarcinoma cells using the oligofectamine method (Invitrogen) and Opti-MEM (Invitrogen), according to the manufacturer's protocol. The siRNA-transfected cells were incubated with growth

medium for 72 h to allow time to reduce target protein expression.

Cell cycle analysis

To visualize the cell cycle transition from the G2/M phase to the G1 phase, flow cytometric analysis was performed. A549 cells were cultured with 0.1 μg/ml nocodazole for 16 h in order to synchronize the cells into the G2/M phase. After nocodazole treatment, 85% of the cells were in the G2/M phase. SchB or caffeine was added to medium 1 h prior to the termination of nocodazole incubation. Cell populations in various phases of the cell cycle were analyzed using ModFit LT2.0 software.

G2/M checkpoint analysis

Phosphorylation of histone H3 at Ser10 was used for monitoring mitosis. Asynchronous and synchronous cells were both fixed with ice-cold 70% ethanol and permeabilized with 0.25% Triton X-100 in PBS on ice for 30 min. The cells were then incubated with 1 μg of polyclonal rabbit phospho-histone H3 (Ser10) antibody (Upstate, NY, USA) and FITC-conjugated goat anti-rabbit IgG antibody (Sigma) for 4 h and 1 h at room temperature, respectively. Counter-staining with 10 μM propidium iodide was performed by subsequent incubation for 20 min at room temperature. Cells were analyzed by FACS using a flow cytometer (Beckman-Coulter, CA, USA).

S-phase analysis

The intake of 5-bromo-2′-deoxyuridine (BrdU) during DNA synthesis was estimated to account for the S-phase checkpoint of cells (21). In brief, 10 μM BrdU in medium was incubated for 1 h after UV irradiation. Cells were collected and fixed in 70% ice-cold ethanol, and kept for 2 h at −20°C. Cells were washed and incubated in 2 N HCl for 30 min at room temperature to denature the DNA. The acid solution was then neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5) and subsequently washed with PBS. The cells were incubated with 1 μg of polyclonal rabbit BrdU antibody (Sigma) and FITC-conjugated goat anti-rabbit IgG antibody (Sigma) for 4 h and 1 h at room temperature, respectively. Counter-staining with 10 μM propidium iodide was performed by subsequent incubation for 20 min at room temperature. Cells were analyzed by FACS using a flow cytometer (Beckman-Coulter).

Effect of SchB on the association of ATR with ATRIP

Flag-tagged ATR was expressed in HEK293T cells following transient transfection with a flag-tagged ATR vector (generous gift from Dr R.T. Abraham), and the expressed proteins were immuno-precipitated for 4 h at 4°C with 20 μg of anti-Flag[R] M2 monoclonal antibody (Sigma) from 2 mg of cell lysates. Subsequently, the proteins were subjected to immunoblot analysis as shown above using an anti-ATRIP antibody (Cell Signaling Technologies).

Kinase assay

Flag-tagged ATR was expressed in HEK293T cells using the same method as described above from 5 mg of cell lysates. ATM was purified by immunoprecipitation from A549 cell lysates, using anti-ATM monoclonal antibody (Rockland Immunochemicals). Cell lysates with antibodies were subsequently incubated with Protein-G Sepharose (Amersham) for 1 h at 4°C independently. Immunocomplexes were washed twice with TGN buffer (50 mM Tris, pH 7.4, 50 mM glycerophosphate, 150 mM NaCl, 1% Tween20, 10% glycerol), and once with kinase buffer, containing 10 mM HEPES (pH 7.5), 50 mM glycerophosphate, 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM $MnCl_2$. The phosphorylation reaction was performed by mixing 1 μg of recombinant PHAS-I (Alexis Biochemicals, CA, USA), a substrate for ATR kinase, with 10 μM $^{32}P-ATP$ (50 Ci/mmol; Amersham), as previously described (22). After incubation for 20 min at 30°C, the reaction was terminated by adding 4 volumes of SDS sample loading buffer, and samples were then subjected to 12% SDS–PAGE. Gels were dried and analyzed by autoradiography. A linear relationship between ATR activity and the level of PHAS-I phosphorylation was verified under the above assay conditions. Chk1 (Upstate), PI3K (Echelon Biosciences Inc, UT, USA), DNA-PK (Promega) and mTOR (Calbiochem) activities were measured using respective assay kits according to the manufacturer's instructions.

Effect of SchB on MAPK activity

To examine the specificity of SchB toward different signaling pathways, SchB was tested for its effect on the MAPK kinase signaling pathway. Cells were first incubated with PD098059, an inhibitor of MEKs, for 15 min, or SchB for 60 min after serum starvation for 16 h (22). Then, cells were treated with 100 ng/ml PMA for 5 min, and then harvested for immunoblot analysis of extracellular-regulated kinase (ERK) phosphorylation, using the antibodies for phosphorylated ERK (Thr202/Tyr204) and total ERK (Cell Signaling Technologies).

Statistical analysis

Statistical analysis was performed using the statistical package for social science (SPSS) software. The half-maximal inhibitions (IC_{50}) of ATR kinase by SchB were calculated by solving the respective linear models. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of SchB treatment on cell viability after UV irradiation

We investigated whether SchB (Figure 1) treatment affects cell survival following DNA damage induced by UV and γ-irradiation (IR). The viability of A549 cells was monitored by clonogenic assay for 14 days after UV or IR exposure in the presence or absence of SchB. SchB showed no effect on cell viability (Figure 2A). Although SchB-untreated cells showed a moderate decrease in
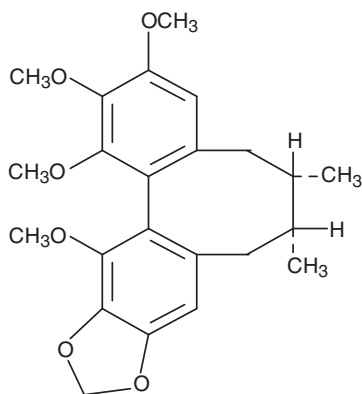
Figure 1. Chemical structure of SchB.

viability following UV irradiation, a significant decline in viability was observed in SchB-treated cells. Notably, the concentrations of SchB treatments at 30 μM or higher were lethal in less than 3 days after UV-induced DNA damage (Figure 2B), and similar effects were observed in UV-irradiated HEK293T and HeLa cells (data not shown). The critical effect of caffeine was not observed at the same concentrations as SchB in the UV-irradiated cells (data not shown). These results suggest that SchB may influence the cellular DNA damage response induced by UV irradiation regardless of the cell type. Moreover, such a marked decline in viability was not seen in cells treated with γIR (Figure 2C). Apparently, DNA damage checkpoint mechanism(s) may be involved in the UV-sensitizing action of SchB in cancer cells.

