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Effects of KRC-108 on the Aurora A activity and growth of colorectal cancer cells

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ABSTRACT

Aurora A is involved in regulating multiple steps of mitosis. Over-expression of Aurora A is related to tumorigenesis and poor prognosis. KRC-108 is a novel multi-kinase inhibitor which has anti-tumor activity *in vivo*. In this study, we identified the inhibitory effects of KRC-108 on Aurora A kinase and growth-inhibitory characteristics of KRC-108. The *in vitro* kinase activity assay, immunoblot, and immunofluorescence analyses demonstrated that KRC-108 inhibited Aurora A activity. KRC-108 exhibited cytotoxicity against human colorectal cancer cell line HT-29. Colony formation assays showed that KRC-108 reduced the colony growth of HT-29 cells. KRC-108 also inhibited migration of HT-29 cells. The expression levels of cyclin B1 and CDC2 were decreased by KRC-108 in HT-29 cells. Cell cycle analysis and flow cytometry indicated that the inhibitory effects of KRC-108 on cell growth are due to induction of G_2/M arrest and apoptosis by inhibition of Aurora A. KRC-108 induces cell-cycle arrest and apoptosis in colorectal cancer cell line by Aurora A inhibition. The reported *in vivo* anti-tumor effects of KRC-108 might partly be due to anti-Aurora A effects. This study suggests that KRC-108 has potential for development as an anti-tumor agent, although further studies are needed.

1. Introduction

The Aurora A kinase belongs to the serine/threonine protein kinases that have a key role in several stages of mitosis [1]. Aurora A is involved in regulating multiple steps of mitosis including centrosome maturation, formation of bipolar spindle, attachment of chromosomal kinetochore to mitotic spindle, and metaphase I spindle orientation [1,2]. Aurora A is activated by binding to proteins such as Ajuba, Bora, PAK1 and TPX2 [3,4]. This leads to the autophosphorylation of a threonine residue (Thr288) in the activation loop [1]. Selective inhibition of Aurora A results in inhibition of autophosphorylation of Aurora A at Thr288, inhibition of p53 phosphorylation, monopolar spindles, and G_2/M arrest [5]. Various Aurora substrates have been identified. Among them the most well characterized substrate is histone H3, a protein involved in chromosome condensation and mitotic entry [6].

Over-expression of Aurora A has been reported for a wide range of solid tumors [7] including colorectal tumors [8,9]. Moreover, Aurora A is amplified in these tumors and localized to an amplicon, AURKA locus (20q13), which is associated with poor prognosis in patients with colorectal tumors [8]. The constitutive activation of Aurora A has been suggested to cause down-regulation of mitotic spindle assembly checkpoint [10] and inhibition of cytokinesis [11], leading to the oncogenic transformation of cells. Aurora A has emerged as an important target for anticancer therapy [3].

Recently, we have synthesized a series of aminopyridine compounds substituted with benzoxazole that inhibit several kinases, with the goal of discovering potential anticancer agents [12]. One of the compounds, KRC-108 showed potent multi-kinase inhibition activities and anti-proliferative activities in several cell lines including colorectal cancer cell line HT-29 *in vitro*. Furthermore, KRC-108 also suppressed tumor growth in mice xenograft models *in vivo* after oral administration [13]. However, the effect of KRC-108 on Aurora A kinase had not yet been evaluated. In this study,

Abbreviations: HTRF, homogeneous, time-resolved fluorescence; TR-FRET, time resolved-fluorescence resonance energy transfer; PARP, Poly (ADP-ribose) polymerase; CDC2, cell division cycle protein 2.

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we identified the inhibitory effects of KRC-108 on Aurora A kinase and cell growth-inhibitory characteristics of KRC-108.

2. Materials and methods

2.1. Cell culture and reagents

Human HT-29 colon cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium (Sigma Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2 in an incubator.

For the cytotoxicity assay, cell viability was measured by a tetrazolium-based assay using EZ-Cytox Cell Viability Assay kit (DaeilLab, Korea). IC_{50} was calculated by nonlinear regression using Prism version 5.01 (GraphPad, La Jolla, CA) [14].

For the anchorage-independent growth assay in soft agar, HT-29 cells (10,000 cells per well) were suspended in 1.5 ml of DMEM medium containing 10% fetal bovine serum and 0.3% Noble agar (BD biosciences, San Jose, CA) and layered over a base prepared in 6-well plates of DMEM medium, 10% fetal bovine serum, and 0.5% agarose supplemented with KRC-108 compound. The plates were incubated for 4 weeks at 37 °C in a humidified CO_2 incubator. Live colonies were stained with 0.005% crystal violet solution, and visualized under a microscope.

