Abstract. Aurora kinase B (AURKB) inhibitors are regarded as potential molecular-targeting drugs for cancer therapy. The present study evaluated the cytotoxic effect of a combination of AZD1152-hQPA, an AURKB inhibitor, and various anticancer agents on the HeLa human cervical cancer cell line, as well as its cisplatin-resistant equivalent HCP4 cell line. It was demonstrated that AZD1152-hQPA had an antagonistic effect on the cytotoxicity of cisplatin, etoposide and doxorubicin, but had a synergistic effect on that of all-trans-retinoic acid (ATRA), Am80 and TAC-101, when tested on HeLa cells. Cisplatin, etoposide and doxorubicin were shown to increase the cellular expression of AURKB, while ATRA, Am80 and TAC-101 downregulated its expression. These results suggested that AURKB expression is regulated by these anticancer agents at the transcriptional level, and that the level of expression of AURKB may influence the cytotoxic effect of AZD1152-hQPA. Therefore, when using anticancer agents, decreasing the expression of AURKB using a molecular-targeting drug may be an optimal therapeutic strategy.

Introduction

The human aurora kinase (AURK) family consists of three genes, including AURKA, AURKB and AURKC. Their gene products are located in different parts of the nucleus and have been suggested to function independently during the mitotic phase (M-phase) of the cell cycle (1-3). AURKB appears in the nucleus at the initial synthesis phase, and is involved in the regulation of cytokinesis by binding to several proteins containing the inhibitor Survivin (4,5). It has been reported that AURKs are overexpressed in tumor cells and, therefore, they are thought to be potential molecular targets for the treatment of malignant tumors (6-9). A number of inhibitors of AURK (ZM447439, VX-680, AT9283, AZD1152, MLN8054 and MLN8237) have been developed (6). These agents inhibit AURKA and AURKB to varying degrees, and some are currently in phase I clinical trials (10). The previously described inhibitor AZD1152 is a produg that changes to the active form AZD1152-hQPA in the cytoplasm, which has a dominant effect on AURKB (11).

Chemotherapy using anticancer drugs, such as platinum-based therapies or taxanes, and radiotherapy are the most commonly employed strategies for the treatment of gynecological malignant tumors (12,13). AURK inhibitors are thought to be an effective molecular-targeting drug for gynecological malignant tumors (6), and clinical trials for their use against leukemia and other cancers are underway (10). In the future, there is a possibility that ARUK inhibitors may be used in combination with anticancer agents. However, it is unknown which anticancer agents would function most effectively in combination with AURK inhibitors. Sun et al (14) reported that the AURK inhibitor, VX-680, downregulated nuclear factor (NF)-κB expression and increased the sensitivity of tumor cells to anticancer agents. Therefore, evaluation of the cellular expression or activity of NF-κB may emerge as an important basis for the use of AURK inhibitors.
Previously, we reported that cisplatin-resistant HCP4 cells, which are derived from the HEla human cervical cancer cell line, overexpressed AURKB. Furthermore, when treated with AZD1152-hQPA, an AURKB inhibitor, the colony formation activity of cisplatin-resistant cells was shown to be significantly decreased, as compared with HEla cells (15). Based on these results, it was hypothesized that a combination of cisplatin and molecular-targeting drugs may have a synergistic cytotoxic effect on malignant tumor cells. However, the present study demonstrated that an AURKB-specific small interfering RNA (siRNA) and AZD1152-hQPA antagonized the cytotoxic effect of cisplatin, whereas it had a synergistic effect on all-trans-retinoic acid (ATRA) and synthetic retinoids. These two different effects were thought to be due to differences in the expression levels of AURKB induced by treatment with specific anticancer agents. The present study aimed to investigate the expression levels of AURKB in the HEla and HCP4 human cervical cancer cell lines, and propose a strategy for combination therapy involving AURKB inhibitors and anticancer agents.

Materials and methods

Cell culture. HEla cells and their derived cisplatin-resistant HCP4 cells were established and kindly gifted by Professor Shin-Ichi Akiyama (Department of Molecular Oncology, Graduate School Medical and Dental Science, Kagoshima University, Kagoshima, Japan) (15). Both cell lines were cultured in RPMI 1640 Medium, GlutaMAX™ supplement (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in 5% CO₂ at 37°C.