Sch B treatment abolishes the UV-activated G2/M and S-phase checkpoints

To examine the effect of SchB on the abrogation of DNA damage checkpoints, we measured the cell cycle transition from the G2/M phase to the G1 phase using flow cytometric analysis (Figure 3A). Cells enriched in the G2/M phase were observed in A549 cells treated with nocodazole for 16 h before UV exposure (indicated by the gray histogram), and an increase in the population of cells in the G1 phase was observed 1 h after removing nocodazole (Figure 3A; left upper and lower panels). UV exposure inhibited the cell cycle transition from the G2/M phase to the G1 phase in a dose-dependent manner (upper panels of Figure 3A and B). In turn, SchB treatment 1 h prior to and after the removal of nocodazole significantly increased the population of G1 cells. Caffeine also increased the percentage of G1 cells, but the increase was not significant compared with the control. To confirm the observations, we examined the effect of SchB on mitosis in UV-irradiated cells using phospho-antibody (Ser 10) against Histone H3 (Figure 3C). The percentage of P-Histone H3-positive cells (indicative of cell population in the G2/M phase) 1 h after removing nocodazole was about 17%. The proportion of mitotic cells decreased in a UV intensity-dependent manner in cells without SchB treatment. As expected, SchB treatment induced significant accumulation of mitotic cells more than caffeine treatment and the control. We further tested a lower
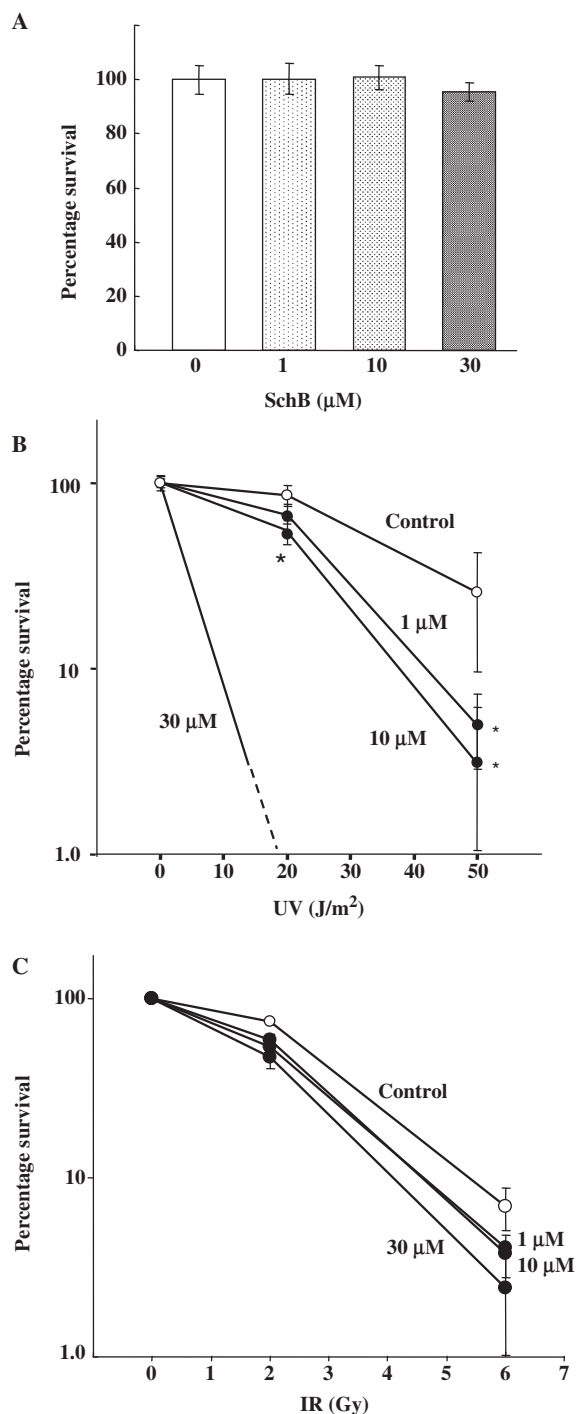

Figure 2. SchB treatment causes a decrease in cell viability after UV irradiation. Relative cell viability was measured by clonogenic assay. A549 cells were seeded as 500 cells/60-mm dish, and then incubated for 24 h before the experiment. The cells were subjected to 1 h pre-incubation and 14 days post-incubation (**A**) with SchB (0, 1, 10 and 30 μM) after (**B**) UV irradiation (0, 20 and 50 J/m^2) or (**C**) IR irradiation (0, 2 and 6 Gy). Values are expressed as mean ± SD (n = 3). *$P < 0.05$ versus SchB untreated control.

