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Effects of AZD1152, a selective Aurora B kinase inhibitor, on Burkitt's and Hodgkin's lymphomas

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ABSTRACT

We studied the effects of AZD1152, an Aurora B kinase inhibitor, on Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL) in human tissues and cell cultures and in a murine xenograft model of lymphoma. Aurora kinase A and B levels were assessed by RT-PCR and immunohistochemistry. They were aberrantly expressed in BL and HL cell lines, and in lymph nodes from patients with BL and HL. Next, activation of the Aurora B promoter was detected by reporter gene assays. The promoter activity of Aurora B kinase was high in BL cell lines and the Aurora B promoter contained a positive regulatory region between −74 and −104 from the transcription initiation site. AZD1152-hQPA had antiproliferative effects in the BL and HL cell lines studied; inhibited the phosphorylation of histone H3 and retinoblastoma proteins, and resulted in cells with >4 N DNA content. AZD1152-hQPA induced caspase-dependent apoptosis of some cell lines, demonstrated by loss of mitochondrial membrane potential, activation of caspase-9, followed by activation of caspase-3. This effect was accompanied by the inhibition of survivin expression. *In vivo* efficacy was determined in NOD/SCID/γc^{null} mice implanted with the Ramos human BL cell line. AZD1152 had anti-tumour effects in this murine xenograft model. There preclinical data suggest that the inhibition of Aurora B kinase is a potentially useful therapeutic strategy in BL and HL.

1. Introduction

The Aurora kinases comprise three isoforms in mammalian cells, Aurora A, B and C, and members of this family have been extensively studied in different model organisms [1,2]. The protein kinase activity of each member is cell cycle-dependent, with the activity gradually increasing at the S phase, reaching a peak level at the G_2/M phase in parallel with increased expression levels of their mRNA and protein [3–6]. Subsequently, the kinases are degraded by the proteasome upon exit from mitosis through the ubiquitin-dependent activator of the anaphase promoting complex/cyclosome pathway [7–9].

Although their catalytic domains show a high degree of sequence identity, the Aurora kinases exhibit different subcellular locations and functions [1,2]. Aurora A is localized in the duplicated centrosomes and in the spindle poles in mitosis [10], and is considered to function in several processes required for the generation of the bipolar spindle apparatus, including centrosome maturation and separation. Aurora B is a chromosomal passenger protein in complex with at least three other proteins, including the inner centromere protein, survivin and borealin. It is localized to the centromeric regions of the chromosomes in the early stage of mitosis, but changes its location at the onset of anaphase to the microtubules at the spindle equator [11]. As the spindle elongates and undergoes cytokinesis, Aurora B accumulates in the spindle midzone and at the site of cleavage furrow ingression before concentrating at the midbody. During mitosis, Aurora B is required for the phosphorylation of histone H3 on serine 10 and is thought to be important in chromosome condensation. Aurora B has been shown to regulate kinetochore function as it is required for correct chromosome alignment and segregation. Aurora B is also required for spindle checkpoint function and cytokinesis [2,12,13]. Aurora C was originally thought to have a limited function in meiosis, but more recent findings suggest that it is more closely related to Aurora B with overlapping functions and similar intracellular distribution [14].

Targeting the progression of mitosis is a highly successful strategy for anticancer treatment [15]. Recent studies have focused

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on the Aurora kinases as targets of novel anti-mitotic drugs, since Aurora A and B are frequently overexpressed in human cancer [16–19]. However, little is known about the Aurora kinases in Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL).

BL and HL represent clonal malignant expansions of B cells and are associated with Epstein–Barr virus (EBV) infection. BL is a high-grade non-HL that occurs sporadically worldwide, but is endemic in Papua New Guinea and in the lymphoma belt of Africa, where malaria and EBV, recognised cofactors for endemic BL, are ubiquitous [20]. The frequency of BL has increased in low-incidence countries since the 1980s, following the advent of human immunodeficiency virus/acquired immunodeficiency syndrome. Patients with human immunodeficiency virus-associated lymphoma pose additional therapeutic challenges, particularly the risk of overwhelming opportunistic infections [21].

Advances in chemotherapy and radiotherapy regimens for treatment of HL represent a significant breakthrough in clinical oncology and have increased the long-term survival rate. Today, the late side-effects of chemotherapy, such as secondary malignancies, myelodysplasia, cardiotoxicities, as well as resistance to chemotherapy, associated with poor prognosis, have become important issues that need to be resolved [22]. Advances in molecular biology have provided many new insights into the biology and treatment options for BL and HL. Recently, a strategy that targets the molecules critical for maintenance and growth of tumour cells has been considered important to the development of more effective treatment with less undesirable effects [23]. This strategy should enhance the specificity of the chemotherapeutic agents against tumour cells and minimize undesirable effects on normal cells.

The results of the present study indicate that Aurora A and B are suitable targets for the treatment of BL and HL. AZD1152 is a recently developed potent and selective inhibitor of Aurora B and is currently being evaluated in clinical trials. AZD1152 is an acetanilide-substituted pyrazole-aminoquinazoline dihydrogen phosphate prodrug with good solubility, making it suitable for parenteral administration. After administration, AZD1152 is rapidly converted in the circulation to the active drug moiety AZD1152-hQPA, which inhibits Aurora A, B and C but displays ~3700-fold lower affinity for Aurora A compared with B and C [24]. In the present preclinical study, we have evaluated AZD1152 as a novel chemotherapeutic agent for the treatment of BL and HL.

2. Materials and methods

2.1. Chemicals

AZD1152 and the active metabolite, AZD1152-hQPA, were obtained from AstraZeneca (Macclesfield, UK). AZD1152-hQPA was used in the *in vitro* experiments, while AZD1152 was used in the murine experiments described below.

2.2. Cells and cultures

Raji and Daudi are EBV-positive BL cell lines. BJAB and Ramos are EBV-negative BL cell lines. B95-8/Ramos is Ramos infected with the B95-8 strain of EBV. L428, KM-H2, HDLM-2 and L540 are HL cell lines. All cell lines were cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% or 20% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Peripheral blood mononuclear cells (PBMC) from healthy volunteers were also analyzed. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden) and washed with phosphate-buffered saline (PBS). $CD19^+$ B cells were purified from PBMC by positive selection with magnetic cell sorting (Stemcell Technologies Inc., Vancouver, British Columbia, Canada) after labelling with anti-CD19 microbeads. All biological samples were obtained after informed consent.