Antibodies and anticancer agents. Rabbit anti-AURKB (cat. no. 1788-1) and mouse anti-β-actin (cat. no. sc-47778) monoclonal antibodies were purchased from Epitomics (Burlingame, CA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. Cisplatin, etoposide and ATRA were purchased from Sigma-Aldrich and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. Doxorubicin was obtained from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). AZD1152-hQPA was purchased from Chemietek (Indianapolis, IN, USA); it was dissolved in dimethyl sulfoxide to a concentration of 10 mM. AZD1152-hQPA was kindly gifted by Professor Shin-Ichi Akiyama (Department of Molecular Oncology, Graduate School Medical and Dental Science, Kagoshima University, Kagoshima, Japan) (15). Doxorubicin was obtained from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). AZD1152-hQPA was purchased from Chemietek (Indianapolis, IN, USA); it was dissolved in dimethyl sulfoxide to a concentration of 10 mM and stored at -20°C. Synthetic retinoids, Am80 (Tambabotene) and TAC-101 were kindly gifted by Dr Shudo Koichi of the Research Foundation ITSUU Laboratory (Tokyo, Japan).

Knockdown analysis using siRNAs. Knockdown of AURKB in HEla cells was performed using AURKB-specific siRNA, as described previously (15). The following 25-bp double-stranded RNA oligonucleotides were commercially generated (Invitrogen; Thermo Fisher Scientific, Inc.): AURKB-specific siRNA: 5'-UUAGGGUCACCACUGACGAUGGCGC-3' and 5'-GCCGCAUCUGCAAGUGGACCUA-3'. A total of 200 pmol siRNA was mixed with 5 µl Lipofectamine 2000 (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. After 20 min, 5x10⁴ cells were gently mixed and incubated for additional 20 min. Transfected cells were used for western blotting and colony formation assay. Negative control siRNA was purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

Cell viability assays and combined effects of anticancer agents. Briefly, HEla cells (1x10⁴) were seeded into 96-well plates for 24 h, and subsequently, cisplatin, etoposide, doxorubicin, ATRA, Am80, TAC-101 and AZD1152-hQPA were added to the cells at a maximum concentration at 20, 40, 1, 500, 100, 200 and 100 µM, respectively, and 2-fold serial dilutions were performed. For combination treatment, fixed combination ratios and 2-fold serial dilutions were employed. For AURKB, specific siRNA-transfected HEla cells (2x10⁴) were seeded into 96-well plates at 24 h post-transfection, and the above indicated single agents were added to the cells with 2-fold serial dilutions. After 72 h, the surviving cells were stained with the water-soluble tetrazolium salt-8 (TetraColor ONE; Seikagaku Corporation, Tokyo, Japan) for 2-3 h at 37°C, according to the manufacturer's protocol. The absorbance was then measured at 450 nm. To measure the half maximal inhibitory concentration (IC₅₀) in each experiment, CalcuSyn software version 2.0 (Biosoft, Cambridge, UK) was used. To evaluate the synergism or antagonism of the combination of an anticancer agent and AZD1152-hQPA, HEla cells were treated with an anticancer agent alone, AZD1152-hQPA alone, or a fixed combination ratio of the anticancer agent and AZD1152-hQPA, as decided by the IC₅₀ values. The experiments were performed in duplicate, with 2-fold serial dilutions. To assess whether there was a synergistic effect on cytotoxicity, the combination index (CI) was calculated using CalcuSyn software version 2.0. This method enables the quantification of synergism (CI<1) and antagonism (CI>1) at different concentrations and effect levels (16). Based on the median effective dose (ED₀, ED₁, and ED₂₀ of the drug combinations, isobolograms were generated and synergy was evaluated using CalcuSyn software version 2.0.

Western blot analysis. Preparation of whole cell lysates and western blot analysis were performed as described previously (15). The cells were washed with PBS twice, and then lysed in buffer containing 50 mmol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA, 120 mmol/l NaCl, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, 1 mmol/l phenylmethylsulfonyl fluoride and 1 mmol/l dithiothreitol. The lysates were centrifuged at 21,000 x g for 10 min at 4°C, and the supernatants (50 µg) were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. The blotted membranes were treated with 3% (w/v) skimmed milk in 10 mmol/l Tris, 150 mmol/l NaCl and 0.2% (v/v) Tween 20, and then incubated for 1 h at room temperature with the corresponding primary antibodies (1:1,000 dilution of rabbit anti-AURKB and 1:10,000 dilution of mouse anti-β-actin). Next, the membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody [anti-rabbit immunoglobulin (Ig) G, peroxidase-linked species-specific whole antibody from donkey (NA934; GE Healthcare Life Sciences, Chalfont, UK) and anti-mouse IgG, peroxidase-linked species-specific whole antibody from sheep (NA933; GE Healthcare Life Sciences)] at 1:7,500 dilution. The bound antibody was visualized using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and the
The signal intensity was quantitated using Multi Gauge software version 3.0 (Fujifilm, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was purified from the transfected- and non-transfected HeLa cells using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), and RT-qPCR was performed as described previously (17). Briefly, RT from total messenger RNA (mRNA) with random primers (Applied Biosystems; Thermo Fisher Scientific, Inc.) was performed with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), and then PCR with TaqMan® Universal Master Mix II with UNG (Thermo Fisher Scientific, Inc.) was conducted on the StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following cycling parameters: 2 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C, according to the manufacturer's protocol. The following primer sets for PCR were used: Hs01060665_g1 for \( \beta \)-actin, Hs00945858_g1 for AURKB and Hs01582072_m1 for AURKA (Applied Biosystems; Thermo Fisher Scientific, Inc.). The comparative Cq method was used to quantify the gene expression (18). Values were normalized to those for human \( \beta \)-actin. All samples were analyzed in duplicate in each experiment.