UV condition which is to minimize the influence of DSB response. SchB treatment completely abolished the G2/M checkpoint even in lower UV irradiation as 10 J/m^2 in asynchronous cells (Figure 3D). These results clearly
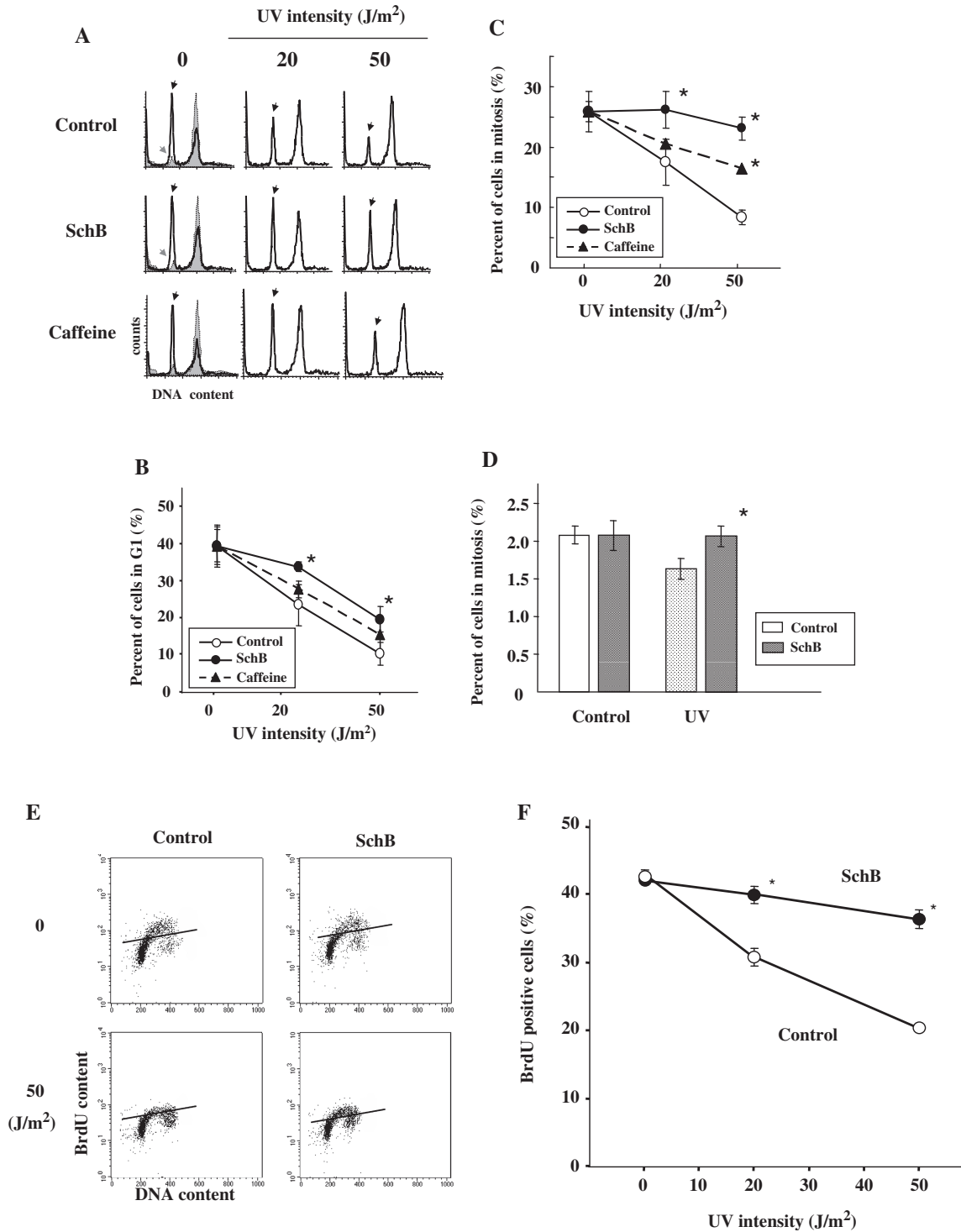
Figure 3. SchB treatment abolishes cell cycle checkpoints induced by UV irradiation. (**A**) Effect of SchB on the G2/M checkpoint after UV-induced DNA damage using G2/M synchronized cells. Cell-cycle distributions were analyzed by FACS after 1 h of UV exposure, followed by data analysis using ModFit software. The histogram in gray represents cells synchronized at the G2/M phase by nocodazole. A549 cells were treated with 30 μM SchB or 10 mM caffeine 1 h before UV irradiation (0, 20 and 50 J/m^2). The arrow shows the cells distributed in the G1 phase. (**B**) Data were expressed as the percentage of G1 cells in relation to the total number of cells. (**C**) The percentage of mitotic cells was estimated by phosphorylation of histone H3 at Ser10. (**D**) The mitotic percentage of asynchronous cells was estimated by phosphorylation of histone H3 in 10 J/m^2 of UV irradiation followed by 1 h incubation. (**E**) The percentage of DNA synthesizing cells was visualized by BrdU intake of cells. (**F**) Data obtained in Figure 3E were analyzed using XL2 software to determine the percentage of mitotic cells. Values are expressed as mean ± SD ($n = 3$). *$P < 0.05$ versus SchB untreated control.
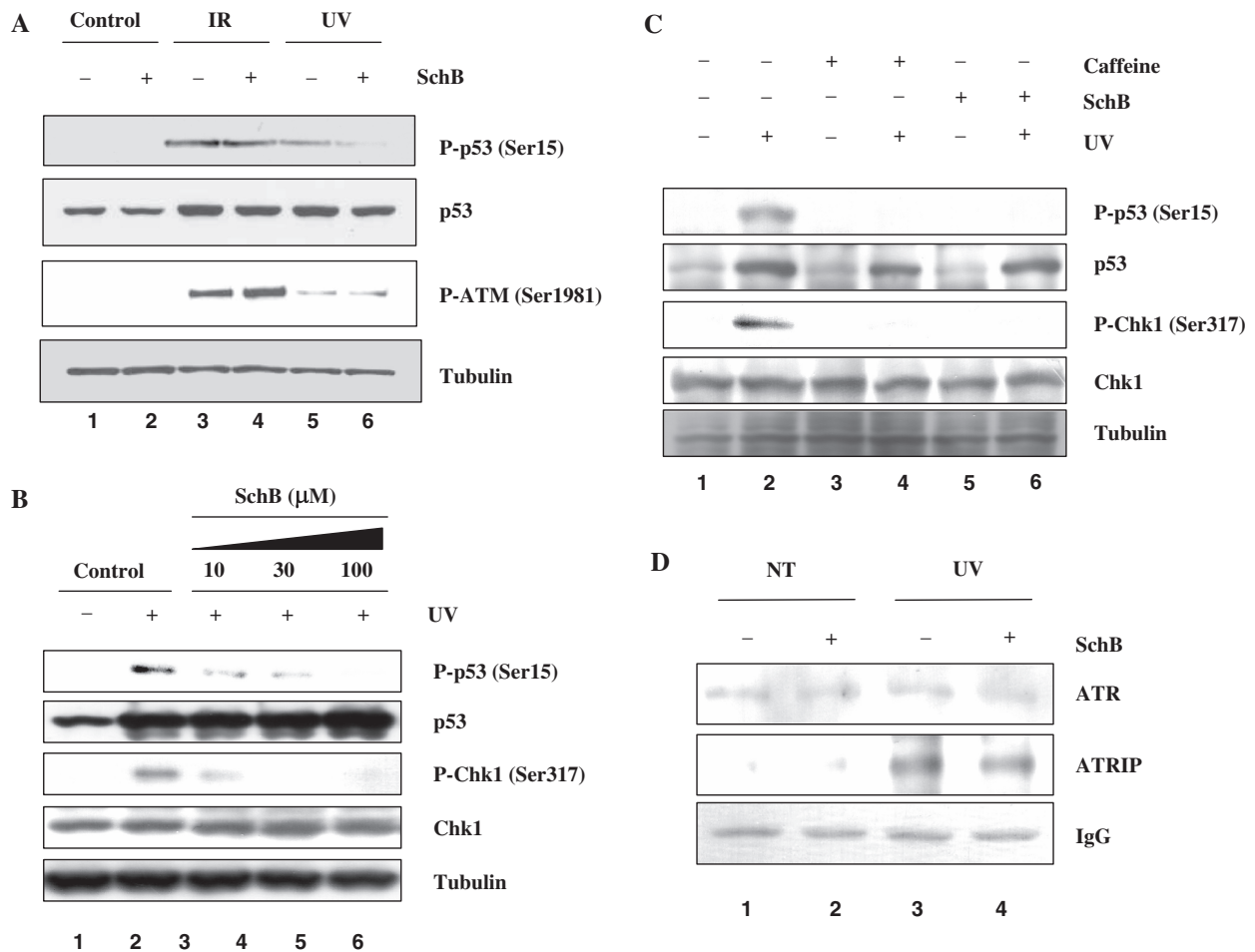
Figure 4. Inhibitory effects of SchB treatment on checkpoint proteins. (**A**) A549 cells were pre-incubated with or without 30 μM SchB for 1 h prior to induction of DNA damage. After pre-incubation, cells were subjected to 10 Gy of IR or 20 J/m^2 of UV irradiation, and cultured at 37°C for 1 h and 3 h, respectively. (**B**) Dose-dependent effects of SchB on the phosphorylation level of p53 and Chk1 were observed by adding increasing concentrations of SchB before and after induction of DNA damage by UV irradiation (20 J/m^2). A549 cells were incubated with a proteasome inhibitor (50 μM LLnL) throughout the experiments in A, B and C to avoid proteasome-dependent protein degradation. Equal loading of extracted proteins was confirmed by determining immuno-stained tubulin. (**C**) A549 cells were pre-incubated with or without 30 μM SchB or 10 mM caffeine for 1 h prior to induction of DNA damage. After pre-incubation, cells were subjected to UV irradiation (20 J/m^2), and cultured at 37°C for 3 h. (**D**) Flag-tagged ATR was expressed in HEK293T cells following transient transfection with a flag-tagged ATR, and the expressed proteins were immunoprecipitated. The association of ATR with ATRIP was analyzed by immunoblot analysis.

