Biochemical and Biophysical Research Communications 463 (2015) 222–228

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Potential new chemotherapy strategy for human ovarian carcinoma with a novel KSP inhibitor

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ARTICLE INFO

Article history:
Received 8 May 2015
Available online 19 May 2015

Keywords:
Apoptosis
Kinesin spindle protein
Human ovarian cancer
Neurotoxicity
Securin

ABSTRACT

Among synthetic kinesin spindle protein (KSP) inhibitor compounds, KPYB10602, a six-member lactam-fused carbazole derivative was the most potent *in vitro* against cell growth of human ovarian cancer, A2780. KPYB10602 caused dose-dependent suppression of tumor growth *in vivo*. Mitotic arrest due to KPYB10602 was confirmed *in vitro*, and was characterized by inhibition of securin degradation. Apoptosis after mitotic arrest was associated with an increase in the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2. Increase of reactive oxygen species (ROS) and caspase pathway were also involved. Furthermore, KPYB10602 caused little neurotoxicity *in vivo*. Therefore, KPYB10602 could be a promising candidate as an anti-tumor agent with reduced adverse events for treating human ovarian cancer.

1. Introduction

Ovarian cancer is a major cause of death among gynecological malignancies. Significant improvement of the survival time has been achieved since platinum derivatives (cisplatin and carboplatin) and paclitaxel were introduced as standard postoperative chemotherapy [1]. However, the success rate of treatment for advanced, recurrent, or persistent ovarian cancer has remained largely unchanged for several decades, which means that there is a need to consider second-line chemotherapy for this cancer.

Anti-microtubule drugs such as taxanes (including paclitaxel) and vinca alkaloids [2] bind to tubulin and block cell cycle progression through disruption of microtubule dynamics and activation of the spindle checkpoint, leading to cell death [3]. These anti-microtubule agents are used extensively for chemotherapy, however, often cause adverse events such as peripheral neuropathy [4]. This is because tubulin is an essential cytoskeletal protein that is involved in not only cell division but also cell morphology, motility, and intracellular transport. Therefore, there is an urgent need for new anti-tumor agents that do not affect microtubule dynamics.

A potentially attractive target is kinesin spindle protein (KSP), which is also known as Eg5 and KIF11 [5]. It plays a critical role in bipolar spindle formation and separation of duplicated chromosomes during early mitosis by driving the antiparallel sliding of microtubules with energy obtained from ATP hydrolysis. KSP inhibitors cause mitotic arrest in which condensed chromosomes form a ring around unseparated spindle poles, commonly referred to as a monaster spindle with DNA rosette. Thus, inhibition of KSP arrests dividing cells in mitosis and leads to death without affecting microtubule function.

The first specific KSP inhibitor, monastrol [6], was reported in 1999 as a compound that induced monoastral spindle formation. Subsequent inhibitors of the ATPase activity of KSP that have been reported include the dihydropyrimidine-2-thione derivative dimethylenastron [7,8], the quinazolinone derivatives ispinesib [9] and SB-743921[10], the dihydropyrrole derivative MK-0731 [11], the tetrahydroquinoline derivative EMD-534085 [12], the cysteine derivative *S*-trityl-L-cysteine [13], and a biphenyl derivative [14].

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http://dx.doi.org/10.1016/j.bbrc.2015.05.029
0006-291X/

Some of these KSP inhibitors have undergone clinical trials [10,15–21].

We have also designed a variety of KSP inhibitors [22–24], and have demonstrated that carbazole derivatives exhibit strong anti-tumor activity *in vitro* [25]. Our present study was aimed to evaluate the *in vivo* activity against human ovarian cancer of the most potent KSP inhibitor that we have identified, and investigate the underlying mechanisms *in vitro* and neurotoxicity *in vivo*.

2. Materials & methods

2.1. Reagents

KSP inhibitors were synthesized according to the method by previously reported [25]. The inhibitors tested in this study were KPYB10602 (a six-member lactam-fused carbazole derivative), KPYC12688 (a five-member lactam-fused carbazole derivative), and KPYC12687 (a pyrrole-fused carbazole derivative) (Fig. 1(A)). Paclitaxel and carboplatin were purchased from Sigma–Aldrich Inc. (St. Louis, MO).

