AZD1152 Rapidly and Negatively Affects the Growth and Survival of Human Acute Myeloid Leukemia Cells In vitro and In vivo

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Abstract

Aurora kinases play a critical role in regulating mitosis and cell division, and their overexpression has been implicated in the survival and proliferation of human cancer. In this study, we report the in vitro and in vivo activities of AZD1152, a compound that has selectivity for aurora B kinase, in acute myeloid leukemia (AML) cell lines, primary AML samples, and cord blood cells. AZD1152 exerted antiproliferative or cytotoxic effects in all cell lines studied, inhibited the phosphorylation of histone H3 (pHs H3) on Ser10 in a dose-dependent manner, and resulted in cells with >4N DNA content. THP-1 cells treated with AZD1152 accumulated in a state of polyploidy and showed a senescent response to the drug, in contrast to the apoptotic response seen in other cell lines. Accordingly, AZD1152 profoundly affected the growth of AML cell lines and primary AML in an in vivo xenotransplantation model. However, concentration-dependent effects on cell growth, apoptosis, and cell cycle progression were also observed when human cord blood and primary lineage-negative stem and progenitor cells were analyzed in vitro and in vivo. These data suggest that the inhibition of aurora B kinase may be a useful therapeutic strategy in the treatment of AML and that further exploration of dosing and treatment schedules is warranted in clinical trials. [Cancer Res 2009;69(10):4150–8]

Introduction

The aurora kinases (Aur) have been implicated in the survival and proliferation of both hematologic and solid malignancies (1, 2). These proteins play a critical role in regulating mitosis and cytokinesis, with the activity of both Aur-A and Aur-B peaking during mitosis (1). The Aur genes map to chromosome loci that are frequently altered in human cancers (2), and the expression of Aur-A and Aur-B is often increased in tumor cell lines and common primary tumors. Aur-A and Aur-B have distinct subcellular localization during mitosis, reflecting their different roles in the process. Aur-A localizes to the centrosomes and mitotic spindle apparatus and regulates spindle formation (3), although it can also phosphorylate p53, facilitating Mdm2-mediated ubiquitination (1). In contrast, Aur-B is a chromosomal passenger protein that regulates chromosome segregation and cytokinesis (4). Several aurora substrates have been described, including histone H3 phosphorylated at Ser10 (3). There is also a third aurora family member (Aur-C), another chromosome passenger protein, which has been implicated in the regulation of meiosis in testes (5).

The effects of inhibiting Aur-A and Aur-B activity have been characterized in vitro. Suppression of Aur-A activity with the small-molecule inhibitor MLN8054 leads to G2-M accumulation, spindle defects, and antiproliferative effects in tumor cells (6). Targeting Aur-B prevents chromosomal alignment and compromises spindle checkpoint function, resulting in repeated rounds of DNA synthesis without cytokinesis, thereby generating polyploid cells and eventual loss of viability (7, 8). Interestingly, studies with mixed Aur (A and B) inhibitors results in a phenotype indicative of Aur-B, rather than Aur-A, inhibition (8, 9).

Aurora kinase expression in actively dividing cells makes them attractive therapeutic targets for the treatment of cancer. A number of small-molecule inhibitors of aurora kinases have been developed and are currently in early clinical evaluation, including AZD1152 (10). AZD1152 is rapidly converted into the active moiety, AZD1152-hydroxyquinazoline-pyrazol-aniline (AZD1152-HQPA), following parenteral administration in vivo. AZD1152-HQPA, a reversible ATP-competitive inhibitor, is a highly potent and selective inhibitor of Aur-B (Ki 0.36 nmol/L) compared with Aur-A (Ki 1369 nmol/L) and has a high specificity in a panel of 50 additional serine-threonine and tyrosine kinases (11, 12). AZD1152 has shown highly significant tumor growth inhibition in a diverse panel of solid human cancer tumor xenograft models, including lung and colorectal cancers (10).

Acute myeloid leukemia (AML) is characterized by a relentless accumulation of immature, abnormal hematopoietic cells in the bone marrow and peripheral blood. It has been postulated that AML is a disease maintained by leukemia stem cells. The immunodeficient mouse xenotransplantation assay is currently the model of choice to assay leukemia stem cells. This approach has been crucial to the understanding of human AML by providing reliable determination of the phenotypes of repopulating cells (13).