indicate that SchB abrogated the G2/M checkpoint during UV-induced DNA damage. The intracellular uptake of BrdU during 2 h incubation was measured to estimate the effect of SchB on S-phase checkpoint abrogation (Figure 3E). The cell population in the S phase was about 42% in asynchronous cells, and UV treatment decreased this to 20% in a dose-dependent manner (Figure 3F). The treatment of SchB for 1 or 2 h before and after UV irradiation did not markedly decrease the cell population in the S phase. This result indicates that SchB inhibited not only the G2/M phase but also the S-phase checkpoints in DNA damage response induced by UV.

SchB inhibits the phosphorylation of checkpoint proteins *in vivo*

To examine whether SchB directly affects DNA damage response especially in ATM- or ATR-related signal transduction pathways in cells, we monitored the phosphorylation of p53 at Serine15 (Ser15), a direct target of ATM/ATR during DNA damage response induced by IR or UV irradiation (1,2,23–25), using phosphorylation site-specific antibodies. Indeed, the level of p53 phosphorylation at Ser15 was increased by exposure to IR (10Gy) or UV (20 J/m^2) in A549 cells in the absence of SchB treatment (Figure 4A, top panel, lanes 1, 3, 5). Interestingly, SchB treatment at 30 μM decreased the level of p53 phosphorylation at Ser15 following UV but not IR exposure (lanes 3–6), suggesting that SchB inhibited ATR- but not ATM-dependent phosphorylation of p53 at Ser15. Consistent with the inability of SchB to inhibit ATM-dependent Ser15 phosphorylation, SchB did not alter the auto-phosphorylation of ATM at Ser1981, which is associated with ATM activation following DNA DSBs (Figure 4A, third panel from top, lanes 3-6). In order to gain a mechanistic insight into the inhibitory effect of