2.3. Reverse transcriptase polymerase chain reaction

Total cellular RNA from cells was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular RNA using a PrimerScript reverse transcriptase polymerase chain reaction (RT-PCR) kit (Takara Bio, Otsu, Japan) with random primers. The specific primers used were 5′-GTCTTGTGTCCTTCAAATTCTTC-3′ (forward) and 5′-TCTTTTGGGTG TTATTCAGTGGC-3′ (reverse) for Aurora A, 5′-AGCTGAAGATTGCT-GACTTCG-3′ (forward) and 5′-ATAGGTCTCGTTGTGTGATGC-3′ (reverse) for Aurora B, 5′-GTGACTGGACTGGAGGAGCC-3′ (forward) and 5′-GAGGGAGTCATCGTGGTGGTG-3′ (reverse) for EBV latent membrane protein (LMP)-1, and 5′-GTGGGGCGCCCCAGGCACCA-3′ (forward) and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (reverse) for β-actin. The semiquantitative RT-PCR for each gene was set as follows; 29 cycles for Aurora A and B, 40 cycles for LMP-1, and 28 cycles for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

2.4. Immunohistochemical analysis

Biopsy samples were taken from the lymph nodes of each of 10 patients with BL and HL. In addition, 2 specimens of lymph nodes from normal subjects were included. The study was approved by the Ethics Committee of University of the Ryukyus, and complied with the Helsinki Declaration. Serial sections were deparaffinized in xylene and rehydrated using a graded ethanol series. For better detection, sections were pretreated with ready-to-use proteinase K (Dako, Carpinteria, CA) for 10 min at 37 °C. This procedure increased the number of antigenic sites available for binding by the antibody. Sections were washed 4 times in PBS for 5 min each. In the next step, the tissues were placed in absolute methanol containing 3% hydrogen peroxide for 5 min to reduce endogenous peroxidase activity, followed by washing 4 times in PBS for 5 min each. Next, the tissue sections were incubated with a monoclonal mouse anti-Aurora A or anti-Aurora B antibody (BD Transduction Laboratories, San Jose, CA) for 3 h at 37 °C. After washing 4 times with PBS for 5 min each, the sections were covered with EnVision plus (Dako, Santa Barbara, CA) for 40 min at 37 °C and washed 4 times in PBS for 5 min each. Antigenic sites bound by the antibody were identified by reacting the sections with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl buffer and 0.01% hydrogen peroxide for 7 min. Sections were washed 3 times in distilled water for 5 min each and then counterstained with 1% methyl green in phthalate buffer pH 4.01 solution (Wako Pure Chemicals, Osaka, Japan) for 10 min, dehydrated through a graded ethanol series, cleared in xylene, and mounted with Permount® (Fisher Scientific, Fair Lawn, NJ). The stained cells were examined under a light microscope.

2.5. Cell viability and apoptosis assays

The effect of AZD1152-hQPA on cell viability was examined using the cell proliferation reagent, WST-8 (Wako Pure Chemicals). This method relies on mitochondrial dehydrogenase cleavage of WST-8 to formazan dye to estimate the level of cell viability. Briefly, cells were incubated in a 96-well microculture plate in the absence or presence of various concentrations of AZD1152-hQPA. After 72 h of culture, WST-8 (5 μl) was added for the last 4 h of incubation and the absorbance at 450 nm was measured using an automated microplate reader. WST-8 solution was added to the media-only wells to correct for background. Apoptotic events in

cells were detected by staining with phycoerythrin-conjugated APO2.7 monoclonal antibody (Beckman Coulter, Marseille, France) [25] and analysis by flow cytometry on a Coulter EPICS XL (Beckman Coulter, Fullerton, CA). Analysis of DNA fragmentation by fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) was performed using a commercial kit (Takara Bio) as described in the instructions provided by the manufacturer.

2.6. In vitro measurement of caspase activity

Cell extracts were recovered using cell lysis buffer and assessed for caspases-3, -8 and -9 activities using colorimetric probes (Medical & Biological Laboratories; MBL, Nagoya, Japan).

2.7. Measurement of mitochondrial potential using JC-1

In all, 2×10^6 cells were treated with AZD1152-hQPA for 24, 48 or 72 h. Cells were suspended in JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) Staining Solution (Cayman Chemical Company, Ann Arbor, MI) then incubated at 37 °C in the dark for 15 min. Cells were harvested and the mitochondrial membrane potential (% of green and red aggregates) was determined by flow cytometry.

2.8. Cell cycle analysis

Cell cycle analysis was performed with the CycleTEST PLUS DNA reagent kit (Becton Dickinson, San Jose, CA). Briefly, 1×10^6 cells were washed with a buffer solution containing sodium citrate, sucrose and dimethyl sulfoxide, suspended in a solution containing RNase A and stained with 125-μg/ml propidium iodide for 10 min. Cell suspensions were analyzed on a Coulter EPICS XL using EXPO32 software.

2.9. Western blot analysis

Cells were lysed in a buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Samples were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with specific antibodies. Mouse monoclonal antibodies to Aurora A and Aurora B were purchased from BD Transduction Laboratories. Mouse monoclonal antibodies to XIAP and phospho-retinoblastoma (Rb) protein (Ser780) were purchased from MBL. Mouse monoclonal antibodies to caspase-8 and caspase-9, rabbit monoclonal antibodies to phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198), cleaved caspase-3 and survivin, and