2.2. In vitro kinase assay

Inhibition of kinase activity against Aurora A recombinant kinases was measured using homogeneous, time-resolved fluorescence (HTRF) assays [15]. Recombinant proteins containing the Aurora A kinase domain were purchased from Millipore (Billerica, MA). Optimal enzyme, ATP, and substrate concentrations were established using an HTRF KinEASE kit (Cisbio, France) according to manufacturer instructions. Assays consist of enzymes mixed with serially diluted compounds and peptide substrates in a kinase reaction buffer (250 mM HEPES (pH 7.0), 100 μM ATP, 0.5 mM orthovanadate, 5 mM $MgCl_2$, 1 mM DTT, 0.05% BSA, 0.1% NaN_3). Following the addition of reagents for detection, the TR-FRET signal was measured with a Victor multi-label reader (Perkin Elmer, Waltham, MA). IC_{50} was calculated by nonlinear regression using Prism.

2.3. Molecular docking simulation

The molecular docking was performed by using CDOCKER interfaced with Accelrys Discovery Studio 3.5. The high resolution (2.30 Å) crystal structure of human Aurora kinase A in complex with VX-680 was downloaded from the protein data bank (PDB id: 3E5A) [16]. For the preparation of protein structure, water and hetero atoms were removed, the hydrogen atoms were added, incomplete side chain residues were corrected and finally energy minimization were accomplished. The active site for docking was selected as the VX-680 binding pocket from the co-crystal structure and the CHARMm force field was applied. The 3D structure of KRC-108 as the ligand for analysis was generated with an energy minimization process using Discovery Studio 3.5. The VX-680 was used as a reference compound to verify our docking protocol, resulting in well-docked poses having almost the same binding mode compared with the orientation of the co-crystal structure.

2.4. Flow cytometric analysis

Flow cytometric analysis was used to evaluate cell apoptosis as described previously [17]. Cells were grown in 6-well plates (100,000 cells per well) and treated with KRC-108 compound for 48 h. Cells were detached, fixed, and treated with RNase A (4 mg/ml). Cells were then stained with propidium iodide (PI) (Sigma) and subjected to flow cytometry using Accuri C6 (BD Biosciences). Data were analyzed by BD Accuri C6 software. For Annexin V staining, cells were seeded in 6-well plates (1×10^6 cells per well) and treated with KRC-108 for 48 h. Cells were then stained with Dead Cell Apoptosis Kit with Alexa Flour 488 Annexin V (Life Technologies, Carlsbad, CA) and subjected to flow cytometry (Accuri C6).

2.5. Immunoblot and immunofluorescence

An immunoblot analysis was performed as previously described [15]. Antibodies against phospho-Aurora A (p-Aurora A) and PARP were purchased from Cell Signaling Technology (Danvers, MA). Anti-histone H3 antibody is from Abcam (Cambridge, MA) and Anti-phospho-histone H3 (Ser10) antibody is from Millipore (Billerica, MA).

An immunofluorescence analysis was performed as described in previous report [18]. Briefly, cells were seeded in an 8-well culture slide (20,000 cells per well) and treated with KRC-108 as indicated. Cells were fixed with 4% formaldehyde solution and then permeabilized with 0.5% Triton X-100 solution. Slides were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (0.1 M phosphate buffer, pH 7.2) containing 0.2% Tween-20 (PBST), and were incubated overnight with the primary antibody at 4 °C. The following day, the slides were washed in PBST, incubated with Alexa Flour 546 labeled secondary antibody (Life Technologies) for an hour at room temperature, then washed with 1% BSA in PBST. Slides were mounted with Prolong Gold Antifade reagent with DAPI (Life Technologies), and visualized using a fluorescent microscope Axiovert 200 (Carl Zeiss, Germany). The number of cells with positive stain in twenty randomly selected fields was counted using NIH Image J software (ver 1.48, Bethesda, MD).

2.6. Wound healing assay

HT-29 cells were plated on 6-well culture plates at 90% confluence. A wound of 2 mm width was made in the cell monolayer using a 10 μl tip. After wounding, the detached cells were removed and treated with various concentrations of KRC-108. Cells were allowed to migrate for 24 h and then photographed using a phase contrast microscope (Carl Zeiss).

