A Novel KSP Inhibitor, KPYB10602, Induces Mitotic Arrest and Cell Death in Breast Cancer Cells

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Abstract

Purpose: Taxane-based combination chemotherapy remains the predominant treatment for breast cancer. However, taxane-related drug resistance and neurotoxicity have prompted us to develop substitute treatment strategies. Kinesin spindle protein (KSP), which is essential for formation of the bipolar mitotic spindle and cell cycle progression through mitosis, has been identified as an attractive target for cancer chemotherapy. We investigated, both in vitro and in vivo, the anti-cancer effect of KPYB10602, a novel KSP inhibitor, in breast cancer.

Materials and Methods: Anti-proliferative activity of KPYB10602 in breast cancer cells was assessed by cell viability assay. The anti-cancer effect and inhibitory mechanism of KPYB10602 in breast cancer cells was further explored by immunofluorescence microscopy, flow cytometry, ELISA, and western blotting. In addition, the anti-tumor effect of KPYB10602 was evaluated in subcutaneous xenograft models.

Results: KPYB10602 showed anti-cancer activity in breast cancer cells. The KPYB10602 treatment was shown to result in induction of mitotic arrest with monopolar spindle formation and then apoptotic cell death. This might occurred via a mitochondria-mediated pathway. KPYB10602 also effectively suppressed tumor growth in a subcutaneous xenograft model.

Conclusion: KSP is a good target for breast cancer chemotherapy, and KPYB10602 has a potential as a novel anti-cancer agent for breast cancer.

Key words
kinesin spindle protein, KPYB10602, breast cancer, chemotherapy, anti-microtubule agent

Introduction

Breast cancer is the most commonly diagnosed cancer in women, with 1 in 12 women in Japan developing breast cancer during the course of a lifetime¹. In addition, breast cancer mortality in Japan has increased annually². A multidisciplinary approach to breast cancer treatment, combining surgical oncology, radiation oncology, and medical oncology, has been shown to improve both disease-free survival and overall survival, and this approach was linked, in a large, registry-based study in Scotland, to a reduction in breast cancer mortality².

At present, taxane-containing chemotherapy, administered either as neo-adjuvant or adjuvant therapy in patients with breast cancer, is the standard of care worldwide³. Taxanes bind microtubules directly, resulting in potent suppression of microtubule dynamics, increased microtubule stability, and interphase microtubule bundling; consequently, cells undergo
mitotic arrest and subsequent apoptotic cell death. Microtubule-targeting agents such as taxanes are effective against a variety of cancers including breast cancer. However, microtubules are not only essential for mitosis; they are required for other critical physiological functions, such as intracellular transport and organelle positioning. Therefore, anti-microtubule agents can cause peripheral neuropathy by interfering with microtubule-based axonal transport. In fact in breast cancer treatment, chemotherapy including the taxanes, especially paclitaxel, causes peripheral neuropathy frequently. Peripheral neuropathy can be very painful, and thus the patient’s quality of life is decreased, and it becomes necessary to modify the drug dosage or even discontinue the drug altogether. Neurotoxicity is one of several dose-limiting toxicities of the taxanes. Therefore, there is an urgent need for new anti-tumor agents that will not affect microtubule dynamics.

Kinesin spindle protein (KSP), a member of the mitotic kinesin family, is involved in the early stages of mitosis. KSP is responsible for centrosome separation, which is required for formation and maintenance of the bipolar spindle. Inhibition of KSP activity blocks cells in mitosis, and this results in the formation of a monopolar spindle. Inhibition of KSP activity is an attractive, candidate chemotherapeutic approach because KSP is expressed predominantly in proliferating cells and is absent from post-mitotic neurons. Therefore, KSP inhibitors are expected not to induce peripheral neuropathy usually observed with traditional microtubule disrupting agents such as paclitaxel.

We evaluated, both in vitro and in vivo, the anti-cancer effect of a novel KSP inhibitor that we have identified for possible application to human breast cancer, and we investigated the mechanism explaining its effect.