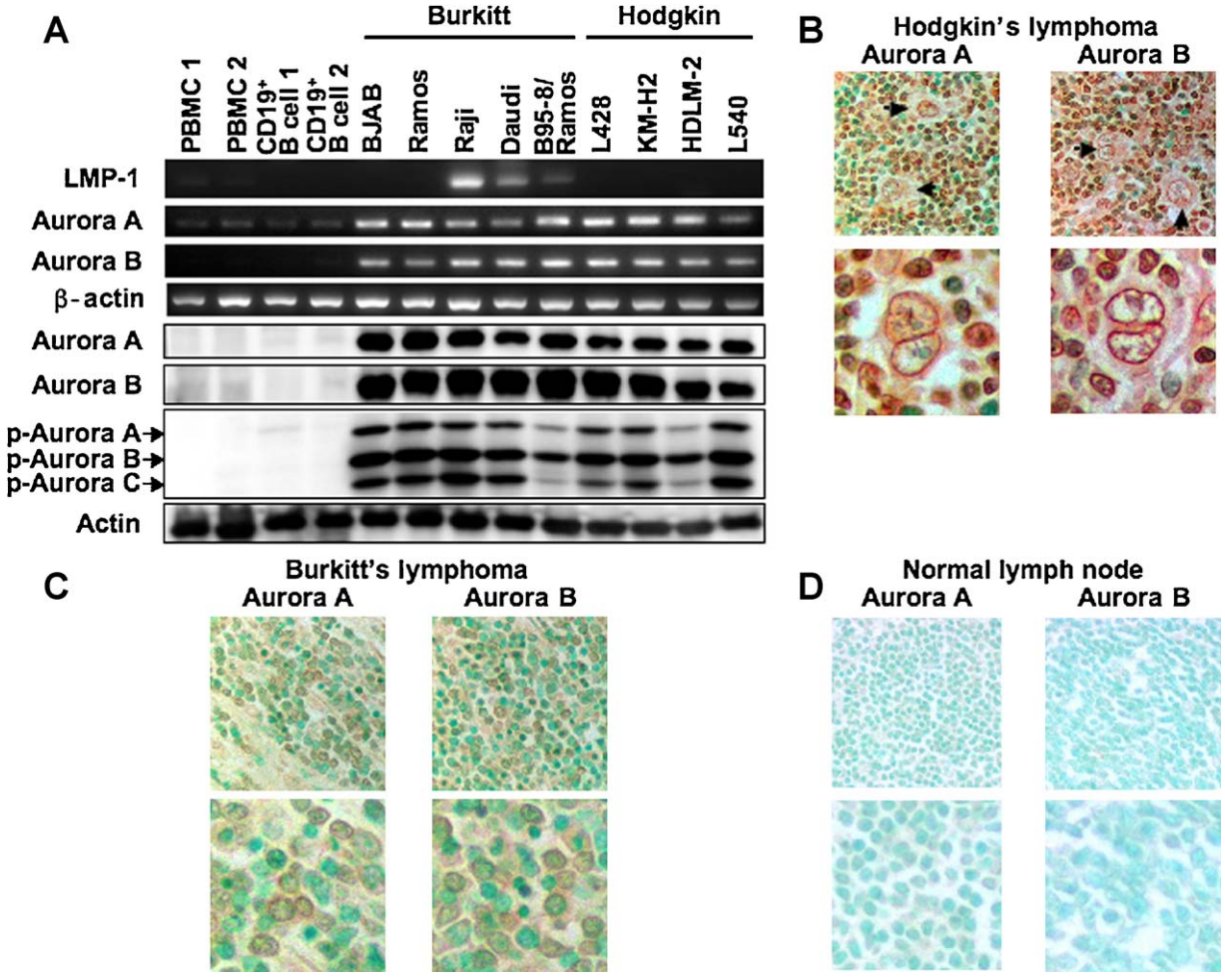

Fig. 1. Expression of Aurora A and B in BL and HL cell lines and primary BL and HRS tumour cells. (A) Expression and autophosphorylation of Aurora A and B in BL and HL cell lines. The expression levels of Aurora A and B mRNAs and proteins were determined by RT-PCR and Western blot analysis, respectively. Aurora A and B proteins phosphorylated on Thr288 or Thr232, respectively, were detected on Western blots. EBV LMP-1 mRNA expression was determined by RT-PCR. As loading controls, β-actin mRNA and actin protein expression were included. (B-D) Primary HRS tumour cells and surrounding bystander cells in lymph nodes of HL patients and BL tumour cells in lymph nodes of BL patients are characterized by high expression of Aurora A and B. Immunostaining for Aurora A and B in primary tissue samples (B, HL; C, BL; D, normal lymph nodes). Arrows indicate HRS cells. Original magnification, ×800 (B-D, top panels), ×2240 (B, bottom panels), ×1870 (C and D, bottom panels).

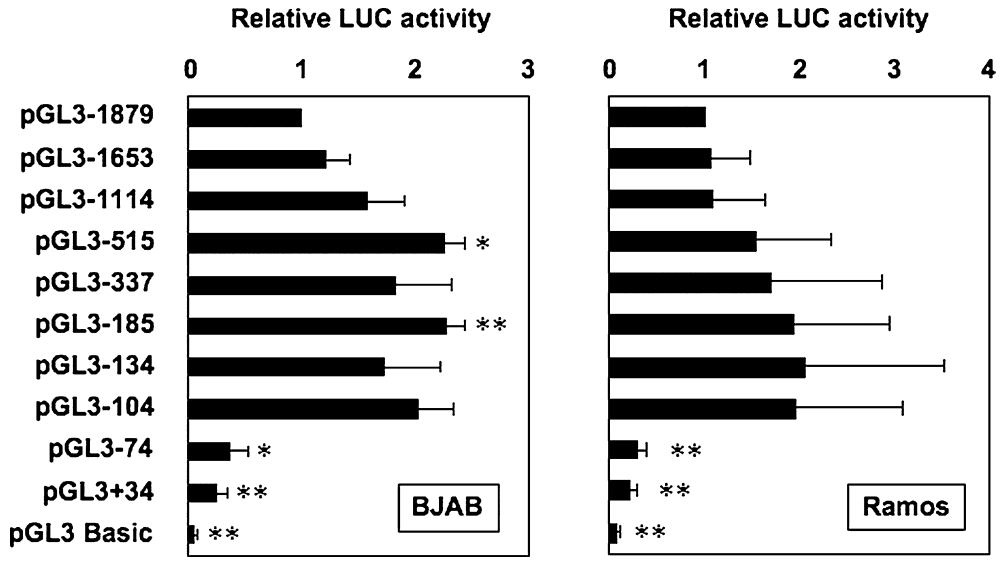

Fig. 2. A series of deletion constructs of the Aurora B promoter region and their relative promoter activities in BL cell lines. A series of deletion constructs ligated into the pGL3-basic luciferase reporter plasmid was used to transiently transfect BJAB and Ramos cells. The relative luciferase activities of the deleted constructs to the pGL3-1879 (−1879 to +359) are shown. Data are mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the pGL3-1879.

rabbit polyclonal antibodies to histone H3, phospho-histone H3 (Ser10), cleaved poly (ADP-ribose) polymerase (PARP), Bcl-xL, Bak and Bax were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies to Bcl-2, p53, p21 and actin were purchased from NeoMarkers (Fremont, CA). The bands were visualized with the Enhanced Chemiluminescence kit supplied by GE Healthcare, Buckinghamshire, UK.

2.10. Transfection and reporter assay

A series of Aurora B deletion promoter-luciferase constructs, in which the number after pGL3 indicates the nucleotide start number from the Aurora B 5′ flanking region have been described previously [26]. Transient transfections were performed in BJAB and Ramos cells using a MicroPorator MP-100

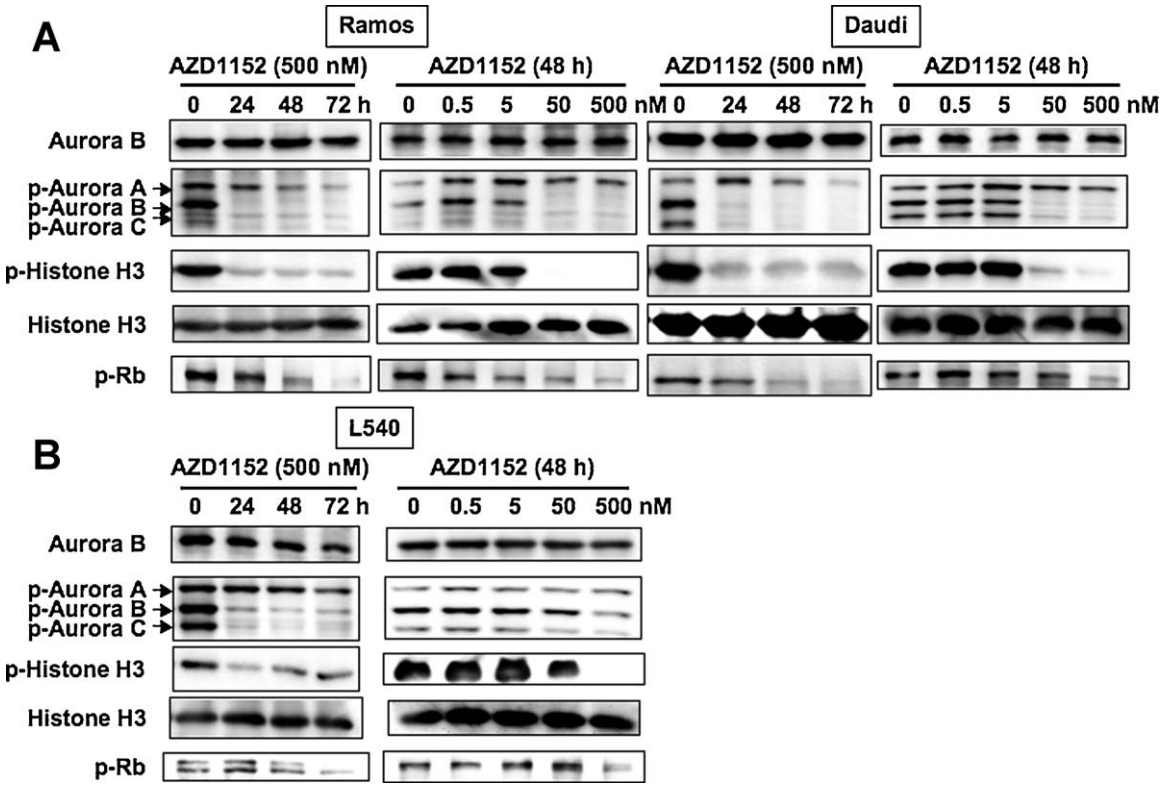

Fig. 3. Effects of AZD1152-hQPA on phosphorylation of Aurora B, histone H3 and Rb. Ramos and Daudi cells (A) and L540 cells (B) were treated with various concentrations of AZD1152-hQPA. At the indicated time points, cells were harvested and proteins were extracted, and subjected to Western blot analysis. Representative examples of three experiments with similar results.