SchB on ATR, we next investigated whether inhibition is limited to the phosphorylation of Ser15 of p53. We found that SchB also dose-dependently inhibited the phosphorylation of another ATR substrate, Ser317 of Chk1 (26) (Figure 4B, top and 3rd panel). Since a proteasome inhibitor LLnL was present in our UV irradiation experiments (Figure 4A and B), the UV irradiation−induced degradation of p53 protein was suppressed (Figure 4B, second panel from top). To investigate the significance of the inhibition of ATR protein kinase activity by SchB, the phosphorylation of p53 and Chk1 was examined in SchB- and caffeine-treated cells after UV irradiation (Figure 4C). SchB markedly inhibited the phosphorylation of p53 (top panel, lanes 5 and 6) and Chk1 (3rd panel, lanes 5 and 6) *in vivo*, as was the case for caffeine. To exclude the possibility of a decrease in phosphorylation of p53 and Chk1 through cell cycle synchronization induced by SchB, the cell cycle distribution was analyzed with or without SchB treatment. We found that SchB treatment at $30\,\mu M$ did not affect cell cycle distribution, even after 6 h of incubation or 24 h of incubation (data not shown). Therefore, the decreased levels of p53 and Chk1 phosphorylation were not due to the differential distribution of cells in various phases of the cell cycle. We further examined whether or not SchB prevents the binding of ATR to ATRIP, a critical interacting protein for ATR activation during DNA damage response (Figure 4D). After UV irradiation, over-expressed ATR-wt in HEK293 cells was strongly associated with ATRIP (2nd panel, lane 3); however, SchB did not reduce the binding amounts of ATRIP to ATR *in vivo* (2nd panel, lane 4). These results suggest that the suppression of UV-induced phosphorylation of p53 at Ser15 and Chk1 at Ser317 by SchB treatment may be a direct inhibition of ATR by SchB, and is not due to the association with ATRIP.

SchB inhibits ATR kinase activity *in vitro*

To directly address the question of whether SchB inhibits ATR protein kinase activity, Flag-tagged ATR was expressed in HEK293T cells by transient transfection, and then isolated for *in vitro* kinase activity assay. After purification of Flag-tagged protein kinase by immunoprecipitation with Flag-M2 antibody, we measured *in vitro* ATR kinase activity toward PHAS-I, a specific substrate of these kinases. ATM kinase activity was measured with endogenous ATM purified by anti-ATM antibody. To verify the specificity of SchB toward ATR, activities of Chk1, phosphoinositide 3-kinase (PI3K), DNA-dependent protein kinase (DNA-PK) and the mammalian target of rapamycin (mTOR) were also measured. The activity of ATR was markedly inhibited by increasing concentrations of SchB, with an IC_{50} of $7.25\,\mu M$ (Figure 5A). In contrast, the activity of immunopurified ATM protein kinase was not affected by SchB treatment at concentrations in the micromolar range, with an IC_{50} of $1.74\,mM$ (Figure 5B). Moreover, the activities of Chk1, PI3K, DNA-PK and mTOR were not affected by SchB treatment (Figure 5C–F). These results indicate that SchB preferentially inhibited the protein kinase activity of ATR rather than ATM, Chk1 and other phosphoinositide 3-kinase

(PI3K) family members *in vitro*, regardless of their similar roles to PI3K at DNA damage checkpoints.

SchB prevents ATR-dependent signaling pathway

If ATR is a direct target of SchB *in vivo*, the inhibitory action of SchB should not be limited to the phosphorylation of p53 and Chk1. To address this point, we studied whether SchB could also affect the phosphorylation of other ATR substrates. AT2KY cells, which are ATM-deficient fibroblasts, were used to exclude the possibility of a redundant function of ATM, which might phosphorylate ATR substrates when ATR was inhibited by SchB. Following UV irradiation, we monitored phosphorylations of p53 (Ser15), Chk1 (Ser317), SMC1 (Ser966) and BRCA1 (Ser1423) using a series of phosphorylation site-specific antibodies in western blot analysis. We found that the phosphorylation of all tested ATR substrates were inhibited by SchB treatment in the ATM-deficient cells, supporting the direct inhibition of ATR activity by SchB (Figure 6A, lanes 3 and 4). To further confirm these results, we next used siRNA to deplete ATM in A549 cells (Figure 6B). SchB treatment was found to inhibit UV-induced phosphorylation of p53 (Ser15) and Chk1 (Ser317) in both control siGFP- and siATM-treated cells (Figure 6B top and 3rd panel; lanes 3, 4, 7 and 8). To ensure the inhibitory potential of SchB in ATR-dependent DNA damage checkpoint signaling, we performed the cell viability test with ATR-deficient Seckel syndrome fibroblasts. Since this cell line remains proficient in other ATR-independent DNA damage responses (27), the survival curve in UV exposed control culture was similar (Figure 6C) to that of other cell lines such as A549 (Figure 2B). As expected, SchB did not show any effect on cell viability following UV irradiation, (Figure 6C). The results indicate that SchB inhibited the signal transduction from ATR to phosphorylation of all tested ATR substrates *in vivo*, which is in agreement with the inhibition of affinity-purified ATR *in vitro* (Figure 5).