Statistical analysis. Student's t-tests were performed for statistical analysis of the variables between the two groups with GraphPad StatMate statistical software version IV (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation.

### Results

AZD1152-hQPA antagonizes the cytotoxic effect of cisplatin on HeLa cells. In our previous study, we reported that the AURKB protein was overexpressed in cisplatin-resistant PCDP5 and HCP4 cells, as compared with the parent PC3 and HeLa cells, respectively, and was induced by cisplatin treatment in a concentration- and dose-dependent manner (15). Furthermore, the AURKB inhibitor, AZD1152-hQPA, sensitized cisplatin-resistant cells, but not parent cells, to cisplatin. To confirm whether cisplatin and AZD1152-hQPA have a synergistic cytotoxic effect on cancer cells, the present study calculated the CI using CalcuSyn software. First, the IC\(_{50}\) values of cisplatin and AZD1152-hQPA with HeLa cells were calculated (Table I), and were 1.87 and 14.8 \( \mu \)M, respectively. Based on these results, we employed a fixed combination ratio of cisplatin-to-AZD1152-hQPA of 1:5 (Table II), and treated HeLa cells with these agents using a single or a fixed combination concentration to calculate the CI. As is shown in Table III, the CIs at ED\(_{50}\), ED\(_{75}\) and ED\(_{90}\) were 1.35, 1.46 and 1.62, respectively, which suggested that, as the concentration of the drugs was elevated, the CI increased. These results indicated...
that cisplatin and AZD1152-hQPA did not have a synergistic cytotoxic effect, but an antagonistic cytotoxic effect, on HeLa cells.

Subsequently, the effect of the combined administration of cisplatin and AZD1152-hQPA on cisplatin-resistant HCP4 cells derived from HeLa cells was investigated. From the result of IC$_{50}$ values for HCP4 (Table I), HCP4 cells were 35-times more resistant to cisplatin than HeLa cells. Therefore, a fixed combination ratio of cisplatin-to-AZD1152-hQPA of 2:1 was employed (Table II). As is shown in Table III, the CI of cisplatin and AZD1152-hQPA was very high, especially when used at ED$_{75}$ and ED$_{90}$, indicating that they had a strong antagonistic cytotoxic effect on HCP4 cells. The antagonistic cytotoxic effect on HCP4 cells was much higher than that of HeLa cells.

**Effect of anticancer agents on AURKB expression.** Consistent with our previous report (15), cisplatin treatment of HeLa cells induced the protein expression of AURKB in a concentration-dependent manner (Fig. 1). To confirm whether AURKB protein expression was transcriptionally induced by cisplatin, RT-qPCR was performed, and demonstrated that AURKB mRNA expression was upregulated by cisplatin (Fig. 2). It was hypothesized that the level of AURKB protein induced by anticancer agents might influence the cytotoxic effect of AZD1152-hQPA. Therefore, the effect of several anticancer agents on AURKB expression was examined.

Notably, etoposide and doxorubicin increased both the protein and mRNA expression levels of AURKB (Figs. 1 and 2). Conversely, ATRA, synthetic retinoids, Am80 and TAC-101 decreased the protein and mRNA expression levels of AURKB (Figs. 1 and 2). In addition, the mRNA expression levels of AURKA in HeLa cells treated with various anticancer agents were evaluated. Cisplatin, etoposide and doxorubicin decreased the mRNA expression levels of AURKB (Figs. 1 and 2). In cisplatin-resistant HCP4 cells, the CI values of ATRA, Am80 and TAC-101 were not shown).