2.7. RT-PCR

Cells were harvested and total RNA was extracted using the ReliaPrep RNA cell miniprep system (Promega, Madison, WI). cDNAs were synthesized by using the cDNA reverse-transcription kit (Life Technologies). One microgram of cDNA was amplified with 0.5 μM EF1α, CDC2, and cyclin B1 primers, using polymerase chain reaction (PCR): 94 °C for 5 min, 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 74 °C for 90 s. The PCR products were analyzed by electrophoresis and stained with Redsafe (iNtRON) on a 1.5% agarose gel. The following are sequences of primers used in the study: EF1α: forward 5′-AGG TGA TTA TCC TGA ACC ATC C-3′, reverse 5′-AAA GGT GGA TAG TCT GAG AAG C-3′, size 234 bp; CDC2: forward 5′-GGT TCC TAG TAC TGC AAT TCG-3′, reverse 5′-TTT GCC AGA AAT TCG TTT GG-3′, size 709 bp; cyclin B1: forward 5′-AAG AGC TTT AAA CTT TGG TCT GGG-3′, reverse 5′-CTT TGT AAG TCC TTG ATT TAC CAT G-3′, size 319 bp.

3. Results

3.1. KRC-108 inhibits the kinase activity of Aurora A

KRC-108 (Fig. 1A) has been reported to inhibit *in vitro* and *in vivo* tumor growth [13]. KRC-108 inhibited several kinases such as Ron, Flt3, and TrkA as well as c-Met, with high potency. In order to investigate other possible mechanisms of anti-tumor activity of KRC-108 in HT-29 cells, KRC-108 was tested against Aurora A kinase activity. Using the purified recombinant Aurora A protein, inhibition by KRC-108 was measured using the TR-FRET method as described in Materials and Methods. KRC-108 exhibited strong inhibition of Aurora A kinase activity in a concentration-dependent manner with an IC_{50} of 0.59 μM (Fig. 1B).

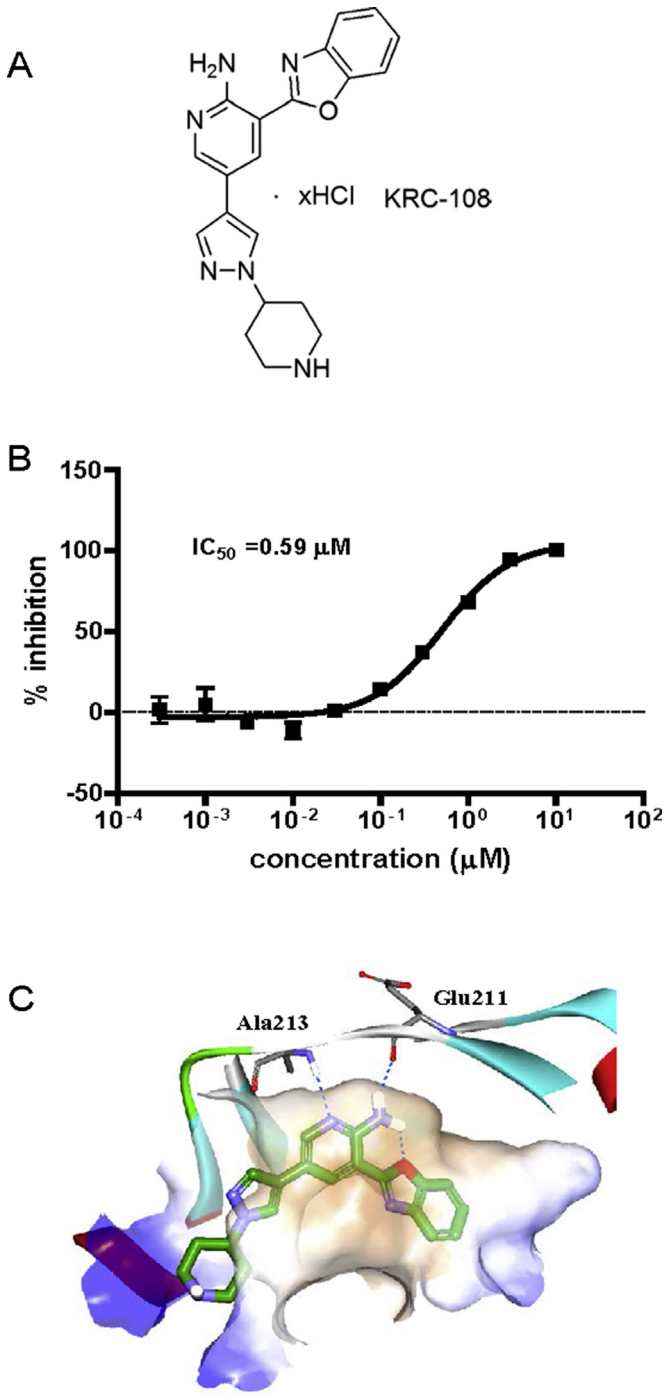


Fig. 1. Direct inhibition of Aurora A kinase by KRC-108. (A) Structure of KRC-108. (B) Effects of KRC-108 on the *in vitro* kinase activity of Aurora A. Direct inhibition of kinase activity was measured using purified recombinant Aurora A using the TR-FRET method. (C) The docking structure of KRC-108 (green) in Aurora kinase A (PDB id: 3E5A).