(Digital Bio Technology, Seoul, Korea) according to the instructions supplied by the manufacturer for optimization and use. In all cases, the reference plasmid phRL-TK, which contains the *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase promoter, was co-transfected to correct for transfection efficiency. After 24 h, the cells were collected by centrifugation, washed with PBS, and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase assays were performed using the Dual-Luciferase Reporter System (Promega), in which the relative luciferase activity was calculated by normalizing transfection efficiency according to the *Renilla* luciferase activities.

2.11. Treatment of NOD/SCID/γc^{null} mice with AZD1152

Six-week-old female NOD/SCID/γc^{null} (NOG) mice obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) were maintained in containment level 2 cabinets and provided with autoclaved food and water *ad libitum*. 1×10^7 Ramos cells were injected subcutaneously into the post-auricular region of mice. The mice were monitored daily for the development of palpable tumours, at which time, drug treatment was initiated, which comprised AZD1152 dissolved in 0.3 M Tris (pH 9.0) at a concentration of 30 mg/ml, injected intraperitoneally at 30 mg/kg body weight, every other day. Tumour size was monitored twice a week. All mice were sacrificed on day 28, and then the tumours were dissected out and weighed. This experiment was performed according to the guidelines for the Animal Experimentation University of the Ryukyus and was approved by the Animal Care and Use Committee, University of the Ryukyus.

2.12. Analysis of in vivo mechanism of action

Tumours were fixed for paraffin embedding and tissue sectioning. Analysis of DNA fragmentation by fluorescent TUNEL was performed using a commercial kit (Takara Bio).

2.13. Statistical analysis

Data are expressed as mean ± standard deviation. Promoter activities from deletion mutant plasmids were compared to that of the pGL3-1879 by the Student's *t* test. Volume and weight of tumours from AZD1152-treated mice were compared to those of the controls by the Mann–Whitney *U* test. A *P* value less than 0.05 was considered statistically significant.

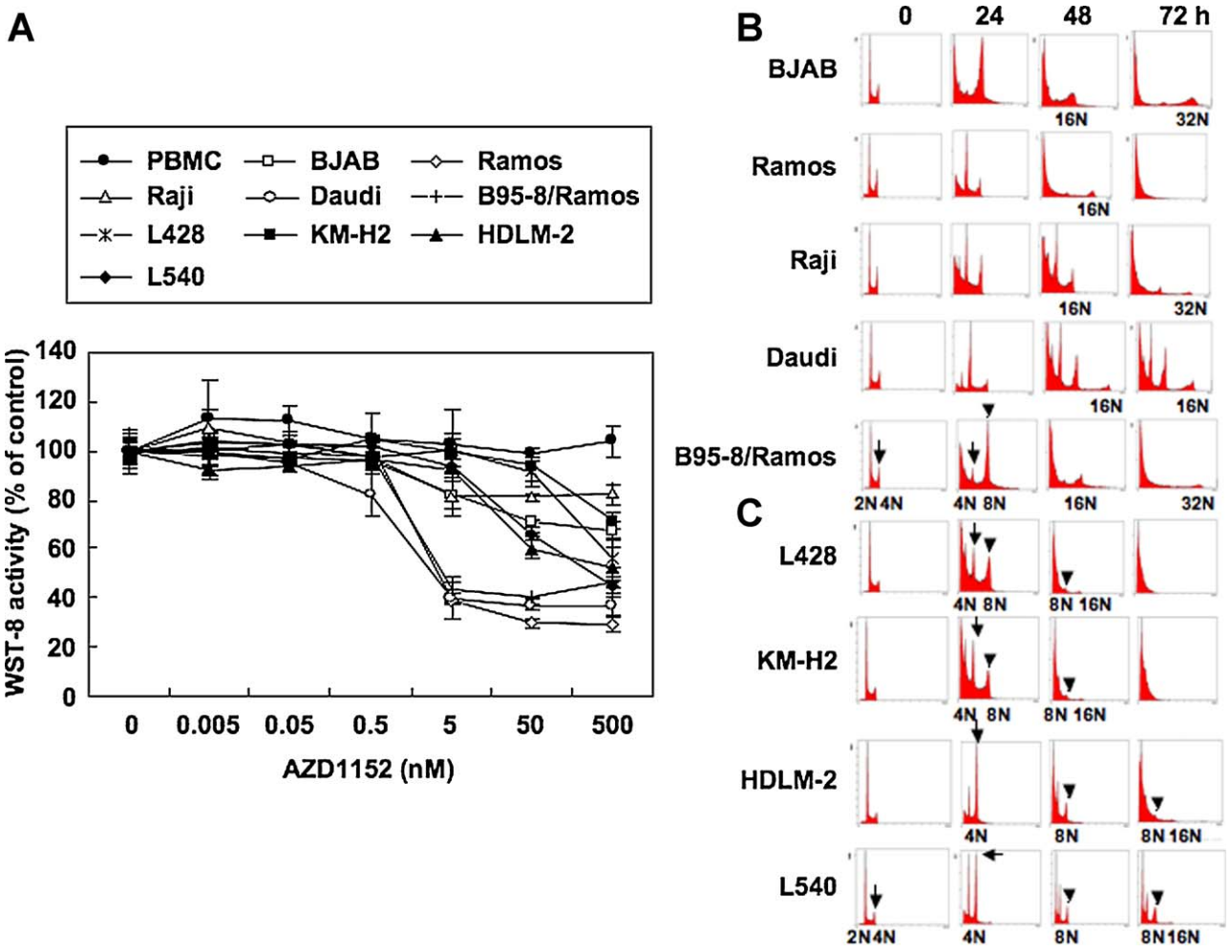

Fig. 4. AZD1152-hQPA induces growth arrest and increase of cells with 4 N and >4 N DNA contents. (A) Inhibitory effects of AZD1152-hQPA on cell growth of BL and HL cell lines and PBMC from a healthy volunteer. Cells were incubated in the presence of various concentrations of AZD1152-hQPA for 72 h, and *in vitro* growth of the cultured cells was measured by WST-8 assay. The relative growth of cultured cells is presented as the mean of cell lines and PBMC from a healthy volunteer ($n = 3$). A relative growth of 100% was designated as the total number of cells that grew in the 72 h-cultures in the absence of AZD1152-hQPA. Data are mean ± SD. (B and C) Cell cycle analysis. BL (B) and HL (C) cell lines were cultured with 500 nM AZD1152-hQPA. After 24, 48 or 72 h, cells were harvested and cell cycle distribution was analyzed by flow cytometry. Arrows and arrowheads indicate 4 N (G_2/M) and 8 N (polyploid) cells, respectively.

3. Results

3.1. Expression of Aurora A and B in BL and HL cell lines and lymph node samples

RT-PCR was utilized to determine Aurora A and B mRNA expression in BL and HL cell lines. The analysis showed significant detectable levels of Aurora A and B transcripts in BL and HL cell lines (Fig. 1A). The protein levels of Aurora A and B expression in the cell lines were confirmed by Western blot analysis (Fig. 1A). The autophosphorylation status within the activation loops of Aurora A and B was evaluated using Western blotting to confirm the presence of phosphorylated Aurora A and B in BL and HL cell lines. No correlation was noted between the expression and phosphorylation levels of Aurora A and B, and EBV infection. Analysis of PBMC and B cells from healthy volunteers showed that these cells were negative for the expression of Aurora A and B (Fig. 1A).