2.2. Cells

The human ovarian carcinoma cell line A2780 (Health Protection Agency Culture Collections, Salisbury, UK) was used. Culture was done in RPMI 1640 medium (Sigma–Aldrich) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 1% antibiotic-antimycotic (Invitrogen), and cells after two passages were used in this study.

2.3. Animals

Animal experiments were performed in accordance with the Guidelines for Animal Experimentation of St. Marianna University Graduate School of Medicine. Pathogen-free female BALB/c-nu mice (Charles River Laboratories Japan, Yokohama) were housed in an isolated animal room at a constant temperature (23 ± 1 °C) and humidity (50–60%) with a 12-h light/dark cycle, and were allowed free access to 30 kGy CL-2 diet (Clea Japan, Tokyo) and sterilized water. Female BALB/c mice were also housed under similar conditions.

2.4. Cell proliferation assay

Cells (1×10^4) were cultured with inhibitors for 48 h, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich) assay and the [^{3}H]-thymidine ([methyl, 1′, 2′-^{3}H]thymidine) (PerkinElmer Japan, Yokohama) incorporation assay were carried out.

2.5. Immuno-staining

Cells (2×10^4) in a Lab-Tek® Chamber Slide (Nalge Nunc International, NY) were cultured with inhibitors for 24 h. Fixed cells were incubated with anti-α/β tubulin (rabbit, Cell Signaling), and reacted with Alexa Fluor® 594 anti-rabbit IgG (Invitrogen). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen).

2.6. Tumor xenograft study

Seven days after cells (5×10^6) were subcutaneously inoculated into female BALB/c-nu mice, and drug treatments were started (day 0). The KSP inhibitor, KPYB10602 (8 or 80 mg/kg) in olive oil was administered subcutaneously on days 0–4 and day 8. The doses and the administration schedule were determined from the results of preliminary studies on tolerability. Paclitaxel (25 mg/kg) was subcutaneously administered on day 0.