**AURKB expression status is affected by anticancer agents, which influences the cytotoxic effect of AZD1152-hQPA.** To investigate whether the AURKB expression status affects the cytotoxic effect of AZD1152-hQPA, CI values were calculated for the combined administration of AZD1152-hQPA with various anticancer agents. In HeLa cells, etoposide and doxorubicin, as well as cisplatin, increased the expression of AURKB and the CI value was >1 in all cases, with the exception of etoposide at ED$_{50}$, indicating that they had a strong antagonistic cytotoxic effect on HCP4 cells. The antagonistic cytotoxic effect on HCP4 cells was much higher than that of HeLa cells.

**Figure 1.** Protein expression of AURKB in HeLa cells is regulated by anticancer agents. (A) HeLa cells were treated with cisplatin, etoposide, doxorubicin, ATRA, Am80 or TAC-101 for 72 h, and the protein expression of AURKB was analyzed by western blotting. x0.5, x1.0, and x2.0 indicate the amplification of the IC$_{50}$ concentration shown in Table II. (B) Relative expression levels of AURKB were determined by densitometry. Each control was set to 1. AURKB, aurora kinase B; ATRA, all-trans-retinoic acid.
<1 at ED$_{50}$ and ED$_{75}$, but were >1 at ED$_{90}$ (Table III). Furthermore, the CI of etoposide and doxorubicin at ED$_{25}$ and ED$_{50}$ was <1 when tested using HeLa cells. As is shown in Table I, cisplatin-resistant HCP4 cells had a multidrug-resistant phenotype, but this mechanism had no effect on the cytotoxicity of ATRA and synthetic retinoids.

Figure 2. Aurora kinase B (AURKB) expression in HeLa cells is regulated by anticancer agents at the transcriptional level. HeLa cells were treated with cisplatin, etoposide, doxorubicin, ATRA, Am80 or TAC-101 for 72 h, after which the mRNA expression levels of AURKB were analyzed by reverse transcription-quantitative polymerase chain reaction. x1.0, x1.5 and x2.0 indicate the amplification of the IC$_{50}$ concentration shown in Table II. ATRA, all-trans-retinoic acid.

Figure 3. Knockdown of AURKB in HeLa cells and their sensitivity to anticancer agents. (A) HeLa cells were transfected with siCtrl or siAURKB for 48 h, followed by treatment with various anticancer agents for 72 h and determination of AURKB expression levels by western blotting and densitometry. AURKB expression was normalized to β-actin, and the expression ratio of the WT cells was set as 1. (B) Relative IC$_{50}$ values were obtained from the concentration-response curves for each anticancer agent. The IC$_{50}$ value for siCtrl-transfected cells was set to 1. *P<0.05 and **P<0.01 vs. siCtrl-transfected cells. AURKB, aurora kinase B; ATRA, all-trans-retinoic acid; WT, wild-type; siCtrl, control small interfering RNA (siRNA); siAURKB, AURKB-specific siRNA; IC50, half-maximal inhibitory concentration.
Knockdown of AURKB and the cytotoxic effects of anticancer agents. AZD1152-hQPA decreases the activity of AURKB (11). To investigate whether knockdown of AURKB affected the cytotoxic effect of ATRA, Am80 and TAC-101, HeLa cells were transfected with AURKB-specific siRNA and treated with these agents. As is shown in Fig. 3A, the expression of AURKB was downregulated in AURKB-specific siRNA-knockdown HeLa cells, as compared with cells transfected with control siRNA. Knockdown of AURKB increased the IC₅₀ values of cisplatin, etoposide and doxorubicin, and decreased the IC₅₀ values of ATRA, Am80 and TAC-101, as compared with the control (Fig. 3B).