The binding mode of compound KRC-108 in Aurora kinase A is shown in Fig. 1C. The docking result indicated that a pyridine ring of KRC-108 made a key interaction with residues in the hinge region. As shown in the Fig. 1, NH_2 group and N of the pyridine ring of compound KRC-108 showed hydrogen bonding interactions with backbone carbonyl group (−C═O) of Glu211 and backbone NH group of Ala213 in the hinge region. The pyridine ring also formed hydrophobic interactions with surrounding residues such as Leu139, Ala213, Tyr212 and Leu263 (not shown in Fig. 1C). The benzoxazole ring was oriented inside the ATP binding cavity beside the alpha helix (as shown in white color surface in the Fig. 1C). CDOCKER interaction energy values for VX-680 and KRC-108 were −53.00 kcal/mol and −44.84 kcal/mol, respectively.

3.2. Effects of KRC-108 are consistent with inhibition of Aurora A activity in HT-29 cells

The mitotic kinase Aurora A phosphorylates itself on Thr288 and histone H3 on Ser10 in M phase [6]. In order to evaluate the inhibition of Aurora kinase activity in cells by KRC-108, the levels of phosphorylated Aurora A and histone H3 Ser10 were determined using immunoblot and immunofluorescence analyses. In HT-29 cells, KRC-108 inhibited autophosphorylation of Aurora A and phosphorylation of histone H3 (p-histone H3) in a concentration-dependent manner, while the total expression level of histone H3 was not changed (Fig. 2A). Next, the inhibition of Aurora A activity by KRC-108 was confirmed, as measured by p-histone H3 inhibition with time course experiments in HT-29 cells. Treatment with KRC-108 induced a time-dependent decrease in p-histone H3 (Fig. 2B). The inhibitory effect on the phosphorylation of histone H3 occurred from 2 h and 1 h after treatment of 1 μM and 10 μM KRC-108, respectively. In the immunofluorescence analysis, HT-29 cells treated with KRC-108 for 2 h showed a concentration-dependent decrease in the number of p-histone H3 immunofluorescence staining positive cells (Fig. 2C). KRC-108 significantly decreased the ratio of p-histone H3 positive cells to total cells at all concentrations tested.

3.3. KRC-108 inhibits proliferation of HT-29 cells and induces apoptotic cell death

In order to investigate the effects of KRC-108 on cell proliferation, a cytotoxicity assay was performed using HT-29 cells. KRC-108 displayed anti-proliferative activity against HT-29 cells with a GI_{50} value of 1.33 μM which was similar to the reported value (Fig. 3A) [13].

In order to detect apoptotic cells, Annexin V staining was performed. As shown in Fig. 3B, cells were treated with KRC-108 for 48 h and analyzed with flow cytometry after staining with Alexa Flour 488 Annexin V and PI. The number of Annexin V-positive cells was increased by KRC-108 treatment. The percentage of early apoptotic cells (bottom right quadrant) increased by KRC-108 treatment. The cell fraction in the top right quadrant (cells undergoing late apoptosis or already dead cells) increased approximately two-fold with KRC-108 treatment. These results are consistent with growth inhibition (Fig. 3A) and cell cycle analyses (Fig. 4A) and indicate apoptotic cell death by KRC-108.