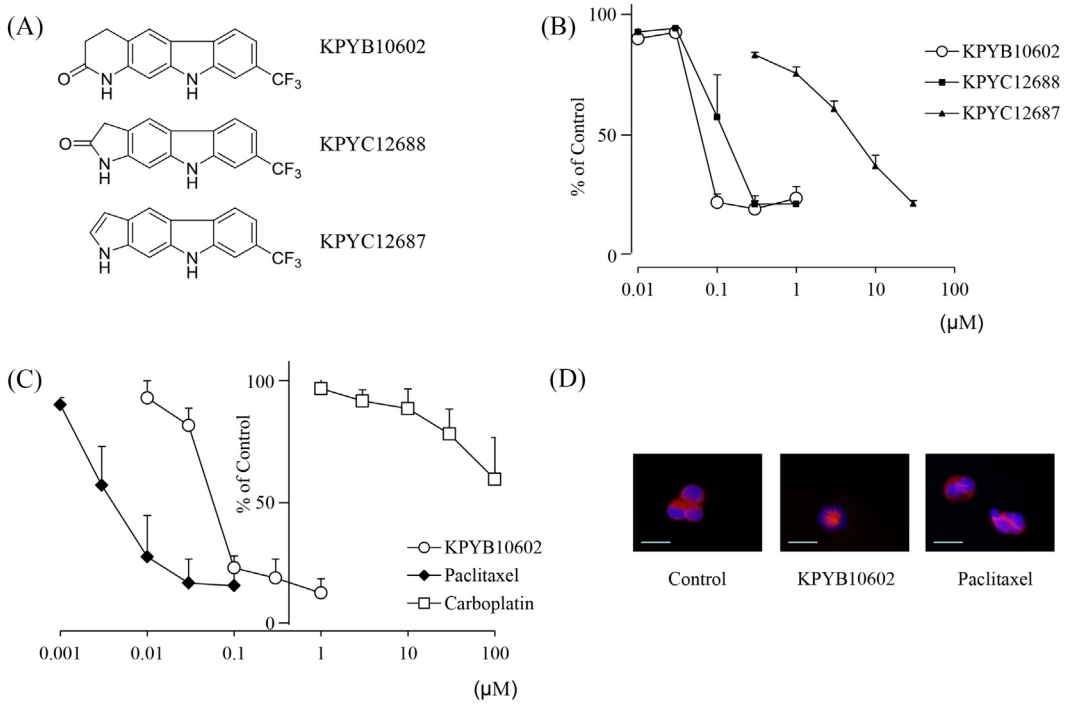

Fig. 1. KSP inhibitors, and their effect on cell growth *in vitro*. (A) Chemical structures of the KSP inhibitors. (B, C) Cells (1×10^4) were cultured with KSP inhibitors, paclitaxel, or carboplatin for 48 h, and MTT assay was performed. Mean ± SD, $n = 6$. (D) Medium alone (Control), KPYB10602 (1 μM), paclitaxel (0.1 μM). α/β tubulin (red). Nuclei (blue). Scale bars = 25 μm.

Tumor volumes (mm^3) were calculated with the standard formula: length × width^2 × 0.5 [26]. Evaluation was stopped on day 18, since the vehicle control (Control) group, the mean tumor volume reached over 5000 mm^3 and some of them died. After *in vivo* evaluation, tumor specimens were used for hematoxylin-eosin (HE) staining.

2.7. Rota-rod test

Female Balb/c mice was subcutaneously treated with KPYB10602 (80 mg/kg) on days 0–4, or with paclitaxel (25 mg/kg) on day 0. Rota-rod test (MK-610A, Muromachi Kikai Co., Ltd. Tokyo) was carried out at a constant speed (6 rpm). The time being placed on the rod was measured during a period of 300 s, and the total time was calculated.

2.8. Cell cycle

Adherent cells (2×10^6) were replaced with serum-free fresh medium and cultured for 15 h. After further incubation with inhibitors, cells were harvested, fixed with ice-cold 70% ethanol, and kept at −20 °C overnight. Then, cells treated with 20 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase were subjected to flow cytometer (BD LSR II, BD Biosciences, San Diego, CA). At least 30,000 cells were analyzed in each assay.

2.9. Apoptosis assay

Cells were cultured with KPYB10602 as described above, and stained with an FITC annexin V/dead cell apoptosis kit (Invitrogen). Cells with early apoptosis (only stained by FITC) or late apoptosis (stained by both FITC and PI) were analyzed with FlowJo software (Tree Star, Ashland, OH).

2.10. Intracellular reactive oxygen species (ROS)

KPYB10602-treated cells were reacted with 100 μM dichlorofluorescein diacetate (DCFH-DA, Sigma–Aldrich) for 10 min at 37 °C. ROS levels were quantified by determining the mean fluorescence intensity (MFI).

2.11. Western blot analysis

Cell lysates (20 μg) were subjected to electrophoresis on 5–20% SDS polyacrylamide gels. After transferred to PVDF membranes (Clearblot P™, Atto Corporation, Tokyo), anti-securin antibody (clone 19H16L48) (rabbit, Invitrogen), anti-Mad2 antibody (mouse, BD Transduction Laboratories), anti-Bax antibody (rabbit, Cell Signaling) anti-Bcl-2 antibody (rabbit, Cell Signaling), anti-caspase 9 antibody (rabbit, Cell Signaling), anti-caspase 3 antibody (rabbit, Cell Signaling), or anti-β-actin antibody (mouse, Sigma–Aldrich) was applied. Then the membranes were incubated with ECL HRP-labeled anti-mouse IgG or HRP-labeled anti-rabbit IgG (GE Healthcare Japan).

2.12. Statistical analysis

Data are expressed as the mean ± SD (or SE). Mean values were compared by one-way analysis of variance. Statistical analysis was performed by using the Mann–Whitney U-test (*in vivo*) or the unpaired Student's *t*-test (*in vitro*), and significance was accepted at $P < 0.05$.

3. Results

3.1. In vitro anti-tumor activity of KSP inhibitors

All of the KSP inhibitors suppressed the cell growth in a concentration-dependent manner (Fig. 1(B)). KPYC12687 was far less potent than the others. The concentration required to reduce cell growth by 50% (IC_{50}) was 0.078 μM for KPYB10602, 0.105 μM for KPYC12688, and 8.325 μM for KPYC12687 (Table 1). Similar results were obtained in the thymidine uptake assay. Since KPYB10602 was the most potent, it was selected for evaluation of *in vivo* anti-tumor activity.