Despite the clear importance of the leukemia stem cells in the genesis and perpetuation of leukemia, existing therapies largely target the bulk leukemic blasts. Because the survival of only a small number of leukemia stem cells may facilitate disease relapse, any new treatment should be tested on the growth potential of these rare cells.

In this study, we have used AZD1152-HQPA and AZD1152 to evaluate the effects of inhibiting Aur-B in vitro and in vivo,
respectively, in AML cell lines, primary AML cells, and primary cord blood stem/progenitor cells.

**Materials and Methods**

**AZD1152.** AZD1152 and the active moiety AZD1152-HQPA for *in vitro* studies were provided by AstraZeneca.

**AML cell lines and primary cell cultures.** HL-60, MV411, THP-1, and U937 AML cell lines were obtained from the Cancer Research UK Cell Bank. These cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂/95% air. Primary AML cells were cultured in RPMI 1640 containing 17% heat-inactivated FBS, 2 mmol/L glucose, penicillin, and streptomycin at 37°C in 5% CO₂/95% air. Leukemic blast cell count in the primary sample before Histopaque separation was >80% for all samples.

**Cord blood and primary AML cells.** AML samples were collected from St. Bartholomew’s Hospital, and cord blood was collected from mothers attending the Royal London Hospital (London, United Kingdom) after informed consent and via a protocol approved by the local Research Ethics Committees. Mononuclear cells were obtained by Ficoll-Paque density centrifugation and ammonium chloride red cell lysis. Density-separated cord blood mononuclear cells were depleted for lineage marker–positive cells via the StemSep system (Stem Cell Technologies) according to the manufacturer’s instructions to generate Lin⁻ cells.

**Cell proliferation, viability, and apoptosis assay.** Cell number and percentage viability were determined in 96-well plate format using the Guava ViaCount assay on a Guava PCA-96 system (Guava Technologies, Inc.) following the manufacturer’s instructions. Apoptotic cells were quantified by Annexin V/7-amino-actinomycin D dual labeling using the Guava Nexin assay.

**Cell cycle distribution.** Cells were collected by centrifugation; the pellet was washed with HBSS, resuspended in 80% ethanol, and fixed overnight at −20°C. After fixation, cells were centrifuged to remove the ethanol, washed twice with HBSS, and resuspended in staining solution containing 50 μg/mL propidium iodide and 50 μg/mL RNase A in HBSS. The cellular DNA content was then analyzed within 1 h on a FACSCalibur (Becton Dickinson) flow cytometer.

**Senescence-associated β-galactosidase staining.** Senescence-associated β-galactosidase activity was determined using the senescence cell staining kit (Sigma). Briefly, cells were seeded in six-well plates followed by 72-h drug treatment and then cultured in the absence of the drug for 7 d. Cells were washed in HBSS and fixed for 6 min at room temperature. After fixing, cells were washed with HBSS and stained overnight at 37°C in the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining mixture. Cells were then washed and observed under the microscope.

**Immunocytochemistry.** Cells were collected by centrifugation; the pellet was washed with HBSS, resuspended in 80% ethanol, and fixed overnight at −20°C. After fixation, the cells were centrifuged to remove the ethanol, permeabilized in a solution of 0.2% Tween 20, washed in a solution containing 2% bovine serum albumin and 0.05% sodium azide, and incubated in 5 μg/mL anti–phospho-histone H3 (Ser10) antibody (Upstate) for 2 h. Cells were then washed and incubated with FITC-conjugated secondary antibody for 1 h in the dark. After incubation, cells were washed and applied onto a microscope slide, air-dried, and counterstained with 4,6-diamidino-2-phenylindole (DAPI) stain. The percentage of cells positive for pHis H3 was assessed by fluorescence microscopy and/or flow cytometry using propidium iodide, pHis H3 dual labeling.

**Western blot analysis.** Cell lysates were prepared in Tritton X-100 lysis buffer and 20 μg protein [determined using the Bradford reagent (Sigma-Aldrich Co.)] were resolved by SDS-PAGE. Gels were then electroblotted onto nitrocellulose membranes and probed for Dcr2, p15 (Upstate Biotechnology), p16, and β-actin (Calbiochem). Protein bands were visualized using an enhanced chemiluminescence visualization system (ECL Plus, Amersham Life Sciences).