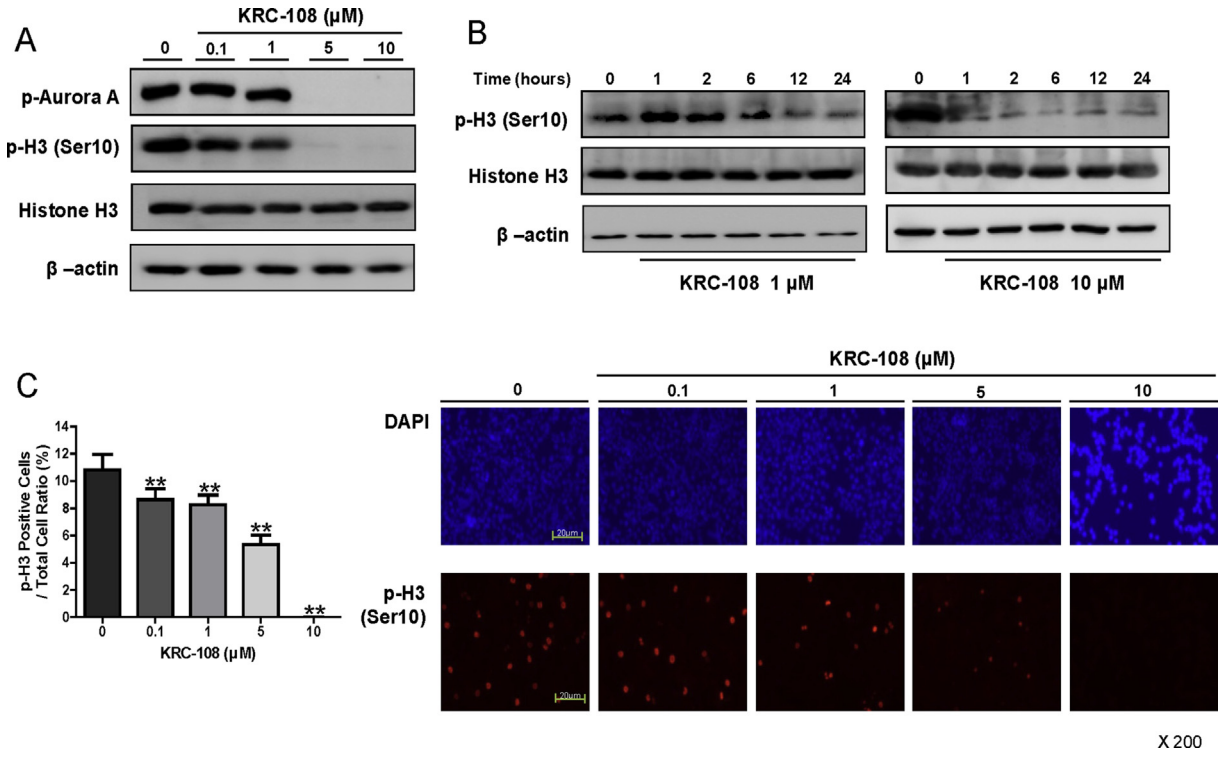


Fig. 2. Inhibitory effects of KRC-108 on phosphorylation of Aurora A in HT-29 cells. HT-29 cells were treated with KRC-108 for 2 h (A) or various times (B). Cell lysates were subjected to western blotting using antibodies against phosphorylated Aurora A (p-Aurora A), phosphorylated histone H3 (p-H3) on Ser10, and total histone H3. As loading controls, β-actin antibody was used. (C) Representative images of p-H3 positive cells treated with KRC-108. Cells were treated with KRC-108 for 2 h and the numbers of p-H3 positive cells and total cells were counted by fluorescence-based microscopy. Cells were stained with p-H3 antibody (red) and DAPI (blue). The ratios of p-H3 positive cell numbers to total cell numbers were compared after treatment of KRC-108. The data are expressed as mean ± standard deviation. $^{**}P < 0.01$ statistically significant compared to control group using the unpaired *t*-test.

Apoptosis induced by KRC-108 was confirmed by PARP cleavage. As shown in Fig. 3C, a dose-dependent decrease of PARP with a corresponding increase in cleaved PARP was observed after KRC-108 treatment.

To investigate the effect of KRC-108 on the anchorage-independent growth of HT-29 cells, soft agar assay was employed. HT-29 cells were grown in soft agar containing KRC-108 for 14 days. As shown in Fig. 3D, colonies of HT-29 cells almost completely disappeared after treatment of KRC-108 at 1 and 10 μM. These results indicate that KRC-108 inhibits the anchorage-independent growth of HT-29 cells.

Cell migration was assessed by the wound healing assay under the treatments of various KRC-108 concentrations. Migration was checked after 24 h. Treatment with KRC-108 disturbed the migration of the HT-29 cells in a dose-dependent manner when compared to untreated cells (Fig. 3E).

3.4. KRC-108 induces G_2/M phase arrest in HT-29 cells

HT-29 cells were treated with the indicated concentrations of KRC-108 for 48 h, and then the effects of KRC-108 on cell cycle distribution of HT-29 cells were tested. KRC-108 increased cell populations in G_2/M phase and sub-G_1, indicating cell cycle arrest in G_2/M phase leading to apoptosis by KRC-108. The G_2/M population of HT-29 cells was increased by KRC-108 from 16.3% in the control group to 32.1% in the 10 μM treatment group (Fig. 4A). KRC-108 induces arrest of cells in G_2/M phase in a dose-dependent manner. These results are consistent with the function of Aurora A in mitosis. The sub-G_1 population of HT-29 cells was also increased after KRC-108 treatment, indicating cell death by KRC-108 treatment.