3.2. In vitro potency of KPYB10602 compared with that of paclitaxel and carboplatin

Prior to *in vivo* evaluation, the *in vitro* effect of KPYB10602 was compared with that of paclitaxel and carboplatin. Cell growth was dose-dependently suppressed (Fig. 1(C)). Based on these IC_{50} values (Table 1), KPYB10602 was 10-fold less potent than paclitaxel, but over 1600-fold more potent than carboplatin. Different from paclitaxel-treated cells, KPYB10602-treated ones were characterized to form a monaster spindle with DNA rosette (Fig. 1(D)).

3.3. In vivo effect of KPYB10602 in A2780 tumor-bearing BALB/c-nu mice

Seven days after cell inoculation, the tumor volume reached around 50 mm^3 (day 0). Tumor volume increased in the vehicle-treated group (Control), reaching about 5000 mm^3 on day 18. KPYB10602 (80 mg/kg) achieved significant suppression compared with the Control (Fig. 2(A)), and complete tumor regression occurred in 3 animals. While KPYB10602 (8 mg/kg) was less effective, the V/V_0 ratio was similar to that in the high dose group up to day 11 (Table 2). Anti-tumor activity diminished after treatment was stopped, however, the high dose of KPYB10602 was able to eradicate some tumors.

The body weight was a little higher in both KPYB10602-treated groups than in the Control (Fig. 2(B)), where solid tumors weighed 3.92 ± 0.90 g (day 18). Extensive nuclear condensation was observed in KPYB10602-treated tumors, suggesting that mitotic arrest was caused (Fig. 2(C)).

Paclitaxel suppressed tumor growth until day 8, after which tumor growth was similar to that in the low-dose KPYB10602 group (Table 2).

3.4. In vivo effect of KPYB10602 on motor coordination in BALB/c mice

Rota-rod test was performed to examine whether KPYB10602 showed neurotoxicity. Paclitaxel induced significant impairment of

Table 1
IC_{50} (μM) of KSP inhibitors, paclitaxel, and carboplatin.

KSP inhibitors	MTT assay	Thymidine incorporation assay
KPYB10602	0.0783 ± 0.0094	0.0405 ± 0.0076
KPYC12688	0.105 ± 0.036	0.0570 ± 0.0072
KPYC12687	8.33 ± 1.40	2.35 ± 0.684
Agents	**MTT assay**	**Thymidine incorporation assay**
KPYB10602	0.056 ± 0.0048	0.035 ± 0.0160
Paclitaxel	0.0051 ± 0.0003	0.001 ± 0.0006
Carboplatin	93.0 ± 15.6	16.8 ± 2.29

A2780 cells were cultured with KSP inhibitors, paclitaxel, or carboplatin for 48 h. Three separate experiments were repeated, and the respective IC_{50} was obtained. Data are expressed as the mean ± SD.