Materials and Methods

Agents

Five anti-tumor agents were used in the study: KSP inhibitors KPYB10602, KPYC12688, and KPYC12687 were synthesized according to a previously described method, and S-(methoxytrityl)-L-cysteine was purchased from Watanabe Chemical Industries, LTD (Hiroshima, Japan). Paclitaxel was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Cell lines and cell culture

Three breast cancer cell lines were used in the study: MCF-7 (luminal A type), MDA-MB-231 (claudin-low type), and HCC1937 (basal like type). Cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific). MDA-MB-231 cells were grown in Leibovitz L15 medium (Sigma-Aldrich Inc.) supplemented with 15% FBS, 2 mM L-glutamine (Thermo Fisher Scientific), 1% NaHCO₃ (Thermo Fisher Scientific), and 1% antibiotic-antimycotic solution. HCC1937 cells were grown in RPMI-1640 (ATCC) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% room air.

Animals

Animal experiments were performed in accordance with the Guidelines for Animal Experimentation of St. Marianna University Graduate School of Medicine. Thirty-two female BALB/c-nu mice were purchased from Charles Liver Laboratories (Yokohama, Japan). The mice were housed in an isolated animal room that was maintained at a constant temperature (23±1°C) and 50-60% humidity under a 12-hour light/dark cycle and were allowed free access to 30 kGy CL-2 diet (Clea Japan, Tokyo, Japan) and sterilized water. The experimental protocol was approved by the Animal Research Committee, Institute for Animal Experimentation, St. Marianna University Graduate School of Medicine (approval number 1506012).

Cell viability assay

Cells (3 × 10⁴) were seeded on 96-well plates and then incubated for 24 hours to allow cell attachment. The cells were then treated with various concentrations of the KSP inhibitors or paclitaxel for 48 hours or 72 hours. For control, cells were exposed to medium alone. After incubation, 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Inc.) was added to each well, and the plates were incubated for 4 hours. The formazan crystals that formed were dissolved with a stop solution (20% sodium dodecyl sulfate in 0.01N HCl). Absorbance was measured at 570 nm and ref-
ereference wavelength of 630 nm with the use of a microplate reader (vient nano, DS Pharma Biomedical Co., Ltd, Osaka, Japan).

**Immunofluorescence imaging**

Cells ($1 \times 10^5$) were seeded on glass bottom dishes. Twenty-four hours after seeding, the cells were treated with 1 µM KPYB10602 for 24 hours and then fixed with 4% paraformaldehyde, permeabilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, and blocked in 1% bovine serum albumin (BSA) in PBS. The cells were incubated with anti-α-tubulin antibody (diluted 1:500 in 1% BSA-PBS; T6199, Sigma-Aldrich Inc.) in combination with goat anti-mouse secondary antibody conjugated with Alexa Fluor 488 (diluted 1:1000 in 1% BSA-PBS; A21202, Thermo Fisher Scientific). Nuclei were counterstained with 2 µg/mL Hoechst 33342 (Dojindo, Kumamoto, Japan). Fluorescent images were captured with a KEYENCE BZ-9000 fluorescence microscope (KEYENCE Corp., Osaka, Japan).

**Cell cycle analysis**

Cells ($1 \times 10^5$) were seeded on 6-well plates, incubated for 24 hours, and treated with 1 µM KPYB10602 for 12, 24, or 48 hours. After incubation, cells were harvested and fixed with ice cold 70% ethanol at −20°C, then treated with 10 µg/mL propidium iodide (PI, Sigma-Aldrich Inc.) and 0.1 mg/mL RNase (Sigma-Aldrich Inc.) for 10 minutes. The DNA content of at least 8,000 cells was analyzed by using a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and FACSDiva software (BD Biosciences).

**Enzyme-linked immunosorbent assay (ELISA)**

Expression levels of securin (MBS947752 Human Securin ELISA kit, MyBioSource, San Diego, CA USA), Bax (ab199080 Human Bax ELISA Kit, Abcam plc, Cambridge, UK) and survivin (7169C PathScan Total Survivin Sandwich ELISA Kit, Cell Signaling Technology, Danvers, MA, USA) were determined according to the kit manufacturer’s instructions. Briefly, cells ($1 \times 10^5$) were seeded on 6-cm dishes, incubated for 24 hours, and treated with 1 µM KPYB10602 for 12, 24, or 48 hours. Following incubation, the floating and adherent cells were harvested for analysis. The total protein concentration was measured (23227 BCA protein assay kit (Thermo Fisher Scientific). Samples containing 25 µg total proteins were used for each assay.