**Cell line colony formation assay.** Cells for colony-forming assay were cultured in the presence of the drug for 72 h. Surviving cells were then counted, washed, and resuspended in drug-free medium. Cells (2 × 10³) in 1.3% methylcellulose–based medium (R&D systems) were then plated onto a 35-mm culture dish and incubated for 9 d at 37°C in a humidified atmosphere of 5% CO₂/95% air. At the end of the incubation period, the numbers of colonies formed were counted under the microscope.

**Xenotransplantation assays.** All animal experiments were done in compliance with Home Office and institutional guidelines. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were originally obtained from Dr. Leonard Schultz (The Jackson Laboratory, Bar Harbor, ME) and bred at Charles Rivers Laboratories. Mice ages 8 to 12 wk were irradiated at 375 rad (137Cs source) up to 24 h before i.v. injection of human cells.

For analysis, the femurs, tibias, and pelvises were dissected and flushed with PBS. RBC were lysed using ammonium chloride. Cells were stained with human specific FITC-conjugated anti-CD19, phycoerythrin (PE)-conjugated anti-CD33, and PE-Cy5-conjugated anti-CD45 antibodies (all from Pharmingen). Dead cells and debris were excluded via DAPI (Sigma) staining. A BD LSR flow cytometer (BD Biosciences) was used for analysis. More than 100,000 DAPI negative events were collected.

AZD1152 was administered *in vivo* by the use of subcutaneous Alzet osmotic pumps according to the manufacturer’s instructions (model 2001).³ Pumps were weighed at the end of AZD1152 treatment to confirm delivery of the full dose. Treatment regimens are summarized in Supplementary Fig. S3.

**Murine cell phenotyping.** Antibody labeling was done in PBS, 2% FCS, 10 mmol/L HEPES for 30 min with appropriate isotype-matched controls. The following antibodies were used (all from Pharmingen): FITC-conjugated anti–Thy-1, anti-CD3, and anti-CD34; PE-conjugated anti-CD2, anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD133, anti-B220, anti–Tier-119, anti–GR-1, and anti-NK1.1; biotinylated anti–Sca-1; and alloporycin conjugated anti-CD117. The lineage cocktail used throughout contained PE-conjugated anti-CD5, anti-CD11b, anti-B220, anti–GR-1, and anti–Tier-119 antibodies.

**Primary cell progenitor assays.** To assay human primary hematopoietic progenitors, Lin⁻ cells were resuspended in 300 μL of Iscove’s modified Dulbecco’s medium (IMDM) and added to 2,700 μL of MethoCult H4434 (Stem Cell Technologies). Cultures were plated in duplicate. Plates were incubated at 37°C, 5% CO₂ for 14 d and colonies were scored according to standard criteria.

**Delta assay.** Lin⁻ cells (5 × 10⁴) were seeded in 1 mL of IMDM-10% FCS supplemented with cerebrospinal fluid, interleukin (IL)-3, IL-6, erythropoietin, and granulocyte colony-stimulating factor. Cells were incubated at 37°C in 5% CO₂ for 1 wk. At the end of the first week, cells were enumerated and subjected to methylcellulose progenitor assays (10⁴/mL), propidium iodide cell cycle analysis, and Annexin V analysis. Each week, 5 × 10⁴ cells were seeded back into the assay for regrowth in cytokine-supplemented IMDM-10% FCS as before. At weeks 2 and 3 of the assay, 3 × 10⁴ and 1 × 10⁵ cells/mL, respectively, were plated in methylcellulose.

**Annexin V labeling.** Annexin V labeling was used to quantify the effects of AZD1152 on apoptosis. One hundred microliters of 10× Annexin V binding buffer (BD Pharmingen) were added to 900 μL of the resuspended cells and mixed. Then, 5 μL of directly conjugated Annexin V Alexa Fluor 647 (Molecular Probes) were added to the cells before incubation at 37°C for 15 min. DAPI was added to the cells, as above, before analysis on a BD LSR-2 flow cytometer.