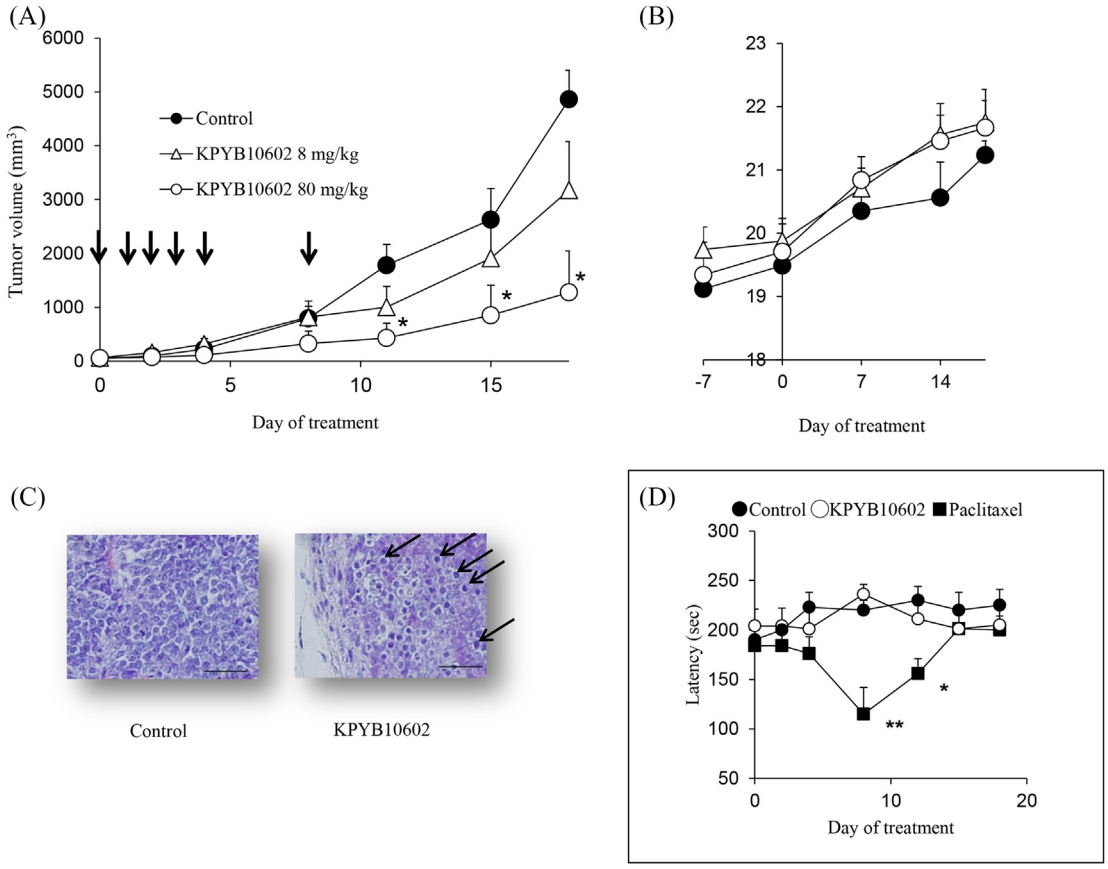

Fig. 2. *In vivo* effect of KPYB10602 on tumor-bearing BALB/c-nu mice (A, B, C), and on motor coordination in BALB/c mice (D). Seven days after cells (5×10^6) inoculation to BALB/c-nu mice, treatment was started (day 0). Subcutaneous treatments were done on days 0–4 and day 8 (arrows). Mean ± SE, $n = 7$. (A) Tumor volume, (B) Body weight, (C) HE staining. Nuclear condensations (arrows). Scale bars = 50 μm. (D) Balb/c mice received KPYB10602 (80 mg/kg) on days 0–4, or paclitaxel (25 mg/kg) on day 0. Rota-rod test was performed twice a week. Mean ± SE, $n = 5$. *p < 0.05, **p < 0.01 vs. Control.

coordination on days 8 and 12 (Fig. 2(D)). In contrast, there was no significant difference in performance between mice treated with KPYB10602 and the Control group.

3.5. *In vitro* effect of KPYB10602 on cell cycle, ROS production, and expression of cell cycle- or apoptosis-related proteins

Since nuclear condensation was prominent in KPYB10602-treated tumors (Fig. 2(C)), the *in vitro* effect was focused.

In the absence of drugs, the proportion of 4 N cells in G_2/M phase was relatively constant at around 15–16% (Table 3). KPYB10602 (10 μM) increased the proportion of G_2/M phase cells at 15 h (35.0%), indicating mitotic arrest (Fig. 3(A)). Paclitaxel (2.5 μM) also caused accumulation of G_2/M phase cells, which reached 32.9% In contrast, carboplatin (100 μM) had little effect on G_2/M phase cells (15.2%). The proportion of >4 N cells was also assessed, but there was little difference compared with the Control.

Apoptotic cells (subG_1) gradually increased after exposure to the drugs. KPYB10602 (10 μM) increased apoptotic cells from 4.55% to 22.3%. The increase was both time- and concentration-dependent. Also, the proportion of early apoptotic cells was 0.21% in the Control, while it increased to 17.8% in KPYB10602-treated cells (Fig. 3(B)).