**Quantitation of DNA fragmentation**

DNA fragmentation, as a marker of apoptosis, was measured with The Cell Death Detection ELISA<sup>pos</sup> kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, cells ($3 \times 10^5$) were seeded on 96-well plates and incubated for 24 hours. The cells were then treated with 1 µM KPYB10602 and grown for an additional 12, 24, 48, or 72 hours. After incubation, the cytoplasmic fraction was used for assay.

**Western blot analysis**

Cells ($1 \times 10^5$) were seeded on 6-cm dishes, incubated for 24 hours, and treated with 1 µM KPYB10602 for 12, 24, or 48 hours. Protein extraction and quantification were performed as described above. Samples containing 30 µg total proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibodies for caspase-3 (sc7272, Santa Cruz Biotechnology Inc., Dallas, TX, USA) (diluted 1:500) and β-actin (Sigma-Aldrich Inc.) (diluted 1:1500) were applied, followed by incubation with horseradish peroxidase-conjugated secondary antibody (MP Biomedicals, Santa Ana, CA, USA). The blots were developed under enhanced chemiluminescence (ImmunoStar Basic, Wako Pure Chemical Industries), and the protein bands were detected with an LAS-3000 medical imager (Fujifilm, Tokyo, Japan).

**Breast cancer tumor xenograft studies**

Thirty-two mice were injected subcutaneously in the flank with $1 \times 10^7$ MDA-MB-231 cells in 100 µL PBS. Seven days after inoculation, the mice were arbitrarily divided into 3 groups. KPYB10602 (20 mg/kg) dissolved in dimethyl sulfoxide (DMSO) diluted with olive oil, was administered intraperitoneally on 3 consecutive days and then twice per week. The dose and the administration schedule were determined on the basis of data obtained in previous studies<sup>11,12</sup>. Paclitaxel (25 mg/kg), dissolved in DMSO diluted with olive oil, was administered intraperitoneally once a week. As a control, vehicle, DMSO in olive oil, was administered to tumor-bearing mice in a manner like that of the KPYB10602 administration. Tumor volume and animal weight were determined twice a week. Tumor volume (mm<sup>3</sup>) was calculated with a standard formula: length × width<sup>2</sup> ×
1/2. All mice were sacrificed on day 24 after initial treatment.

**Statistical analysis**

All values are expressed as mean + SE. Dunnett’s control-comparison test was used to examine differences in securin, DNA fragmentation, Bax, and survivin levels and in tumor volume and body weight, and analysis of covariance (ANCOVA) was used to examine differences in tumor growth rate. All statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA), and \( P < 0.05 \) was accepted as statistically significant.

**Results**

**Anti-proliferative activity of KSP inhibitors in breast cancer cells**

The anti-proliferative activity of the novel synthesized KSP inhibitors (KPYB10602, KPYC12687, and KPYC12688) was evaluated in breast cancer cells. Cell growth was suppressed, no matter which KSP inhibitor was applied; the most efficient anti-proliferative activity was seen with KPYB10602 (Figure 1a, b). Therefore, KPYB10602 was used for

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**Figure 1.** Effects of KSP inhibitors on breast cancer cell growth.

MCF-7 cells (a) and MDA-MB-231 cells (b) were treated with varying concentrations of KSP inhibitors for 48 hours, and MTT cell proliferation assay was performed. Values are shown as mean + SE (n = 12). (c) MCF-7, MDA-MB-231, and HCC1937 cells were treated with KPYB10602 for 72 hours, and MTT cell proliferation assay was performed. MCF-7 cells (d) and MDA-MB-231 cells (e) were treated with KPYB10602 or paclitaxel for 72 hours, and MTT assay was performed. Values are shown as mean + SE (n = 12).
subsequent experiments. To assess the effects of KPYB10602 on breast cancer cell lines MCF-7, MDA-MB-231, and HCC1937, the cells were treated with varying concentrations of KPYB10602 for 72 h. KPYB10602 suppressed the proliferation of these cells in a concentration-dependent manner (Figure 1c) and at the same potency as paclitaxel (Figure 1d, e).