**Statistics.** Student’s paired *t* test was used to compare the effects of different treatments.

**Results**

**Effects of AZD1152-HQPA in AML cell lines *in vitro* and the phosphorylation of histone H3 on Ser10.** The cytotoxic and antiproliferative effects of AZD1152-HQPA were evaluated in exponentially growing HL-60, THP-1, U937, and MV-411 cell lines.
treated with 0 to 1,000 nmol/L of AZD1152-HQPA for up to 96 hours. MV411 and THP-1 cells treated with 10 nmol/L show a marked inhibition in proliferation, as did HL-60 and U937 cells but to a lower extent (Fig. 1A). At 100 nmol/L, growth inhibition and cytotoxicity were observed in all cell lines except THP-1. The cytotoxic effect of AZD1152-HQPA was both concentration and time dependent (Fig. 1B). In HL-60, MV411, and U937 cells, a 96-hour exposure to 1,000 nmol/L resulted in an 80% loss of viability. Although AZD1152-HQPA had an antiproliferative effect in THP-1 cells, the effect of the drug on cell viability was minimal.

The inhibition of Aur-B activity was confirmed by a decrease in the phosphorylation of histone H3 on Ser10. This was observed in all cell lines studied, with complete inhibition of phosphorylation at 100 nmol/L (Fig. 1C and D).

**Cell cycle effects of AZD1152-HQPA on AML cells.** The effect of AZD1152-HQPA on cell cycle distribution was investigated in all cell lines, with data shown for HL-60 and THP-1 cells (Fig. 2A). The effects shown in HL-60 cells also reflect the observations made in U937 and MV411 cells. AZD1152-HQPA induced polyploidy in all AML cell lines studied. By 48 hours, cells had gone through a round of DNA replication without cytokinesis, giving rise to a concentration-dependent increase in polyploid (≥8N DNA content) cells (Fig. 2A). By 96 hours, cells seemed to progress from polyploidy to apoptosis, with a concentration-dependent increase in the

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**Figure 1.** AZD1152-HQPA inhibited cell proliferation, induced cytotoxicity, and inhibited phosphorylation of histone H3 (Ser10) in AML cell lines. A and B, effects of AZD1152-HQPA on cell number (A) and cell viability (B) at 48 and 96 h. C, changes in histone H3 phosphorylation in HL-60 and THP-1 cells by flow cytometry at 18 h; IgG staining and colcemid-treated cells were used as negative and positive controls, respectively. The decrease in H3 phosphorylation was concentration dependent in all cell lines and was fully inhibited at 100 nmol/L AZD1152-HQPA (D).
proportion of cells with <2N DNA content (data not shown) and an increase in Annexin V staining (Fig. 2B).

AZD1152-HQPA also induced polyploidy in THP-1 cells (Fig. 2A). However, more than 80% of the cells treated with 100 nmol/L AZD1152-HQPA remained polyploid at 96 hours (data not shown), with <5% apoptotic cells (Fig. 2B). Because THP1 cells showed such a low apoptotic response to AZD1152, we investigated the effect of aurora kinase inhibition on the ability of these cells to form colonies. THP-1 cells treated with 100 and 1,000 nmol/L of AZD1152-HQPA lost colony-forming capability (data not shown). On further investigation, 80% and 95% of THP1 cells treated with 100 and 1,000 nmol/L of AZD1152, respectively, for 72 hours and then cultured in drug-free medium for 7 days showed clear senescence-associated β-galactosidase activity (Fig. 2C). No β-galactosidase activity was apparent in HL-60 cells. Senescence is commonly associated with an increase in the cell cycle inhibitory proteins p15 or p16, which was not seen for THP-1 cells treated with AZD1152-HQPA (Fig. 2D). In contrast, a marked increase in the senescence-associated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) decoy receptor Dcr2 was observed, whereas Dcr2 showed a marked decrease in HL-60 cells in which AZD1152-HQPA induced apoptosis.

Effects of AZD1152-HQPA on AML primary cells in vitro.

The effects of AZD1152-HQPA on viable cell number, cell cycle distribution, and pHis H3 staining were studied in 12 human primary AML samples (see Supplementary Table S1 for clinical parameters). After 96-hour exposure to 1,000 nmol/L AZD1152-HQPA, viable cell number was reduced by a median of 34% (range, 0–68%) relative to control (untreated cells). In most samples, there was little effect on cell cycle distribution or the