Activation of kinase Aurora A induces the expression of downstream target proteins including cyclin B1 and CDC2 during mitosis [19] and cell cycle progression [20]. The expression level of cyclin B1 and CDC2 mRNA was examined to confirm the effect of KRC-108 on the cell cycle through Aurora A inhibition. The HT-29 cells were treated with KRC-108 for 2 h and subjected to RT-PCR. As shown in Fig. 4B, KRC-108 decreased mRNA levels of cyclin B1 and CDC2 in a dose dependent manner. The results indicate that reduced Aurora A activity by KRC-108 resulted in decreased cyclin B1 and CDC2 expression followed by cell cycle arrest at G_2/M phase.

4. Discussion

Classical anti-microtubule drugs such as the taxanes and vinca alkaloids are the currently best approach in targeting mitosis to treat a variety of tumors. However, these compounds cause significant side effects such as neutropenia and neurotoxicity. Moreover, anti-microtubule agents have limited efficacy as single agents. There has been continuous interest to search for mitosis-targeting drugs for tumor treatment with enhanced therapeutic potency and fewer side effects. Currently, mitosis specific kinases such as Aurora A have been suggested to be essential regulators of mitosis and promising novel targets for anti-tumor therapy.

In this study, we found that KRC-108 exhibited inhibitory effects against Aurora A kinase, which consequently resulted in cell cycle arrest in G_2/M phase and apoptosis. We previously