We also evaluated the expression of Aurora A and B protein in lymph nodes of BL and HL patients by immunohistochemistry. Aurora A and B expression was examined in 10 specimens each of lymph nodes from BL and HL patients. Representative results are shown in Fig. 1B and C. Strong nuclear expression of Aurora A and B was detected in all cases of HL analyzed (Fig. 1B), especially in mononuclear Hodgkin and multinuclear Reed–Sternberg (HRS) cells as well as in the surrounding bystander cells (Fig. 1B). Aurora A and B immunoreactivity was also observed in all samples of BL lymphoma (Fig. 1C). In contrast, no staining was observed in normal lymph nodes (Fig. 1D).

3.2. Promoter activity of 5′ flanking region of human Aurora B gene in BL cell lines

Levels of mammalian Aurora B protein are regulated by transcription and protein degradation during the cell cycle [26]. Aurora B is overexpressed at the mRNA and protein levels in a variety of human cancers, but the regulation mechanism of the Aurora B promoter has not been fully studied. To investigate the promoter activity of Aurora B in cell lines, a series of deletion mutant plasmids of the 5′ flanking region of the human Aurora B gene cloned into a luciferase reporter vector pGL3-Basic were transfected into BJAB and Ramos cells. Deletion mutant pGL3-74 had very little promoter activity, suggesting that the Aurora B promoter contains a positive regulatory region between −74 and −104 (Fig. 2).

3.3. Effect of AZD1152-hQPA on phosphorylation of Aurora B and histone H3

We examined whether Aurora B is a suitable target for the treatment of BL and HL using cell lines. Exposure of BL cell lines, Ramos and Daudi, and HL cell line, L540 to AZD1152-hQPA effectively blocked the phosphorylation of Aurora B kinase in time- and dose-dependent manners (Fig. 3). Although phosphorylation

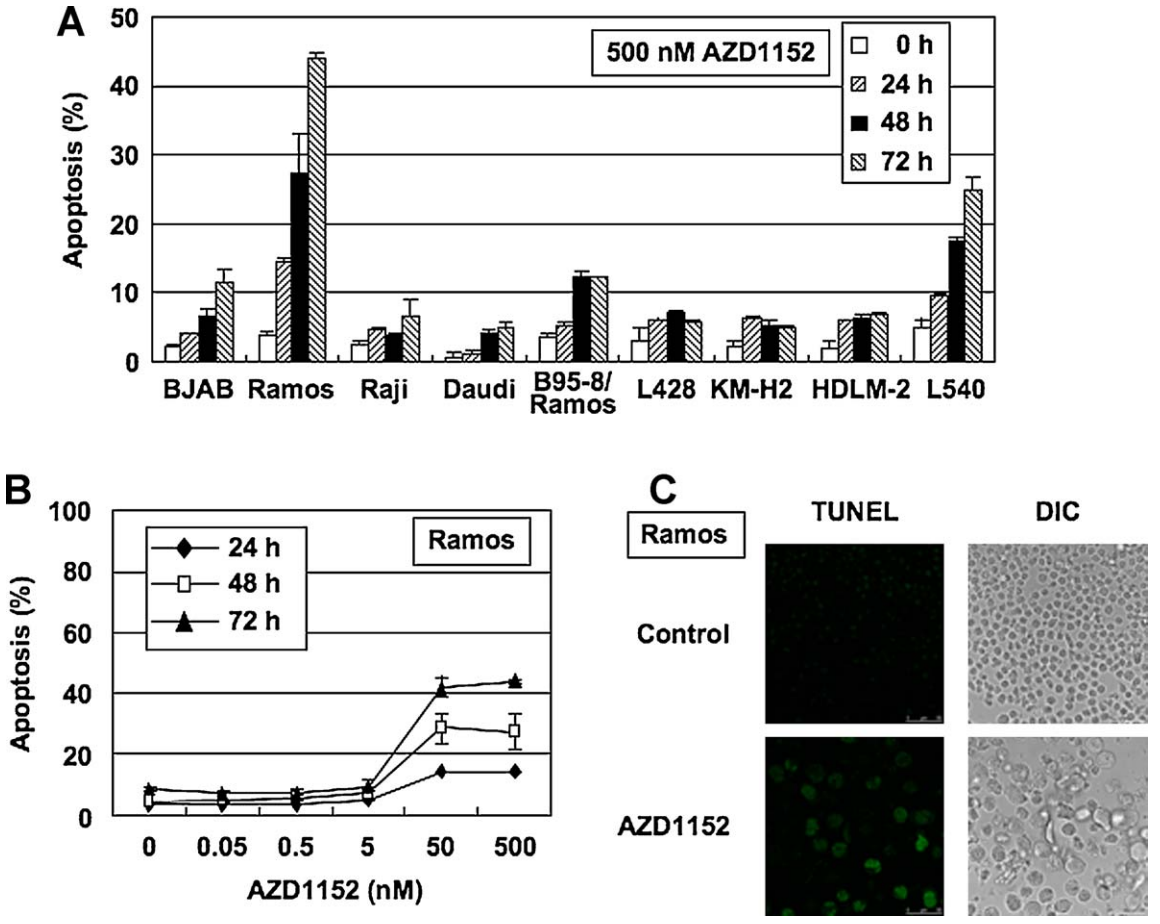

Fig. 5. AZD1152-hQPA induces apoptosis in BL and HL cell lines. (A) Flow cytometric analysis of cell apoptosis. The indicated cells were incubated with 500 nM AZD1152-hQPA for 24, 48 or 72 h, then collected, stained with the APO2.7 monoclonal antibody and subjected to flow cytometry. Data are mean ± SD ($n = 3$). (B) Ramos cells were cultured with various concentrations of AZD1152-hQPA (0.05–500 nM). After 24, 48 or 72 h, the cells were harvested, and APO2.7 staining was analyzed by flow cytometry. Data are mean ± SD ($n = 3$). (C) Ramos cells were either left untreated (control) or treated with 500 nM AZD1152-hQPA for 72 h. Apoptotic cells were detected by TUNEL assay (green fluorescence). DIC: differential interference contrast images. Magnification, 630×.

of Aurora A was blocked at 72-h incubation of 500 nM AZD1152-hQPA, AZD1152-hQPA showed selectivity for Aurora B over Aurora A in all cell lines tested (Fig. 3). Histone H3 is one of the substrates of Aurora B kinase [27], and phosphorylation of histone H3 on Ser10 is thought to play an important role in chromosome alignment during mitosis [27]. We therefore examined whether AZD1152-hQPA inhibits the phosphorylation of histone H3 on Ser10 by Western blot analysis with Ser10-phosphorylated histone H3 specific antibody. As shown in Fig. 3, the phosphorylated histone H3 was significantly reduced in Ramos, Daudi and L540 cells treated with AZD1152-hQPA in time- and dose-dependent manners, suggesting that AZD1152-hQPA effectively inhibits Aurora B kinase in these cells.