Figure 2. Induction of polyploidy by AZD1152-HQPA in HL-60 and THP-1 cells. Changes in cell cycle distribution were investigated after exposure of HL-60 and THP-1 cells to the indicated concentrations of AZD1152-HQPA for 48 h (A). HL-60 cells showed a marked increase in the apoptotic fraction after AZD1152-HQPA for 96 h, whereas little change was seen in THP-1 cells (B). 7-AAD, 7-amino-actinomycin D. AZD1152-HQPA–treated THP1 showed the enlarged flattened morphology of senescent cells (C), stained positive for senescence-associated β-galactosidase activity (C), and showed an increase in the antiapoptotic TRAIL decoy receptor DcR2 with 100 nmol/L AZD1152 (D).
appearance of an apoptotic population (results from two primary samples are shown in Supplementary Fig. S1). It was noticeable that pHis H3 staining in untreated primary AML cells was low (median, 1.5%; range, 0–4.4%) in comparison with AML cell lines, suggesting a low proliferative rate. Based on this limitation, we went on to examine the effect of AZD1152 using an in vivo model of AML.

Effects of AZD1152 in vivo on mouse hematopoiesis. Preliminary experiments were done to determine whether AZD1152 was tolerated in NOD/SCID mice. There was no significant negative effect on the weight of 12-week-old NOD/SCID mice when AZD1152 was administered as an infusion for 2 consecutive weeks at 25 mg/kg/d. The mean (±SD) weight of mice before drug delivery was 17.9 (±0.85) g (n = 5) and after treatment was actually slightly higher at 19.5 (±1.9) g (n = 5). This suggests that the overall health of NOD/SCID mice is not severely affected by AZD1152 treatment.

AZD1152 treatment had a minimal effect on the absolute numbers of CD45+ cells present in the marrows of NOD/SCID mice. In mice that received PBS pumps, there was a mean (±SD) of $2.5 \times 10^7 \pm 0.9 \times 10^7$ CD45+ cells. One week of AZD1152 treatment at 25 mg/kg/d resulted in a reduction of absolute numbers of CD45+ cells to $1.7 \times 10^7 \pm 0.3 \times 10^7$ (n = 6 for each group; P = 0.09; Supplementary Fig. S2).

![Figure 3](image_url). In vivo effect of AZD1152 treatment on HL-60 and primary AML. One million HL-60 cells were injected i.v. into sublethally irradiated mice. Four weeks later, one group of mice were treated with AZD1152. One week after treatment, the level of HL-60 xenograft was compared with that of untreated controls (A). B, example of FACS analysis that displays human, myeloid cell content in the murine marrows. Top, AZD1152-treated mice; bottom, untreated mice. Two bar charts that summarize the frequency of human cells in murine marrows as a percentage of total nucleated cells. n, number of mice in each group. C and D, summary of the effect of 1 wk (C) and 2 wk (D) of 25 mg/kg/d AZD1152 treatment on the human cell content from various AML cases.
A comparison of the percentages of various subsets of CD45+ cells found no significant difference in the percentage of lineage (Lin)/Sca-1+/c-kit+ stem/progenitor cells, B220+ B-cells, CD2+ T cells, Gr-1+ cells, or CD11b+ cells between the treated and untreated groups of mice (Supplementary Fig. S2; n = 6 for all).

When combined, this initial toxicity assessment indicates that even in the presence of an apparent cytotoxic or cytostatic effect on murine hematopoietic cells, there does not seem to be any particular cell subset that is preferentially targeted, and the mice tolerate the AZD1152 treatment.

AZD1152 rapidly and profoundly reduces an established xenograft of HL-60 cells. To explore the effect of AZD1152 on the growth of human AML cells in vivo, we first examined the growth of the AML cell line HL-60. The HL-60 cells grow aggressively in the NOD/SCID model.
As for all our in vivo experiments, we first established the xenograft before treatment with AZD1152 and subsequent analysis of the murine bone marrow for human cell content. Preliminary experiments identified 5 weeks as the maximum time of HL-60 marrow engraftment from 10⁶ injected log-phase cells without serious adverse effects.

The results of 1 week of 25 mg/kg/d AZD1152 treatment on HL-60 xenografts in vivo are summarized in Fig. 3A. The mean (±SD) percentage of HL-60 engraftment in controls was 64.5% (±19.0%; n = 11). After 1 week of 25 mg/kg/d AZD1152 treatment, the percentage of HL-60 cells was 0.29% (±0.74%; n = 9). An example of a fluorescence-activated cell sorting (FACS) analysis for the human cell content is provided in Fig. 3B. A small group of mice were kept for a further 2 weeks after AZD1152 treatment to see if the xenograft would start to grow back. In all mice processed in this fashion, the xenograft did not regrow (0.008 ± 0.006; n = 3), indicating that AZD1152 treatment effectively eradicated HL-60 engraftment (Fig. 3A).