Securin degradation is essential for the progress from metaphase to anaphase. The securin/mitotic arrest defective 2 (Mad2) ratio was increased over 2-fold by addition of KPYB10602 (Fig. 3(C)). Concentration-dependent suppression was also observed after 6 h and 24 h of culture (data not shown). Similar results were obtained with paclitaxel, but not with carboplatin (data not shown).

Table 2
V/V_0 ratio in each group.

	Dose (mg/kg)	Day of treatment					
		2	4	8	11	15	18
Control	0	1.82 ± 0.40	4.86 ± 0.47	24.0 ± 8.17	63.4 ± 21.5	138.4 ± 26.9	259.9 ± 18.8
KPYB10602	8	1.98 ± 0.52	4.71 ± 1.49	14.1 ± 6.01	21.8 ± 8.39	62.6 ± 22.4	115.6 ± 39.1
KPYB10602	80	1.42 ± 0.26	1.54 ± 0.66*	15.6 ± 10.4	21.1 ± 12.5	42.1 ± 25.2*	63.9 ± 35.0*
Paclitaxel	25	1.40 ± 0.85	1.64 ± 0.73*	7.64 ± 2.89	22.4 ± 7.30	70.7 ± 24.3	100.1 ± 34.0

The V/V_0 ratio = V (tumor volume on the day of evaluation)/V_0 (tumor volume on the first day of treatment).
Data are expressed as the mean ± SE (n = 7). *p < 0.05 vs. Control.

Table 3
Effects of KPYB10602, paclitaxel, and carboplatin on cell cycle.

(a)	Treatment	(μM)	SubG$_1$	G$_1$	S	G$_2$/M	>4N
	Control	0	2.42	29.6	10	15.4	42.4
	KPYB10602	1	2.25	16.0	7.0	31.3	43.0
		10	2.83	15.3	6.9	31.8	44.6
	Paclitaxel	0.25	2.5	22.9	7.8	24.9	41.8
		2.5	2.15	17.2	7.2	29.3	41.9
	Carboplatin	10	2.72	30.1	6.6	16.9	43.7
		100	2.0	32.2	7.3	15.9	42.6
(b)	Treatment	(μM)	SubG$_1$	G$_1$	S	G$_2$/M	>4N
	Control	0	4.55	44.1	6.1	16.7	28.6
	KPYB10602	1	7.83	27.3	3.3	31.0	31.0
		10	22.3	16.5	3.6	35.0	23.5
	Paclitaxel	0.25	11.4	22.1	4.2	32.3	29.5
		2.5	13.3	28.4	4.1	32.9	24.6
	Carboplatin	10	6.7	42.5	6.8	16.7	26.7
		100	7.2	44.3	8.9	15.2	25.2

A2780 cells were incubated with KPYB10602, paclitaxel, or carboplatin for 6 h (a) or 15 h (b). Each assay was performed three times, and the data obtained from one representative experiment were shown. Each value was expressed as %.

Pro-apoptotic Bax protein increased, and anti-apoptotic Bcl-2 protein decreased slightly (Fig. 3(D)). After 3 h of culture with KPYB10602 (10 μM), the Bax/Bcl-2 ratio showed an almost 2-fold increase, and a concentration-dependent increase of this ratio was also observed after 6, 15, and 24 h of culture. In addition, ROS level increased during incubation with KPYB10602 in a concentration-dependent and time-dependent manner (Fig. 3(E)). Western blot analysis revealed that the levels of cleaved caspase-3 and caspase-9 increased in a concentration-dependent manner (Fig. 3(F)).

4. Discussion

The present study demonstrated that a carbazole-derivative KSP inhibitor, KPYB10602, which was the most potent of the KSP inhibitors tested *in vitro*, had *in vivo* anti-tumor activity in an A2780 human ovarian cancer xenograft model.

It was noted that tumor growth was significantly suppressed with KPYB10602, and there were even some animals with diminished tumors. Furthermore, extensive nuclear condensation was observed in KPYB10602-treated tumors. These findings indicate that KPYB10602 could have induced mitotic arrest, leading to tumor cell death.