SchB does not affect mitogen-activated protein kinase pathway *in vivo*

ATR is a member of the phosphoinositide 3-kinase-like kinase (PIKK) family and contains a kinase domain with motifs that are typical of the lipid kinase, PI3K. To further investigate the specificity of SchB as an ATR kinase inhibitor, we examined whether SchB affects the mitogen-activated protein (MAP) kinase pathway, which is unrelated to the DNA damage response. Treatment of A549 cells with phorbol 12-myristate 13-acetate (PMA) after 16 h serum starvation dramatically induced the phosphorylation of ERK in A549 cells, as monitored by p44 and p42 ERK phosphorylation using Western blot analysis (Figure 7). While PD98059, a specific inhibitor for MEKs (MAPK/ERK kinases), inhibited the phosphorylation of ERKs (Figure 7, top panel, lanes 2 and 6), SchB treatment, at a concentration that readily inhibited ATR-dependent phosphorylation, did not change the extent of the phosphorylation of ERKs (Figure 7, lane 4). This result indicates that SchB is not a non-specific inhibitor of protein kinases in A549 cells.
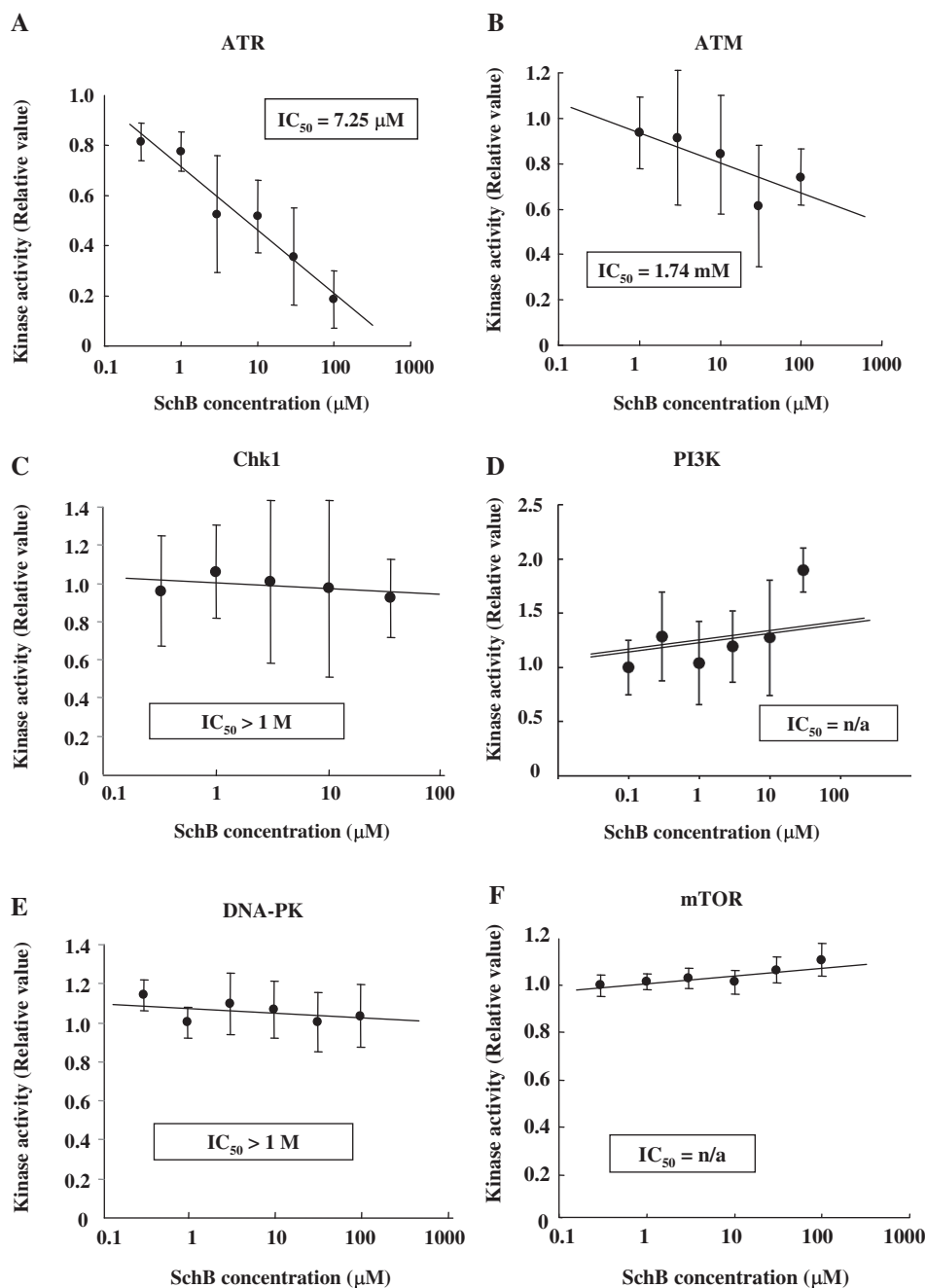
Figure 5. SchB treatment inhibits ATR kinase activity. Kinase activities of ATR (**A**), ATM (**B**), Chk1 (**C**), PI3K (**D**), DNA-PK (**E**) and mTOR (**F**) were measured *in vitro* as phosphorylation activity in the presence of SchB. A flag-tagged ATR-wt plasmid was transfected into HEK293T cells. ATR protein kinases were purified by immunoprecipitation using anti-Flag M2 antibody and Protein-G agarose. The endogenous ATM protein was purified from A549 cell lysates with anti-ATM antibody. Kinase activity was monitored for 20 min at 30°C. Chk1 (Upstate), PI3K (Echelon Biosciences), DNA-PK (Promega) and mTOR (Calbiochem) activities were measured using respective assay kits according to the manufacturer's instructions. Values are presented as mean ± SD ($n = 3$).

DISCUSSION

In order to avoid the carry-over of damaged DNA to the next generation of cells, checkpoint signals provide a safeguard in several critical phases of the cell cycle (1,2). The requirement of ATM and ATR for checkpoint activation and cell survival following UV exposure, IR irradiation or treatment with genotoxic agents is well documented (7–9). ATM and ATR