AZD1152 rapidly and extensively reduces primary AML xenografts in vivo. In an effort to mimic the presentation of human AML, we injected 10⁷ primary AML mononuclear cells and allowed them to establish as xenografts for 10 weeks before treatment with AZD1152 for 1 week and analysis for human cell content at the 11-week time point.

Five primary human AML samples (patients’ details given in Supplementary Table S1) were analyzed in this fashion. After administration of 25 mg/kg/d of AZD1152 for 1 week, a rapid and extensive reduction in the xenografts was observed in all the AML samples analyzed. A summary of the human cell percentage human cell content immediately after 1 week of 25 mg/kg/d of AZD1152 is shown in Fig. 3C.

One cycle of AZD1152 treatment is sufficient to compromise AML xenografts in vivo. The experiment that featured the largest residual AML xenograft after AZD1152 treatment (AML-1) was selected for further analysis, the results of which are summarized in Fig. 3D.

Four weeks after the first cycle of AZD1152 treatment, the xenograft had not grown back [29 ± 3.8% after one pump (n = 12) analyzed at 11 weeks versus 3.8 ± 3.7% 4 weeks after first pump (n = 9) at 15 weeks; P = 0.512]. Further treatment with a second cycle of AZD1152 for 1 week at 14 weeks did not further reduce the residual xenograft percentage (1.8 ± 2.3%; P = 0.628). The cohort of mice that were implanted with primary AML cells and then left untreated for 14 weeks before analysis featured a large percentage of human cells in their marrows (36.2 ± 29.7%; n = 4). Treatment at this 14-week time point with one cycle of AZD1152 for a period of 1 week reduced this xenograft percentage to levels similar to those achieved with two rounds of AZD1152 treatment at weeks 11 and 14 (1.2 ± 1.7%; n = 3).

Effects of AZD1152-HQPA on primary cord blood progenitor cells in vitro. To explore the effect of AZD1152 treatment on the growth of human normal hematopoietic stem/progenitor cells, we performed an in vitro assessment with AZD1152-HQPA. Cord blood Lin⁻ cells were treated with 10, 100, and 1,000 nmol/L AZD1152-HQPA (results summarized in Fig. 4). A dose-dependent effect of AZD1152-HQPA on the growth and differentiation of progenitor cells was observed after a 2-week exposure. Control (PBS-supplemented) assays yielded 250 ± 53 colonies/mL from 1,000 cells/mL (n = 4), which was reduced to 126 ± 35 with 10 nmol/L (n = 4) and 34 ± 18 with 100 nmol/L (n = 4) of AZD1152-HQPA (P < 0.05; Fig. 4A). At 1,000 nmol/L AZD1152-HQPA, no colonies were detected after 2 weeks.

In a similar pattern to the baseline methylcellulose assays, a concentration-dependent effect of AZD1152-HQPA on the liquid culture growth of normal hematopoietic cells was observed. At the end of the first week, the mean total cell growth from 2 delta assays done in triplicate was 2.6 × 10⁵ ± 5.4 × 10⁴ cells per well for controls, 1.8 × 10⁵ ± 5.7 × 10⁴ for 10 nmol/L AZD1152-HQPA, 2.9 × 10⁵ ± 7.1 × 10⁴ for 100 nmol/L AZD1152-HQPA, and below the threshold of accurate counting (<10⁴ cells) for 1,000 nmol/L AZD1152-HQPA.

Surviving cells on day 7 were reseeded and cultured for an extra 4 weeks (not done for 1,000 nmol/L AZD1152-HQPA–treated cells because cell number was too low). As summarized in Fig. 4B, surviving cells continued to proliferate at a similar rate to nontreated cells.

To examine progenitor cell output, cells from the liquid culture were seeded into methylcellulose cultures at the end of each week. The number of colonies per well was reduced by AZD1152-HQPA in a concentration-dependent manner. The production of colonies continued after AZD1152-HQPA removal, indicating the presence of surviving progenitors at weeks 2 and 3 of the assay (Fig. 4C). ( Colony number reduces in all conditions with time as a result of the proliferation/differentiation cocktail used in the liquid culture.)