3.4. AZD1152-hQPA inhibits growth of BL and HL cell lines

We examined the ability of AZD1152-hQPA to inhibit the cell growth of BL and HL cell lines. Culture of cells with various concentrations (0–500 nM) of AZD1152-hQPA for 72 h resulted in the suppression of cell growth in a dose-dependent manner in most of the 9 lines tested as assessed by the WST-8 assay (Fig. 4A). AZD1152-hQPA markedly inhibited the growth of BL cell lines, Ramos, Daudi and B95-8/Ramos. The concentrations of AZD1152-hQPA required to inhibit growth of these 3 BL cell lines by 50% (IC_{50} values) ranged from 3.0 to 4.6 nM. Although the sensitivity to AZD1152-hQPA varied among the cell lines studied, EBV infection did not influence the effect of AZD1152-hQPA on the BL cell lines. HL cell lines were less susceptible to AZD1152-hQPA than BL cell lines. Importantly, normal PBMC were resistant to AZD1152-hQPA.

3.5. AZD1152-hQPA increases the population of cells with 4 N and >4 N DNA contents

To investigate the mechanism leading to AZD1152-hQPA-induced cell growth inhibition, changes in the cell cycle distribution of the BL and HL cell lines treated with the inhibitor were evaluated by flow cytometry. Exposure of BL and HL cell lines to AZD1152-hQPA markedly increased cells with 4 N and >4 N DNA contents in a time-dependent manner (Fig. 4B and C), suggesting that cells exposed to AZD1152-hQPA exited mitosis and subsequently proceeded through the S phase in the absence of cytokinesis (cell division) followed by endo-reduplication and polyploidy formation.

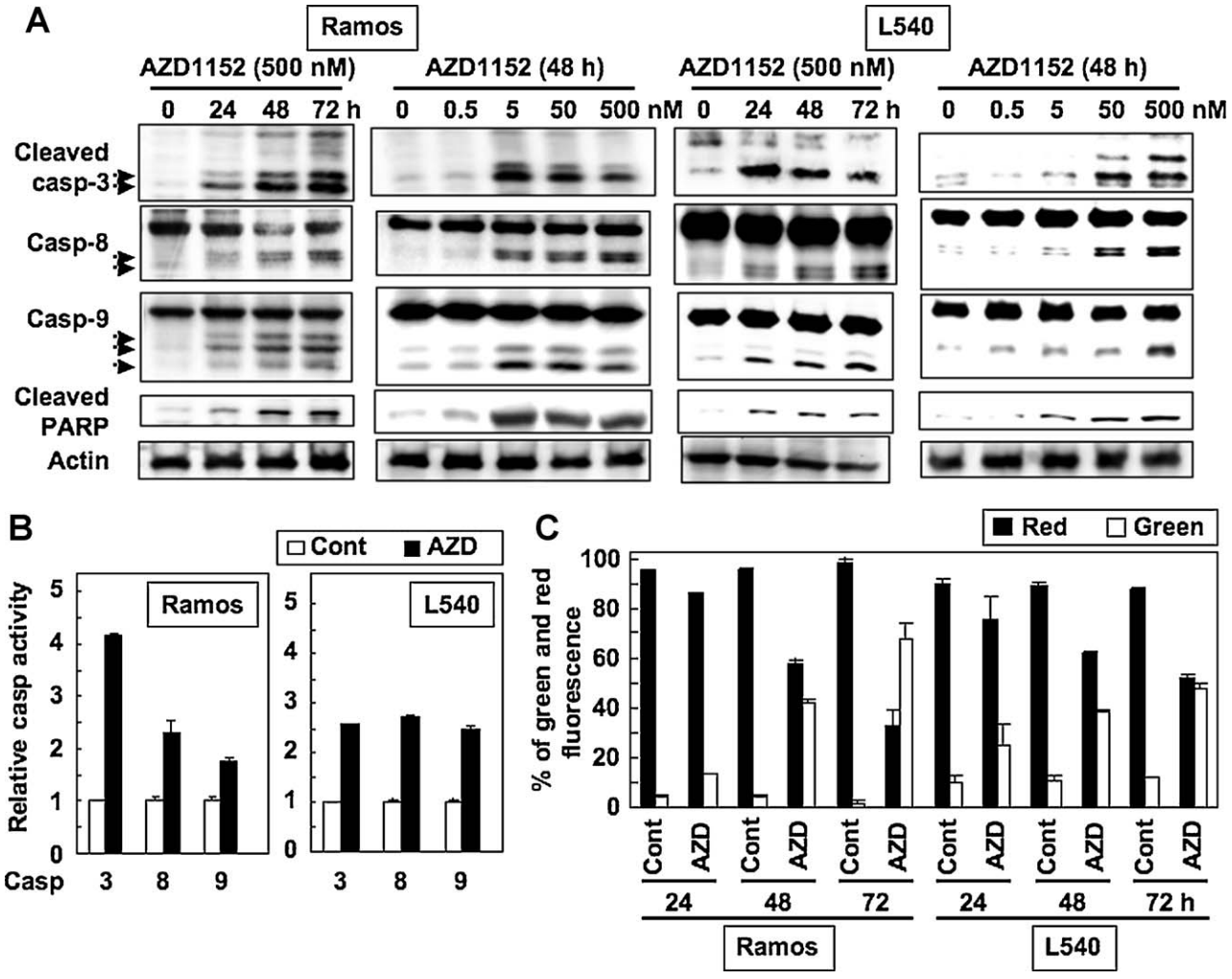

Fig. 6. AZD1152-hQPA induces the activation of caspases in BL and HL cell lines. (A) Ramos and L540 cells were incubated with various concentrations of AZD1152-hQPA for 24, 48 or 72 h. Cells were lysed and equal amounts of proteins were separated by SDS-polyacrylamide gels, transferred to immobilon membrane and immunoblotted with the indicated antibodies. Arrows indicate the cleaved form of caspases. Actin was used as a protein-loading control. (B) Determinant of caspase activity. Ramos and L540 cells were either left untreated (Cont) or treated with 500 nM AZD1152-hQPA (AZD). After 72 h, cell lysates were prepared and incubated with the labelled caspase substrates, and caspase activity was measured using an automated microplate reader. Caspase activity is expressed relative to that of untreated cells, which was assigned a value of 1. Data are mean ± SD ($n = 3$). (C) Mitochondrial staining of Ramos and L540 cells using JC-1 dye. Cells were either cultured without treatment (Cont) or with 500 nM AZD1152-hQPA (AZD) for 24, 48 or 72 h. Live cells with intact mitochondrial membrane potential and apoptotic cells with lost mitochondrial membrane potential were measured by JC-1 staining and analyzed by flow cytometry as described in Section 2. Data are mean ± SD ($n = 3$).

Aurora B prevents endo-reduplication and polyploidy formation by directly phosphorylating Rb at Ser780 [28]. We also asked whether AZD1152-hQPA could block the phosphorylation of Rb at Ser780 in Ramos, Daudi and L540 cells. AZD1152-hQPA reduced the level of phosphorylated Rb in time- and dose-dependent manners (Fig. 3), suggesting that it induces polyploidy in Ramos, Daudi and L540 cells via inhibition of Rb phosphorylation.

3.6. AZD1152-hQPA induces apoptosis of BL and HL cells

The ability of AZD1152 to induce apoptosis was assessed by measuring APO2.7 staining in BL and HL cell lines treated with AZD1152-hQPA (Fig. 5A). APO2.7-positive populations represent early apoptotic cells. Exposure of these cells to AZD1152-hQPA (500 nM) for 24, 48 or 72 h induced apoptosis in a time-dependent manner (Fig. 5A). For example, exposure to AZD1152-hQPA for 72 h induced apoptosis of 44% of Ramos cells and 25% of L540 cells, respectively (Fig. 5A). Exposure of Ramos cells to AZD1152-hQPA induced apoptosis in dose- and time-dependent manners (Fig. 5B). Treatment of Ramos cells with 500 nM AZD1152-hQPA for 72 h resulted in visible morphological changes including an increase (approximately doubling) in cell size at 72 h, relative to untreated cells, with clear morphological evidence of apoptosis in the majority of cells (Fig. 5C). These results were confirmed by the TUNEL assay, which indicated that AZD1152-hQPA induces apoptosis in Ramos cells. The number of TUNEL-positive cells among Ramos cells treated with AZD1152-hQPA was higher than that among untreated cells (Fig. 5C).