**Induction of apoptosis by KPYB10602 in breast cancer cell lines via mitotic arrest**

We performed immunofluorescence analysis to understand the mechanism underlying the anti-proliferative effect of KPYB10602 in the breast cancer cell lines. Untreated cells showed normal microtubule spread during interphase and a typical bipolar spindle at the metaphase plate during mitosis (Figure 2, white arrow). In contrast, the spindle that developed in cells treated with KPYB10602 was monopolar (Figure 2, red arrow), and DNA staining was observed as an outer ring surrounding the microtubule staining, a pattern commonly referred to a rosette formation (Figure 2, yellow arrow), indicating suppression of centrosome separation.

The mitotic arrest associated with KPYB10602 was further characterized by means of flow cytometric cell cycle distribution analysis. With KPYB10602 treatment, the number of G$_2$/M phase cells was significantly increased in comparison to the number seen in the control condition (Figure 3a), indicating mitotic arrest. The portion of mitotic cells peaked at 24 hours after treatment (G$_2$/M phase cells: 77.3% of

![Figure 2](image_url) **Figure 2.** Immunofluorescence staining of breast cancer cells treated with KPYB10602.

After exposure of MCF-7 and MDA-MB-231 cells to 1 µM KPYB10602 for 24 hours, the cells were stained with anti-tubulin antibody (green) and Hoechst 33342 (nuclei, blue). Scale bars represent 20 µm. The experiment was performed in triplicate, and similar results were obtained each time.
MCF-7 and MDA-MB-231 cells were treated with 1 µM KPYB10602 for 12, 24, or 48 hours. (a) Flow cytometric analysis was performed for evaluation of cell cycle distribution. The experiment was done in triplicate. (b, c) The protein levels of securin were evaluated by ELISA. Values are shown as mean + SE (n = 4). **P < 0.01 and ***P < 0.001 vs. control.

MCF-7 cells, 73.7% of MDA-MB-231 cells). The subG1 cells appeared after 48 hours of treatment in MDA-MB-231 cells (3.0 %). In addition, expression of securin, which plays important role in cell cycle progression, increased with the addition of KPYB10602 (Figure 3b).

As shown in Figure 4, DNA fragmentation was markedly enhanced with KPYB10602 treatment, and this enhancement indicated increased apoptosis of the breast cancer cells. Furthermore, expression of pro-apoptotic protein Bax was increased, and expression of anti-apoptotic protein survivin was significantly decreased (Figure 5a, b).

Western blotting revealed absence of caspase-3 expression in MCF-7 cells. MDA-MB-231 cells expressed procaspase-3, but its cleaved forms were not
MCF-7 and MDA-MB-231 cells were treated with 1 µM KPYB10602 for 12, 24, 48, or 72 hours. DNA fragmentation was detected by ELISA. Values are shown as mean ± SE (n = 8). **P<0.01 and ***P<0.001 vs. control.

detected (Figure 5c).

Effect KPYB10602 in the MDA-MB-231 cell xenograft model

By day 7 after injection of the cancer cells into the BALB/c-nu mice, tumor volume reached approximately 120 mm³ (day 0), and KPYB10602 (20 mg/kg), paclitaxel (25 mg/kg), or vehicle at an equal amount was administered. Although the tumor volume increased in all groups, the growth rate was significantly inhibited with the KPYB10602 treatment and with the paclitaxel treatment (P < 0.001) (Figure 6a). No significant weight loss or toxicity was observed in either of these 3 groups of mice (Figure 6b).

Discussion

This study demonstrated that a novel synthesized KSP inhibitor, KPYB10602, had anti-tumor activity in human breast cancer cell lines and xenograft tumor model.

The taxanes, such as paclitaxel and docetaxel, are microtubule-stabilizing agents that have been widely applied as active chemotherapy agents in the treatment of breast cancer. They inhibit spindle microtubule dynamics, which causes cell cycle arrest and apoptosis. However, taxane as an anti-microtubule drug is of limited clinical utility because resistance to the drug develops and patients suffer side effects caused by a mutation of the drug-binding site on β-tubulin, overexpression of P-glycoprotein, and neurotoxicity.

In contrast to taxanes, KSP inhibitors do not directly interfere with the intracellular function of microtubules. KSP inhibitors are seen as candidate chemotherapeutic agents, the use of which would avoid toxicity in post-mitotic neurons. KPYB10602 has been shown to selectively inhibit KSP ATPase activity with an IC50 of 0.031 µM in human cervical cancer cell lines. In our study, the anti-proliferative effect of KPYB10602 in breast cancer cells was more efficient than that of the other inhibitors. Furthermore, KPYB10602 suppressed tumor growth without apparent toxicity in the subcutaneous xenograft tumor model. In a previously reported study, mice treated with KPYB10602 did not exhibit significantly impaired in motor coordination.