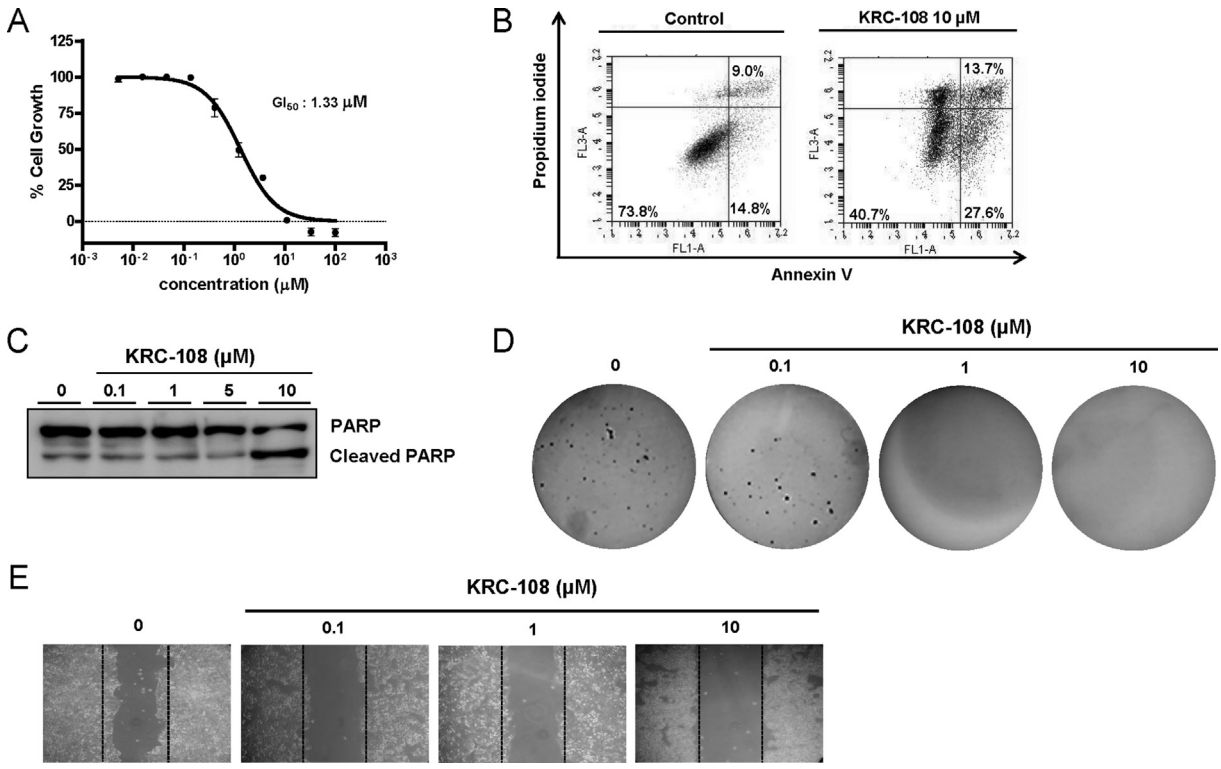
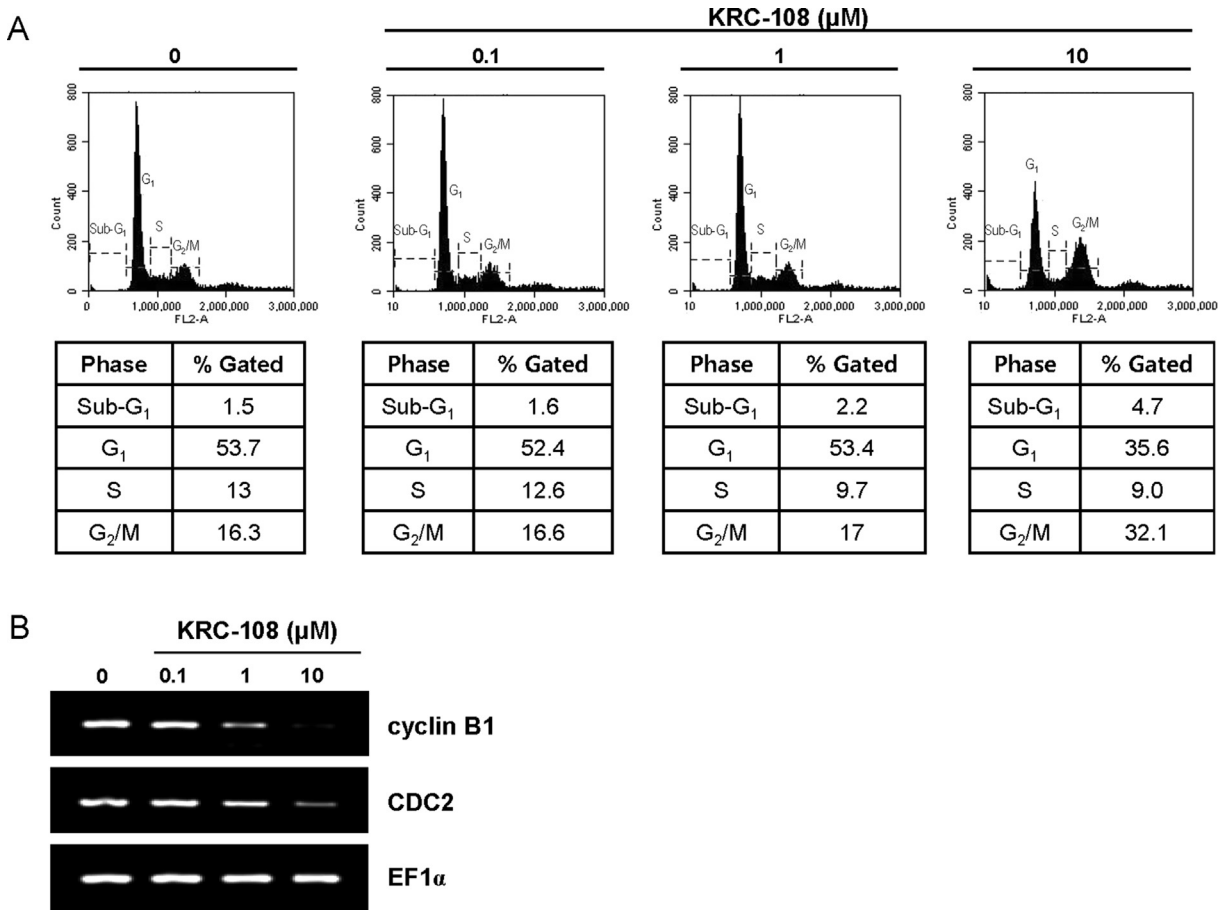


Fig. 3. Effects of KRC-108 on HT-29 cell growth and apoptosis. (A) Effect of KRC-108 on the proliferation of HT-29 cells. Cells were seeded in 96-well plates (2000 cells per well) and treated with KRC-108. Cells were incubated for 72 h and cell viability was measured using a tetrazolium dye assay using EZ-Cytox Cell Viability Assay kit. Cell growth (%) was calculated using 0.5% DMSO treatment as a negative control. (B) Effect of KRC-108 on apoptosis. HT-29 cells were treated with KRC-108 for 48 h and stained with Alexa Flour 488 Annexin V and PI. Bottom right quadrant (Annexin V+ and PI−): early apoptotic cells; top right quadrant (Annexin V+ and PI+): late apoptotic and already dead cells. (C) Effect of KRC-108 on PARP cleavage. HT-29 cells were treated with KRC-108 for 48 h. Then cells were harvested and lysed for measuring PARP and cleaved PARP protein levels by western blot. (D) Effect of KRC-108 on the anchorage-independent growth of HT-29 cells. HT-29 cells (10,000 cells/well) were plated in soft agar containing 0.5% DMSO (control) and KRC-108 in 6-well plates and incubated for four weeks. Live colonies were stained with 0.005% crystal violet and counted under a microscope. (E) Effect of KRC-108 on cell migration. After making the wound, cells were washed and treated with KRC-108 for 48 h. Cell migration into the wound was observed using a microscope.