It should be noted that KPYB10602-treated animals showed little neurotoxicity. Paclitaxel-treated animals showed impairment of motor coordination during the first 8–12 days, although this subsequently recovered. No such incoordination was observed in animals treated with KPYB10602. These results indicate that KPYB10602 primarily acts on mitotic cells, but does not have much influence on microtubule functions, different from paclitaxel.

Cell cycle analysis confirmed that KPYB10602 induced mitotic arrest, similar to anti-microtubule drugs such as paclitaxel. In addition, subG$_1$ (apoptotic) cells were increased in a dose-dependent manner.

KSP is required for bipolar spindle formation in the mitotic phase, so inhibition of KSP generates monopolar spindle, resulting in unaligned condensed chromosomes and activation of the spindle assembly checkpoint (SAC). This activation finally blocks the degradation of securin through activation of Mad2. Therefore,

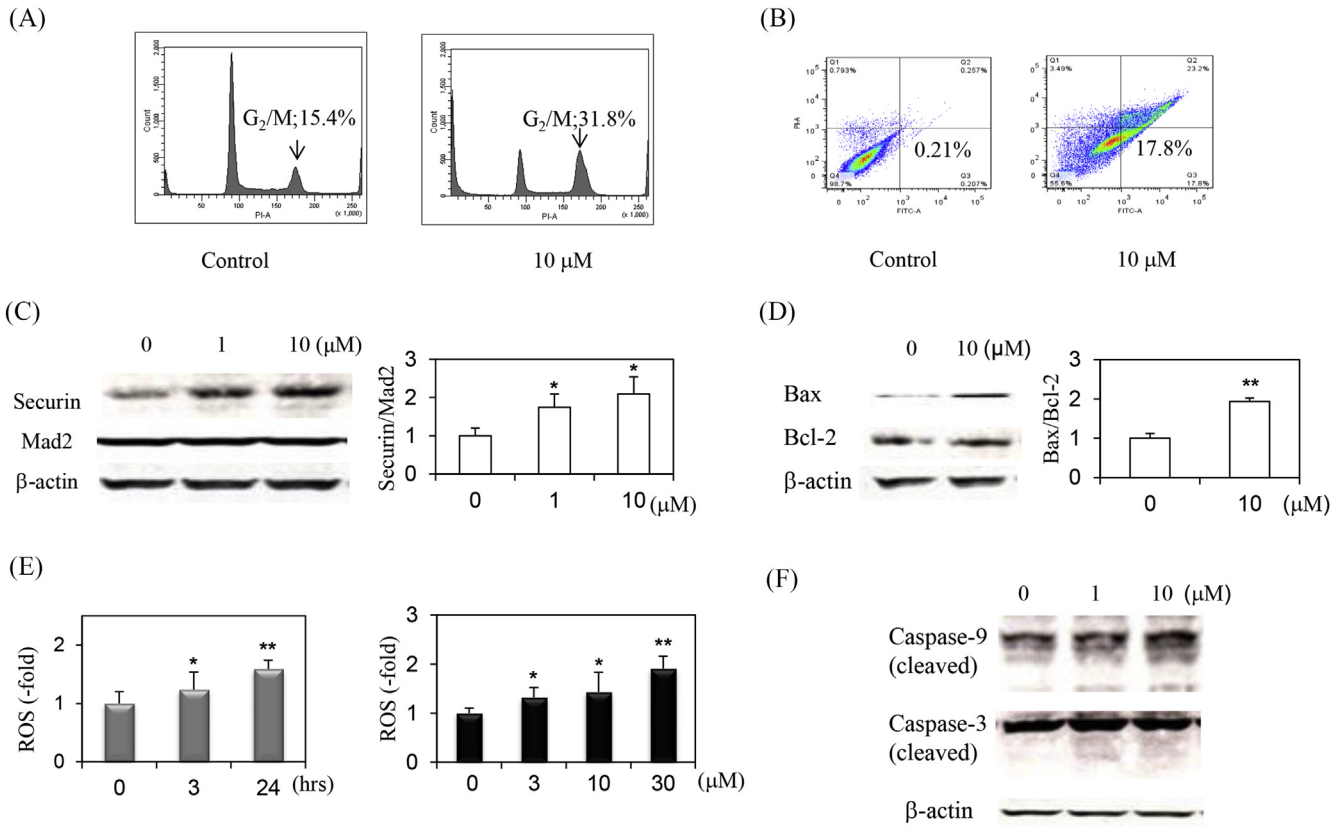

Fig. 3. *In vitro* effect of KPYB10602 on cell cycle (A), apoptosis (B), ROS production (E), and the expression of related proteins(C, D, F). Cells (2×10^6) were cultured with KPYB10602 for 15 h or indicated times. ROS levels (E) were presented as the fold change of MFI. Relative intensity was expressed as securin to Mad2 (C) or Bax to Bcl-2 (D). *$p < 0.05$, **$p < 0.01$ vs. Control.

securin degradation is essential for the separation of duplicated chromosomes after completion of alignment [27]. KPYB10602 suppressed securin degradation in a concentration-dependent manner, which must have prevented the progression from metaphase to anaphase. Metaphase arrest must have led to apoptotic cell death without affecting microtubules dynamics.

Mitotic arrest can result in cell death through mitotic slippage, which is characterized by development of multinucleated (>4 N) cells [28]. Some KSP inhibitors [26] have been reported to induce mitotic slippage, however, it was not observed under our experimental conditions.

ROS production was induced in a time-dependent and concentration-dependent manner. ROS are generated during mitotic arrest mainly via cytochrome *c* release in the mitochondria. Bax is a pro-apoptotic protein that increases the leakage of apoptotic factors from the mitochondria, while Bcl-2 is an anti-apoptotic protein that negatively regulates this leakage. KPYB10602 enhanced the expression of Bax and slightly decreased that of Bcl-2, resulting in a marked increase of the Bax/Bcl-2 ratio. This would have contributed to ROS production via promotion of cytochrome *c* release. In addition, cleaved caspase-9 and -3 were observed. Therefore, not only increased Bax/Bcl-2 but also ROS production would have been involved in caspase activation and apoptosis.