We next investigated the roles of various caspases in AZD1152-hQPA-induced apoptosis by measuring the cleavage of known caspase substrates by immunoblot analysis. Treatment of Ramos and L540 cells resulted in cleavage of the caspase-3-specific substrate PARP into the characteristic 89 kDa fragments (Fig. 6A). In addition, the initiator caspases-9 and -8, and the executioner caspase-3 were processed in both cells after treatment with AZD1152-hQPA in time- and dose-dependent manners.

The immunoblotting allowed us to examine the processing of caspases, but did not indicate whether the cleavage products were enzymatically active. Therefore caspases-3, -8 and -9 activities were determined by the cleavage of DEVD (Asp-Glu-Val-Asp)-ρ-nitroanilide, IETD (Ile-Glu-Thr-Asp)-ρ-nitroanilide and LEHD (Leu-Glu-His-Asp)-ρ-nitroanilide, respectively, in colorimetric assays. Again, Ramos and L540 cells treated with AZD1152-hQPA for 72 h showed markedly increased caspases-3, -8 and -9 activation (Fig. 6B).

We also tested the effect of AZD1152-hQPA on mitochondrial membrane potential in these cells using JC-1 dye. Ramos and L540 cells were labelled with JC-1 dye, and mitochondrial potential was measured by flow cytometry. Incubation of these cells with AZD1152-hQPA resulted in loss of mitochondrial membrane potential as measured by JC-1 stained green fluorescence depicting apoptotic cells (Fig. 6C). These results indicate that the exposure of

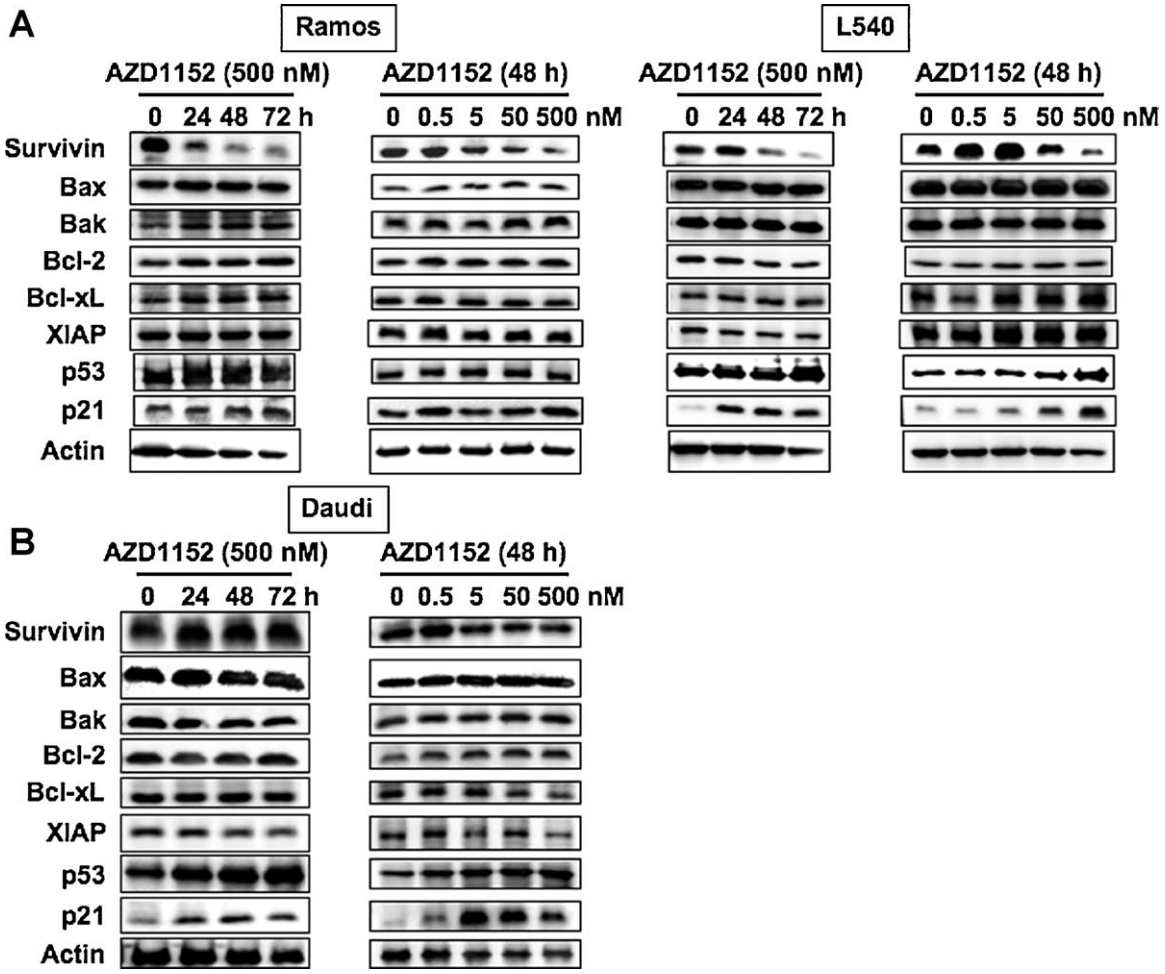

Fig. 7. Effects of AZD1152-hQPA on expression of survivin in Ramos, L540 and Daudi cells. Ramos and L540 cells (A) and Daudi cells (B) were treated with various concentrations of AZD1152-hQPA. At the indicated time points, cells were harvested and proteins were extracted, and subjected to Western blot analysis. Representative examples of three experiments with similar results.

BL and HL cells to AZD1152-hQPA results in apoptosis via the mitochondrial pathway.

3.7. AZD1152-hQPA induces apoptosis in parallel with down-regulation of survivin

AZD1152-hQPA induced apoptosis of Ramos and L540 cells in association with loss of mitochondrial outer membrane potential (Figs. 5A and 6C). On the other hand, AZD1152-hQPA had a negligible effect on apoptosis of Daudi cells (Fig. 5A). Ramos and Daudi cells have established p53 mutant alleles, leading to inactivation of p53 [29], while L540 cells express the wild-type p53 [30]. These results suggest that the mechanism by AZD1152-hQPA-induced apoptosis in BL and HL cell lines probably did not involve p53. A slight induction of p53 expression was noted in L540 and Daudi cells but not in Ramos cells (Fig. 7). Exposure to AZD1152-hQPA induced p21 expression in L540 and Daudi cells, which was probably p53-independent, because p53 protein up-regulation was minimal and p53 protein was not functional in Daudi cells. As expected, the levels of Bax, a target of p53, were not up-regulated in any of the cell lines after exposure to AZD1152-hQPA (Fig. 7). AZD1152-hQPA had no effect on the levels of the anti-apoptotic proteins, Bcl-2, Bcl-xL and XIAP or of the pro-apoptotic protein, Bak, in all cell lines. However, treatment with AZD1152-hQPA decreased the levels of survivin in a time and dose dependent manner in Ramos and L540 cells but not in Daudi cells (Fig. 7). In summary, these results suggest that survivin may play a role in the apoptotic sensitivity following Aurora B kinase inhibition.