reported KRC-108 as a multi-kinase inhibitor with anti-tumor activity in mice xenograft models [13]. KRC-108 showed good inhibitory activity for various kinases. However, the effect of KRC-108 on Aurora A kinase had not yet been evaluated. Therefore, we tested the inhibitory effect of KRC-108 against Aurora A kinase (Fig. 1) and further characterized the anti-tumor effect of this compound. The inhibition of Aurora A kinase activity with KRC-108 using recombinant enzyme was confirmed by the measurement of autophosphorylation of Aurora A and phosphorylation of Histone H3 on Ser10, the direct target of Aurora A kinase (Fig. 2). When KRC-108 was applied to HT-29 cells, KRC-108 not only blocked cell proliferation and migration, but also induced apoptotic cell death (Fig. 3). As cleaved PARP is one of the markers of apoptosis [21], increased levels of cleaved PARP (Fig. 3C) suggested KRC-108-induced apoptosis. The inhibition of Aurora A kinase activity by KRC-108 resulted in cell cycle G_2/M arrest and decreased expression of the cell cycle checkpoint proteins cyclin B1 and CDC2, the downstream target proteins of Aurora A (Fig. 4B). These results were consistent with the reported Aurora A function. Activated Aurora A has been known to cause centrosomal activation of the cyclin B1/CDC2 complex and promote mitotic entry [4]. Moreover, elevated cyclin B1 and CDC2 override G_2 arrest and facilitate spindle assembly checkpoint abrogation [22]. It was reported that over-expression of Aurora A upregulates cyclin B1 expression through increased stability of mRNA [23]. Taken together, inhibition of Aurora A by KRC-108 down-regulated cyclin B1 and CDC2. Consequently, it contributed to the G_2/M arrest in HT-29 cells leading to cell death by apoptosis.

Over-expression of Aurora kinases causes abnormality of spindle assembly checkpoints in cell mitosis and may play a role in carcinogenesis [10]. Thus, in the presence of over-expressed Aurora kinases, anti-microtubule drugs cannot induce apoptosis of aberrant cells and have resistance or limited efficacy. Therefore, drugs that inhibit Aurora kinases may overcome anti-microtubule drug resistance and enhance antitumor efficacy of anti-microtubule drugs. For this reason, numerous small molecule inhibitors of the Aurora kinases have been discovered and developed that exhibit preclinical activity against various solid tumors, with a few currently in phase II testing [24,25]. Although so far no Aurora kinase inhibitor has been approved for clinical use in humans, more than 35 clinical trials of Aurora kinase inhibitors have been registered in the US clinical trials database. Some of them are being tested in Phase III trials [25]. This suggests Aurora A kinase inhibitors as possible treatment options for cancer in the near future.

Multi-target kinase agents that have anti-Aurora A activity such as AT9283 and KW-2449 are also being developed in order to increase the efficacy and reduce side effects [24,25]. Similarly, KRC-108, which is a multi-kinase inhibitor along with anti-Aurora A activity, has advantages for development as an anti-tumor agent, although further studies are needed. Our results also provide valuable chemical and pharmacological information useful in the field of Aurora A research with regard to anti-tumor targets.



Phase	% Gated
Sub-G_1	1.5
G_1	53.7
S	13
G_2/M	16.3

Phase	% Gated
Sub-G_1	1.6
G_1	52.4
S	12.6
G_2/M	16.6

Phase	% Gated
Sub-G_1	2.2
G_1	53.4
S	9.7
G_2/M	17

Phase	% Gated
Sub-G_1	4.7
G_1	35.6
S	9.0
G_2/M	32.1

Fig. 4. Effect of KRC-108 on cell cycle (A) HT-29 cells were treated with KRC-108 for 48 h and stained with PI for cell cycle analysis. The charts show a representative experiment ($n = 3$). (B) Effect of KRC-108 on the expression level of cyclin B1 and CDC2 mRNA. HT-29 cells were treated with KRC-108 for 2 h, and total RNA was extracted from the cells and subjected to RT-PCR. RT-PCR of EF1α was used as a loading control.

Conflict of interest

None.

Acknowledgments

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Transparency document

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