At the end of the first week, cells were also harvested for analysis of cell cycle distribution and percentage of apoptosis or necrosis. In a similar pattern to the in vivo effect of AZD1152-HQPA on AML, there was a concentration-dependent effect on the proportion of

Figure 5. In vivo effect of AZD1152 treatment on umbilical cord blood cells. One round of AZD1152 treatment at 25 mg/kg/d severely reduced the level of human cord blood–derived xenografts. However, when left after treatment for 5 more weeks before analysis, the level of cord blood–derived graft was slightly higher. Further treatment with another round of 1-wk AZD1152 treatment did not completely eradicate the human grafts. *< 0.05.
cells that either were undergoing apoptosis (Annexin V+ or were dead (Annexin V-, DAPI+). This cell death occurred concurrently with a modest increase in the percentage of G2-M cells and polyploid cells. Representative results of this analysis are provided in Fig. 4D.

**Activities of AZD1152-HQPA in primary cord blood stem and progenitor cells in vivo.** To explore a possible toxic effect of AZD1152 on the growth of normal hematopoietic cells in vivo, we examined its effects on xenografts derived from human cord blood Lin- cells. We established the xenograft before treatment with AZD1152 and subsequent analysis. The first cycle of 1-week treatment with AZD1152 was carried out 8 weeks after implantation. One cohort of mice was analyzed 4 weeks after this AZD1152 treatment to determine whether the cord blood xenograft could recover from AZD1152 treatment. Another cohort was given an additional cycle of AZD1152 treatment 4 weeks after the first treatment. The results of this analysis are summarized in Fig. 5.

The level of engraftment after 9 weeks was significantly lower in the mice that were given AZD1152 than in the control mice (9.8 ± 6.6% (n = 4) for AZD1152-treated mice versus 62.5 ± 13.9% (n = 5) for control mice; P < 0.005). Four weeks later, the percentage of human cells was slightly higher in mice that had received one cycle of AZD1152 at 9 weeks, but this did not reach statistical significance [16.7 ± 14.1% (n = 3) at 13 weeks versus 9.8 ± 6.6% (n = 4) at 9 weeks; P = 0.39]. A second cycle of AZD1152 treatment reduced xenograft levels such that they were slightly lower than after the first round of treatment, but this was not statistically significant (7.9 ± 6.4%; n = 3), indicating that residual cells might be more resistant to a second cycle of AZD1152.

**Discussion**

The aurora kinases are emerging as potential therapeutic targets in the treatment of cancer. Although several dual (Aur-A and Aur-B) inhibitors have been described, their effects may be more dependent on the inhibition of Aur-B than that of Aur-A (3). We have therefore investigated the activity of AZD1152, a selective inhibitor of Aur-B, on AML both in vitro and in vivo.

**In vitro** AZD1152-HQPA was shown to effectively inhibit Aur-B activity in AML cell lines and in some human primary leukemic cells, as evidenced by complete inhibition of His H3 phosphorylation at submicromolar concentrations. In all AML cell lines studied, AZD1152-HQPA also induced a marked antiproliferative effect accompanied by the appearance of a polyploid population, which in most cases led to apoptosis. Similar observations have been reported recently with this compound by Yang and colleagues (14). In THP-1 cells, AZD1152-HQPA had little effect on viability or apoptosis but instead induced a senescent phenotype. In THP-1 cells, a senescent response was also induced by the topoisomerase II inhibitor doxorubicin, but not by other antileukemic agents, suggesting a drug-specific response (data not shown). This lack of apoptosis after exposure to AZD1152-HQPA in THP-1 cells may be associated with a marked increase in expression of the senescence-associated TRAIL decoy receptor Dcr2, which has previously been shown to reduce sensitivity to cytotoxic agents (15).

In primary AML cells, the effect of AZD1152-HQPA was mainly cytotactic, possibly due to the low proliferative rate of primary cells ex vivo.

Cord blood Lin- cells responded to AZD1152-HQPA in vitro in a similar fashion to AML cell lines. There was a concentration-dependent toxic effect on cell cycle and cell growth that occurred alongside an increase in apoptosis and cell death. However, once AZD1152-HQPA was removed from the culture system, the surviving cord blood cells still had progenitor cell activity as illustrated by the in vitro proliferation and differentiation of lineage-restricted colony-forming cells. Unfortunately, due to the poor performance of primary AML cells ex vivo, we could not perform the same comparison in AML cells. Therefore, although it is encouraging that nonmalignant progenitors seem to survive AZD1152 treatment, we cannot conclude that there is a differential effect.