Discussion

AURKs are important molecules involved in mitosis, and AURKA and AURKB have been shown to be overexpressed in several types of cancer (1). Therefore, AURKs are potential novel molecular targets for the prevention of cancer proliferation, and clinical trials have been performed (10). It has been reported that AURKB inhibitors are able to enhance the cytotoxic effects of anticancer agents (19-21). Fu et al. (19) reported that the AURK inhibitor VE 465 was able to enhance the anti-tumor activity of carboplatin in human ovarian cancer cells. Fiskus et al. (20) used a combination treatment involving the pan-AURK inhibitor MK-0457 and vorinostat in human breast cancer cells, and demonstrated a synergistic effect in both in vitro and in vivo assays. Therefore, the present study aimed to investigate the cytotoxic effect of the AURKB-specific inhibitor, AZD1152-hQPA, and cisplatin on the HeLa cervical adenocarcinoma cell line, and demonstrated that the effects were antagonistic. Previously, we reported that the expression of AURKB was increased following treatment of cancer cells with cisplatin (15). It was hypothesized that the induction of AURKB by cisplatin treatment may alter the sensitivity of cancer cells to AZD1152-hQPA, and it was decided that we would search for anticancer agents that increased or decreased AURKB expression. In the present study, it was demonstrated that doxorubicin, etoposide and cisplatin increased the expression of AURKB, while ATRA, Am80 and TAC-101 decreased its expression. Subsequently, the combined effects of these anticancer agents with AZD1152-hQPA were investigated, their IC₅₀ were calculated, and it was demonstrated that the combinations that increased AURKB expression showed antagonistic cytotoxic effects on HeLa cells. In contrast, the combination of AZD1152-hQPA with anticancer agents that decreased the expression of AURKB showed synergistic cytotoxic effects on HeLa cells. These results indicated that AURKB expression may influence the cytotoxic effect of AZD1152-hQPA. To the best of our knowledge, there has been no previous report showing a synergistic cytotoxic effect for AZD1152-hQPA used in combination with anticancer agents. Zhang and Zhang (21) reported that ZM447439, an AURKB inhibitor, suppressed the growth of SiHa cervical cancer cells and enhanced their chemosensitivity to cisplatin, which was inconsistent with the results of the present study. Both AZD1152-hQPA and ZM447439 also inhibited AURKA, but the effect of AZD1152-hQPA on AURKB inhibition was greater than ZM447439 (6). Furthermore, AURKA expression was increased by cisplatin in the present study. This discrepancy might be due to differing specificities for AURKs or the type of cells used.

In the present study, cisplatin, doxorubicin and etoposide increased the cellular expression of AURKB, while ATRA, Am80 and TAC-101 decreased it. Notably, these anticancer agents regulated AURKB expression at the transcriptional level, as demonstrated by RT-qPCR. From sequence alignment analysis, the Alu sequence upstream of the transcription start site of the AURKB gene was identified, and it was observed that the promoter region contained ~230 bp (data not shown). The promoter region of the AURKB gene is ~230 bp (data not shown). Kimura et al. (22) reported that the E2 family (E2F) of transcription factors promote the transcription of the AURKB gene via a cell-cycle-dependent element (CDE) in the promoter region. Ianari et al. (23) reported that treatment with cisplatin or doxorubicin increased E2F-1 expression, and E2F-1 Ser403 phosphorylation was induced by doxorubicin (24). Conversely, E2F-1 expression, induced by treatment with estrogen, was inhibited by treatment with a trans-retinoic acid (25). Therefore, the CDE/E2F-1 pathway may be associated with AURKB gene expression and regulated by anticancer agents. Notably, the effects of anticancer agents on the cell cycle in previous studies were different. Cisplatin, doxorubicin and etoposide induced G2/M-phase cell cycle arrest (26-28), while ATRA, Am80 and TAC-101 induced G1 cell cycle arrest (29-31). Further analysis is required to elucidate the associations among AURKB expression and anticancer agents.

Previously, we reported that AURKB expression in cisplatin-resistant HCP4 cells was upregulated, as compared with parent HeLa cells, and demonstrated that HCP4 cells were hypersensitive to AZD1152-hQPA using colony formation assays (15). However, in the present study, cytotoxicity assays demonstrated that HCP4 cells were resistant to AZD1152-hQPA. This discrepancy may be due to differences in the assays performed and the growth rates of each cell line. Furthermore, upregulation of AURKB expression in HCP4 cells may have contributed to induce resistance to AZD1152-hQPA. Unlike HeLa cells, the combined treatment of AZD1152-hQPA and anticancer agents had a biphasic effect in cisplatin-resistant HCP4 cells: the ED₅₀ of ATRA and synthetic retinoids was synergistic, while the ED₅₀ of these agents was antagonistic. This biphasic effect may also be influenced by the slow growth rate of HCP4 cells. Further analysis is required to develop an effective method to overcome cisplatin resistance when the combination of AURK inhibitors and anticancer agents is used.

In the present study, AZD1152-hQPA had an antagonistic effect on cisplatin. Therefore, whether AURKB knockdown was able to inhibit the cytotoxic effect of cisplatin was investigated. AURKB knockdown resulted in the resistance of HeLa cells to cisplatin, doxorubicin and etoposide, while it sensitized the cells to ATRA, Am80 and TAC-101, as well as AZD1152-hQPA. These results suggested that strategies involving AURKB inhibition or knockdown may have similar effects as anticancer agents.

In conclusion, the results of the present study suggested that a combination of molecular-targeting drugs against AURKB and anticancer agents may influence the cytotoxic effects in cells. The optimal combination therapy may be determined by revealing these mechanisms.
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