phosphorylate various substrates including Chk1, p53, NBS1, BRCA1 and SMC1. The disruption of DNA damage checkpoints can lead to the chemo- and radio-sensitization of cancer cells (3,28), thus inhibitors for checkpoint kinases, particularly the ATM/ATR kinases in proximity to the checkpoints, may prove to be beneficial in serving as radio/chemo-sensitizing agents for cancer therapy (3,14)
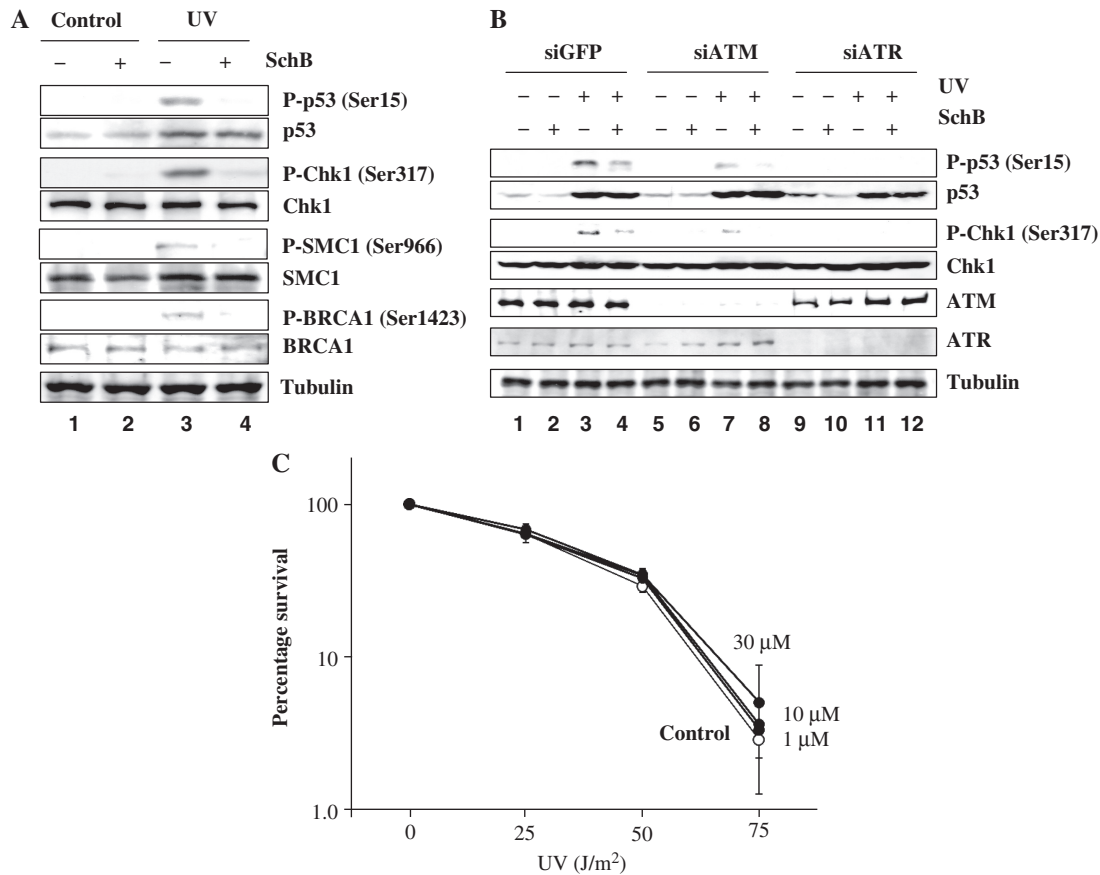
Figure 6. SchB prevents ATR-dependent signaling pathway after DNA damage. (**A**) AT2KY fibroblasts were pre-incubated with or without 30 μM SchB for 1 h, and then subjected to UV irradiation (20 J/m^2). The cells were harvested 4 h later and phosphorylation levels of p53, Chk1, SMC1 and BRCA1 were measured to assess checkpoint function. (**B**) A549 cells were transfected with siRNA for control (siGFP), ATM or ATR using the oligofectamine method, and the cell extracts were immunoblotted with anti-phosphorylated p53, phosphorylated Chk1, total-ATM, total-ATR or anti-tubulin. After 72 h incubation for the knockdown of ATM or ATR, cells were subjected to UV irradiation (20 J/m^2) followed by 4 h incubation with or without SchB (30 μM) pre/post-incubation. The equal loading of an extracted protein was confirmed using anti-monoclonal tubulin antibody. (**C**) The cell viability was measured in ATR-deficient Seckel syndrome fibroblasts with or without SchB (0, 1, 10 and 30 μM) after UV irradiation (0, 25, 50 and 75 J/m^2). Values are expressed as mean ± SD ($n = 3$).
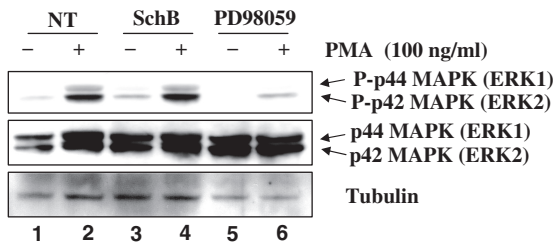

Figure 7. Phosphorylation of ERK at Thr202 and Tyr204. A549 cells were pre-treated with 50 μM PD098059 or 30 μM SchB after serum starvation for 16 h. The phosphorylation of ERK was activated by adding 100 ng/ml PMA followed by a 5 min incubation. The cells were lysed and applied to immunoblot analysis. Equal loading of an extracted protein was confirmed using anti-monoclonal tubulin antibody.

In the present study, we have shown that SchB, an active ingredient of *F. schisandrae*, represents a novel and potent ATR protein kinase inhibitor. SchB has long been used for the treatment of hepatitis and heart failure in China (16). Interestingly, Li *et al.* (18) have recently

reported that SchB significantly enhanced DOX-induced apoptosis in human hepatic carcinoma and breast cancer cells. As DOX can activate both cellular ATR and ATM kinase activities in cultured cells (29,30), our finding of the inhibition of ATR kinase *in vivo* and *in vitro* by SchB suggests that SchB may enhance DOX-induced cancer cell death by inhibiting the ATR-dependent checkpoint signals. The finding of a dose-dependent decrease in cell survival as induced by SchB following UV irradiation (Figure 2) suggests that SchB influence the DNA damage response following UV irradiation. A modest difference in cell survival was observed between untreated and SchB-treated cells after IR irradiation. In relation to this, it is known that ATR and ATM are activated by different types of DNA damage (1,2). Single-strand break (SSB) generated by UV irradiation or DNA-damaging agents primarily activates ATR, whereas DSB produced by IR irradiation activates ATM (1,28). Consistent with the reduction in cell survival by SchB following UV irradiation, SchB treatment disrupted the G2/M checkpoint activated by UV exposure, but had a weak effect