Once we had ascertained the effects of AZD1152-HQPA in vitro, we progressed to analyze the effects of the prodrug AZD1152 on the growth of the AML cell line HL-60, primary AML cells, and primary cord blood Lin- cells in the NOD/SCID xenotransplantation model. In all our in vivo assessments, we first established xenografts before AZD1152 treatment and subsequent analysis. This approach should be a superior test to experiments where the xenograft and test drug are administered at the same time (16–18) because it better reflects the clinical situation where patients present with established leukemias.

AZD1152-HQPA had a profound, rapid, and prolonged effect on the growth of HL-60 cells in vivo at the dose of 25 mg/kg/d. In all five primary AML analyzed, 1 week of AZD1152 treatment led to an extensive and rapid reduction of the xenograft content. Where analyzed, AML xenografts did not regrow, suggesting that the SL-IC activity within the graft had been compromised.

There were, however, small residual populations of primary AML xenografts that persisted after AZD1152 treatment and during a further round of AZD1152 treatment. Attempts at secondary engraftment from these cells were unsuccessful (data not shown), indicating that they may not be capable of repopulating the disease. However, due to the high frequency of false-negative results in secondary transplant experiments, we cannot rule out the existence of residual functional leukemia stem cell populations.

Ongoing studies of the pharmacokinetics of AZD1152 administration in mice have revealed that at steady-state conditions, 25 mg/kg/d of the prodrug AZD1152 results in a free, unbound plasma concentration of ~16.9 to 18.7 nmol/L. Hence, the dose we used in vivo results in slightly higher plasma concentrations than the 10 nmol/L AZD1152-HQPA concentration we used in vitro.6

A 25 mg/kg/d dose in vivo and a 10 nmol/L concentration in vitro were capable of inhibiting AML cell line and primary human AML cell growth in vivo and in vitro. Although a larger proportion of cord blood–derived cells survived the same dose of AZD1152 treatment in vivo and in vitro, significant toxicity was observed; xenografts did not regrow in 3 weeks after initial treatment, and attempts at 6-week secondary engraftment were unsuccessful (data not shown).

Attempts to obtain a clinically relevant therapeutic window with AZD1152 may therefore be limited by hemotoxicity. Phase 1 clinical trial data support this suggestion, as the dose-limiting toxicity is reportedly neutropenia (19).

As a single agent, the effects of AZD1152 compare favorably with those of cytotoxic agents that have been tested on primary

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6 Astrazeneca, unpublished data.
human AML cells grown as xenografts in NOD/SCID mice. For example, the effect of cytosine arabinoside in NOD/SCID mice on the growth of the same number of primary AML cells as used in our own studies (107 cells) is reportedly to have a slight but not statistically significant inhibitory effect on the engraftment after 4, 8, or 12 weeks of transplantation in NOD/SCID mice (18).

The present study describes the use of an established xenograft in the NOD/SCID model for the analysis of the efficacy of a novel anticancer treatment. This approach represents an advance for the preclinical development of novel anticancer treatments. A xenotransplantation model that uses human cells is more representative of the human disease than are alternative animal models and can analyze a range of primary samples from patients with differing outcomes at different stages of the disease. In contrast, clinical trials early in the development of a novel anticancer compound are usually limited to heavily pretreated patients with a poor prognosis.

In summary, we describe the antiproliferative or cytotoxic effects of AZD1152, and the data presented from these studies support the further clinical evaluation of AZD1152 in the treatment of AML and highlight the use of a valuable in vivo model for the preclinical investigation of novel antileukemic agents.

Disclosure of Potential Conflicts of Interest

D. Bonnet: Commercial research grant, AstraZeneca. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 8/20/08; revised 1/26/09; accepted 2/25/09; published OnlineFirst 4/14/09.

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We thank the staff of both the Biological Research Unit and the Flow Cytometry Unit at Cancer Research UK for their invaluable assistance. We thank Nicholas Keen, Kate Byth, Jim Growcott, Kirsten Mundt, and Jane Robertson (AstraZeneca) for helpful discussions.

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Cancer Research 2009; 69: (10). May 15, 2009 4158 www.aacrjournals.org

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