Mitotic arrest can cause the accumulation of P53 [29], and in particular P53 phosphorylated at serine 15 has been reported to induce apoptosis partly via activation of Bax [30]. While P53 expression was increased by carboplatin, KPYB10602 caused little increase of P53 in the present study (data not shown). This might suggest that KPYB10602-induced apoptosis was independent of P53-mediated pathways.

Among KSP inhibitors that we tested *in vitro*, KPYB10602 was the most potent against A2780 human ovarian cancer cells. This potency was reflected by the IC_{50} for KSP ATPase (0.031 μM for KPYB10602, 0.037 μM for KPYC12688, and 0.17 μM for KPYC12687). It should be noted that these compounds are much specific for KSP and do not target other kinesin motor proteins such as CENP-E, Kid, MKLP-1, or KIF-4 [23].

Some KSP inhibitors have already been investigated in preclinical studies [10,15–21]. Some KSP inhibitors have been reported to show inhibitory activity even against drug-resistant ovarian cancer [24], suggesting that these compounds could become a new treatment option for ovarian cancer with less toxicity. Therefore, in the next study, KPYB10602 would have to be examined against drug-resistant ovarian cancer. In parallel, the distribution of a drug can affect its efficacy as well as potency. If *in vivo* drug delivery systems to target tumor cells can be developed, the anti-tumor effect of KPYB10602 could be augmented further.

The specific KSP inhibitor, KPYB10602 showed anti-tumor activity in A2780 tumor-bearing mice. KPYB10602 was proved to suppress securin degradation, leading to mitotic arrest. Mitotic arrest by KPYB10602 must have led to apoptotic cell death without affecting microtubules dynamics, different from paclitaxel. This was supported by the fact that KPYB10602 showed little *in vivo* neurotoxicity. Taken together, KPYB10602 could be a promising candidate as an anti-tumor agent with reduced adverse events for treating human ovarian cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (25462618); Platform for Drug Discovery, Informatics, and Structural Life Science from MEXT, Japan. T.T. is grateful for JSPS Research Fellowships for Young Scientists.

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