on caffeine-treated cells (Figure 3B and C). The result suggests that SchB might have an inhibitory effect on the ATR- rather than ATM-dependent checkpoint. In good agreement with the ATR inhibition by SchB, the G2/M checkpoint-disrupting effect of SchB was more evident with a lower dose of UV, where the percentage of mitotic cells was almost the same as the control cells (Figure 3D). Bakkenist and Kastan (31) reported that ATM activation occurred within minutes after IR irradiation or hours after UV irradiation. Because phospho-Histone H3-positive cells (i.e. mitotic cells) were measured 1 h after UV irradiation to avoid an inflow of cells from the S phase to the M phase in our G2/M checkpoint study (Figure 3C), the effect of SchB on mitosis at a higher UV dose ($50 \, J/m^2$) might be due to the participation of ATM triggered by a higher UV dose, although the ratio of contribution is still not yet clear. Nevertheless, a significant difference in mitotic cell populations between the control and SchB-treated cells was still observed. Taken together, the results unequivocally support the ATR kinase-specific inhibitory effect of SchB in DNA damage response.

To understand the molecular mechanism(s) involved in the above observed effects of SchB, we examined how SchB influences cellular DNA damage responses in detail. We found that the levels of several cellular phosphorylated molecules after UV irradiation were decreased by SchB treatment (Figure 4). Since both ATM and ATR play a critical role in triggering the cascade of DNA damage response (1,2), our result indicates that SchB treatment can lead to the abrogation of DNA damage response induced by UV irradiation via ATM and/or ATR inhibition. It has been reported that p53 is phosphorylated at multiple sites *in vivo* by several different protein kinases (1,2,32). Since the specific and direct phosphorylation of p53 by ATR and ATM occurs at Ser15 in response to UV, IR irradiation, or methylating agents (32), Ser-15 phosphorylation serves as a useful biochemical monitor for ATM and ATR activities *in vivo* after DNA damage. Although Ser15 of p53 was phosphorylated after both IR and UV irradiation (Figure 4A), SchB reduced only the extent of UV-induced phosphorylation. Therefore, the UV wavelength of 254 nm used in the present study was found to mainly activate the cellular ATR activity (1,25,33). Moreover, the inhibition of p53 phosphorylation by SchB did not require the presence of cellular ATM (Figure 6), and SchB did not affect the activation of ATM kinase, as revealed by auto-phosphorylation of Ser1981 (Figure 4A). These observations strongly suggest that SchB specifically inhibits ATR-dependent phosphorylation of p53 at Ser15. On the other hand, our data show that the phosphorylation of Chk1 at Ser317, a specific and direct phosphorylation site of both ATM and ATR, was also suppressed by SchB treatment in a dose-dependent manner (Figure 4B). At the same time, the S-phase checkpoint estimated by DNA synthesis in cells was abrogated by SchB treatment (Figure 3E and F). Therefore, the inhibitory effect of SchB on DNA damage checkpoints is not limited to one particular ATR substrate that functions at the G2/M checkpoint. How does SchB affect ATR-dependent phosphorylation?

The result obtained from the *in vitro* phosphorylation study suggests that ATR, but not ATM, is a direct target for the inhibitory action of SchB (Figure 5), which is in agreement with the observed inhibition of the phosphorylation of all tested ATR substrates. On the other hand, the association between ATR and ATRIP, a typical mechanism of ATR activation after the DNA damage *in vivo* (34,35), was not prevented by SchB treatment (Figure 4D). Pertaining to this, we demonstrated the inhibition of ATR activity by SchB *in vivo*, comparable to the effect of caffeine, a well-known inhibitor of PI3K kinases, including ATR (Figure 4C). With reference to IC_{50} values on ATR and ATM, SchB was more potent than caffeine ($7.25 \, \mu M$ versus $1.1 \, mM$) (22). The inhibition of p53 and Chk1 phosphorylation by SchB implies that SchB is also likely involved in the G1/S and S-phase checkpoint regulation through the specific inhibition of ATR protein kinase activity.

To confirm the ATR-specific inhibition by SchB, we further studied the effect of SchB using AT2KY cells, which are AT patient-derived fibroblasts lacking functional ATM, and siRNAs for ATM and ATR in A549 cells. The results indicate that the inhibitory effect of SchB on the phosphorylation of various ATM/ATR substrates required the presence of ATR but not ATM (Figure 6). Importantly, the inhibitory effect of SchB on the phosphorylation of ATR substrates was observed in both BRCA1 and Chk1. This observation, therefore, further supports the specific action of SchB on ATR, and the G2/M and S-phase checkpoint abrogation by SchB shown in Figure 3 might also be due to the inhibition of the ATR-BRCA1/Chk1/SMC1 pathway. Moreover, SchB did not affect the phosphorylation of ERKs, a signal event mediated by MAP kinase but unrelated to the DNA damage response. Furthermore, it is interesting to note that the G2/M checkpoint-disrupting effect of SchB was not detectable in other dibenzocyclooctadiene derivatives of *F. schisandrae* such as schisandrin C and angeroylgomisin-A (generous gifts from Tsumura Co., Japan) (data not shown). This finding suggests a highly specific action of SchB in the inhibition of the G2/M checkpoint.

SchB may be used as a sensitizer in cancer therapy because of its high specificity toward ATR. Conceivably, SchB may be used for sensitizing malignant cells to anti-cancer drugs such as DNA-damaging methylating/alkylating agents (36,37) and topoisomerase inhibitors (1,20) that induce ATR activation. Finally, by abrogating the G2/M checkpoint, SchB may be used as an adjuvant agent for anti-cancer therapy, particularly for targeting cancer cells that are resistant to chemo- and radiotherapy due to the absence of functional p53 (38). Further in-depth study is required to determine the clinical feasibility of SchB as a sensitizing agent for anti-cancer therapy.

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