KSP is responsible for centrosome separation, which is required for formation and maintenance of the bipolar spindle. Inhibition of KSP function by KPYB10602 resulted in a characteristic monoastral spindle phenotype. Like taxanes, KSP inhibitor leads
Figure 5. Expression of apoptosis-related factors.

MCF-7 and MDA-MB-231 cells were treated with 1 µM KPYB10602 for 12, 24, or 48 hours. The protein levels of Bax (a) and survivin (b) were measured by ELISA. Values are shown as mean + SE (n = 4). *P<0.05, **P<0.01 and ***P<0.001 vs. control. Caspase-3 (c) was determined by Western blotting, and β-actin was used as a loading control.
Figure 6. In vivo effect of KPYB10602 on tumor-bearing BALB/c-nu mice.

Mice bearing subcutaneously implanted MDA-MB-231 cells were treated with KPYB10602 (20 mg/kg, n = 10), paclitaxel (5 mg/kg, n = 10), or vehicle (control, n = 12). Tumor volume (a) and body weight (b) were measured twice a week. Values are shown as mean + SE.
to mitotic arrest and apoptosis. We observed an increase in the number of G2/M phase and sub-G1 cells with KPYB10602 treatment, suggesting that apoptotic cell death resulted after mitotic arrest. At the onset of anaphase, securin is ubiquitinated, leading to its destruction and to the liberation of separase, which results in segregation of the chromosomes\(^{20,21}\). Securin, called an anaphase inhibitor, blocks separase function, preventing segregation of the chromosomes\(^{20}\). KPYB10602 treatment resulted in an increase in securin, indicating the arrest of cell cycle progression from metaphase to anaphase.

Although cell death after prolonged mitosis seems to be a common phenomenon in cells treated with KSP inhibitor, the underlying apoptotic mechanism is not clearly understood. Microtubule-targeting agents are known to induce mitochondrial membrane permeabilization and subsequent induction of apoptosis by modulating Bcl-2 family proteins, including Bax\(^{22,23}\). Bax is a proapoptotic protein, and previous reports have described the induction of apoptosis through elevated Bax expression resulting from KSP inhibition\(^{24–26}\). In addition, survivin, a member of the family of inhibitor of apoptosis proteins (IAPs), seems to be selectively expressed in transformed cells and in most human cancers, including breast cancer\(^{27,28}\). Survivin is released from the mitochondria in the cytosol and inhibits apoptosis by suppression of active caspase-9\(^{27,29}\). In this study, the elevated Bax expression and decreased survivin expression were consistent with an increase in DNA fragmentation following KPYB10602 treatment. Our study data suggest that KPYB10602 induced mitotic arrest, followed by cell death mediated via the mitochondrial pathway.

In this study, MDA-MB-231 cells showed higher level of DNA fragmentation and sub-G1 population than MCF-7 cells did. These differences between the cell lines might be due to its molecular biology. In fact, MCF-7 cells lack caspase-3\(^{30}\), and MDA-MB-231 cells have mutant p53\(^{31}\). These 2 proteins are responsible for the induction of apoptosis. In addition to the MCF-7 cells, MDA-MB-231 cells expressed inactive caspase-3, and this expression was independent of the exposure to KPYB10602. Thus we assume that KPYB100602 can induce apoptosis without the activity of caspase-3 and/or p53.

Another advantage of KSP inhibitors is that they can overcome resistance to taxanes caused by overexpression of P-glycoprotein; KSP is not transported out of cells by P-glycoprotein\(^{7}\). Furthermore, KPYB10602 does not affect microtubules dynamics\(^{18}\), and it avoids the taxane resistance that results from mutation at the drug-binding site on β-tubulin.

In summary, our in vitro and in vivo experiments showed a novel KSP inhibitor, KPYB10602, to have a potent anti-cancer effect on breast cancer cells. To the best of our knowledge, this is the first study to identify this novel KSP inhibitor as a promising treatment for breast cancer. Further evaluation is needed to elucidate of the apoptotic pathways by which KPYB10602 acts in breast cancer cells.

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