3.8. Effects of AZD1152 in Ramos cells in vivo

Finally, we evaluated the antigrowth activity of AZD1152 *in vivo*. When treatment was initiated on the day after cell injection, AZD1152 (30 mg/kg) completely inhibited the proliferation of Ramos cells compared with control tumours (data not shown). Therefore, upon formation of palpable tumours, mice were injected with ($n = 5$) or without ($n = 6$) AZD1152 intraperitoneally every other day. AZD1152 did not affect incidence of tumourigenesis but significantly slowed the growth of the tumours. After 11-day's treatment (after 21-day inoculation), AZD1152 significantly decreased tumour volume compared with control mice ($P < 0.05$) (Fig. 8A). Statistically similar differences were found in tumour weights at necropsy ($P < 0.01$) (Fig. 8B). TUNEL assay showed few apoptotic cells in tumours from untreated mice, whereas apoptotic cells were abundant in the tumours taken from AZD1152-treated mice (Fig. 8C).

4. Discussion

Immunohistochemical examination has shown recently that BL (6/7, 86%) cells highly expressed Aurora B [31]. In this study, we have also shown that Aurora A and B are overexpressed in BL (10/10, 100%) and HL (10/10, 100%) lymph nodes and cell lines. Because none of the low-grade B-cell lymphoma highly expressed Aurora B compared with BL [31], overexpression of Aurora B seems not to reflect only the characteristic of malignant cells and neoplastic transformation. In addition, we found that the two Aurora kinases were aberrantly phosphorylated in BL and HL cell lines. These results suggest that both Aurora kinases are activated in BL and HL.

We also investigated the transcriptional regulation mechanism of human Aurora B gene in BL cell lines. The results identified a positive regulatory region between −74 and −104 upstream of the transcription initiation site in Aurora B. EBV is linked to BL, and EBV oncoprotein LMP-1 activates transcription and promotes cellular transformation through activation of nuclear factor-κB (NF-κB) in B cells [32]. NF-κB is the main transcription factor responsible for biological properties of BL cells [33]. However, LMP-1 did not

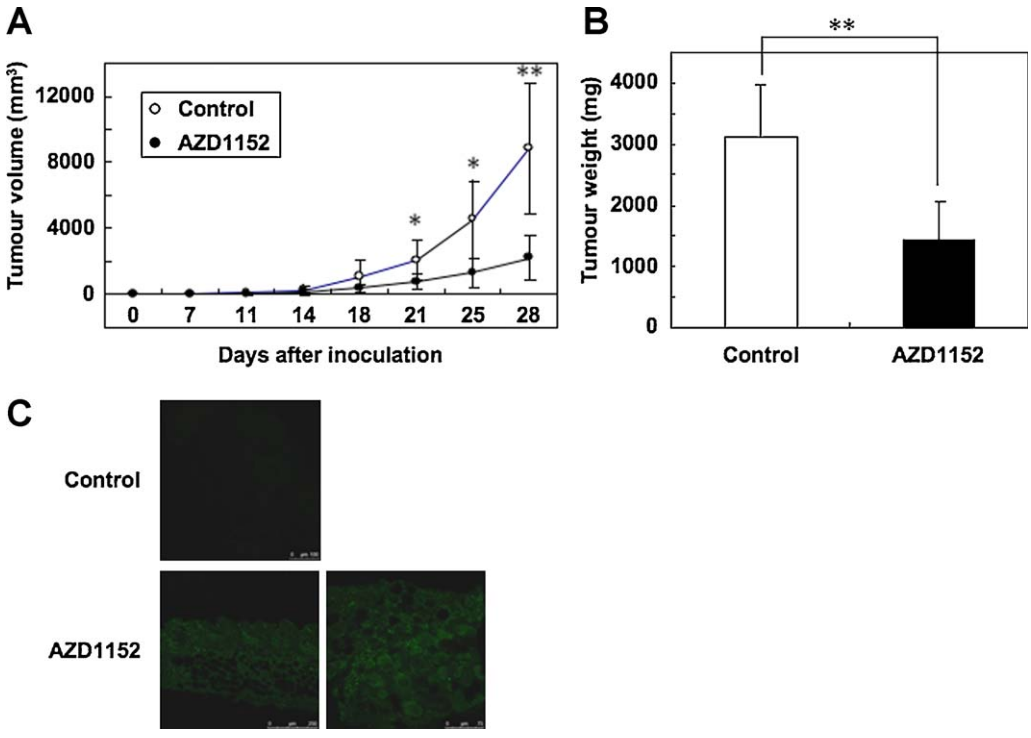

Fig. 8. Effects of AZD1152 on the proliferation of Ramos cells in a murine xenograft model. (A) Ramos cells were injected subcutaneously into NOG mice. Mice were treated with ($n = 5$) or without ($n = 6$) 30 mg/kg of AZD1152 by intraperitoneal injection every other day, and followed for 18 days following formation of palpable tumours. Tumour volume was measured at the indicated days after cell inoculation. Each data point represents the mean ± SD of tumours. (B) Tumours removed from AZD1152-treated mice and untreated mice on day 28 after cell inoculation were weighed. Data are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control. (C) TUNEL assays show apoptotic cells in tumours from mice treated with or without AZD1152. Magnification, 100× (control and AZD1152, left panel) and 300× (AZD1152, right panel).

up-regulate Aurora B promoter activity, and Aurora B promoter sequence between −74 and −104 did not include sequences suggestive to be sites for binding to NF-κB (data not shown). These results suggest that Aurora B is not the primary target of LMP-1 and its transcription is not mediated by NF-κB.

Inhibition of Aurora B kinase by the selective inhibitor AZD1152-hQPA produced growth arrest and polyploidy in all BL and HL cell lines. However, the levels of induction of apoptosis varied among the cell lines studied. Several reports indicate that Aurora A interacts with p53 protein at multiple levels. Aurora A phosphorylates p53 at Ser315 to facilitate MDM2-mediated degradation of p53 [34] and at Ser215 to suppress its transcriptional activity [35]. In addition, Aurora A regulates p53 through Akt/MDM2 mechanisms [36]. Recent studies have shown that p53 is critical for the Aurora B kinase inhibitor-mediated apoptosis in acute myelogenous leukemia cells [37]. However, p53 in BL and HL cells does not appear to be associated with apoptosis. The p53-independent induction of p21 in L540 cells may play a role in the apoptotic changes associated with AZD1152-hQPA. The loss of survivin protein may also be associated with apoptosis. Survivin is considered to inhibit apoptosis and to regulate cell division. It binds with Aurora B kinase and the inner centromere protein to form the chromosome passenger complex [38]. AZD1152-hQPA-induced inhibition of survivin may result in the augmentation of apoptosis and mitotic inhibition.

AZD1152 had a potent and long-term effect on the growth of Ramos cells *in vivo* when treatment was initiated the day after cell injection (data not shown). Initiation of treatment with AZD1152 after the tumours became palpable also resulted in delayed tumour growth (Fig. 8). AZD1152 was well tolerated by the mice and no digestive distress or significant weight loss was observed. AZD1152 treatment increased the number of apoptotic cells in the tumours. The anti-neoplastic activity against BL and HL cells in culture and the *in vivo* anti-neoplastic effect demonstrated in our experiments warrant further investigation of this drug in clinical trials for BL and HL.

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