

Overcoming CML acquired resistance by specific inhibition of Aurora A kinase in the KCL-22 cell model

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Serine/threonine kinase Aurora A is essential for regulating mammalian cell division and is overexpressed in many types of human cancer. However, the role of Aurora A in chemoresistance of chronic myelogenous leukemia (CML) is not well understood. Using the KCL-22 cell culture model we have recently developed for studying mechanisms of CML acquired resistance, we found that Aurora A expression was partially reduced in these cells upon treatment with the tyrosine kinase inhibitor imatinib, which accompanied the acquisition of BCR-ABL mutation for imatinib resistance. Gene knockdown of BCR-ABL also reduced Aurora A expression, and conversely, Aurora A expression increased in hematopoietic progenitor cells after BCR-ABL expression. Inhibition of Aurora A induced apoptosis of CML cells with or without T315I BCR-ABL mutation and suppressed CML cell growth. Inhibition of Aurora A by gene knockdown or a highly specific small molecule inhibitor sensitized CML cells to imatinib treatment and effectively blocked acquisition of BCR-ABL mutations and KCL-22 cell relapse on imatinib, nilotinib or dasatinib. Our results show that Aurora A plays an important role for facilitating acquisition of BCR-ABL mutation and acquired resistance to tyrosine kinase inhibitors in the culture model and suggest that inhibition of Aurora A may provide an alternative strategy to improve CML treatment to overcome resistance.

Introduction

Chronic myelogenous leukemia (CML) is a fatal hematopoietic malignancy caused by an oncogenic fusion gene *BCR-ABL*, a product of a reciprocal translocation of chromosome 9 and 22. Tyrosine kinase inhibitor imatinib mesylate (STI-571; Gleevec) is the frontline treatment for the disease and brings complete cytogenetic responses in most chronic phase patients (1). However, the drug has poor responses in advanced patients with frequent relapse (2,3). Genetic mutations of BCR-ABL kinase domain account for most of the clinical-acquired resistance to STI-571 treatment (4). Numerous BCR-ABL mutations have been identified; among them, T315I mutation is frequent and the most resistant (4–7). T315I mutant BCR-ABL is resistant to inhibition by the second generation tyrosine kinase inhibitors nilotinib (8,9) and dasatinib (10) that inhibit most other BCR-ABL mutations. *In vivo*, CML patients with T315I mutation do not respond to either nilotinib or dasatinib (11,12).

To help better understand the mechanisms of acquired resistance, we have recently developed a novel cell culture model using the blast crisis CML cell line KCL-22, in which cells acquire T315I BCR-ABL mutation within 2 weeks upon treatment with therapeutic concentrations of

imatinib (13). We have shown that acquisition of BCR-ABL mutation is dependent on *BCR-ABL* gene expression and is influenced by the *BCR-ABL* translocation locus. The rapid turn around, high reproducibility and commercial availability of the cell line make this model very useful for studying mechanisms of CML acquired resistance.

Aurora serine/threonine kinases are critically involved in regulating mammalian cell division, with Aurora A essential for bipolar spindle assembly during mitosis and Aurora B for proper chromosome attachment to the mitotic spindle, whereas Aurora C is important for meiosis (14,15). Aurora A is overexpressed in various human cancer cells (16,17). Aurora A overexpression leads to centrosome amplification, aneuploidy and chromosome instability, resulting in transformation of immortalized mouse and rat fibroblasts (16–18). Tissue-specific overexpression of Aurora A in mouse mammary gland produces polyploidy of epithelial cells, hyperplasia and mammary tumors (19,20). Aurora A overexpression overrides the mitotic spindle assembly checkpoint and promotes cancer cells resistance to chemotherapy (21). Aurora kinase inhibitors have been developed for cancer treatment (22). Interestingly, the pan-Aurora kinase inhibitor VX-680 (synonyms MK-0457 and Tozasertib) not only inhibits Aurora kinases but also suppresses T315I BCR-ABL through a distinct structural mechanism (23,24). However, specific roles of Aurora kinases in CML drug resistance are not clear.

In this report, we show that Aurora A is downregulated during the process of acquisition of BCR-ABL mutation for imatinib resistance and plays an important role for newly emerging mutant cells to pass initial mitosis and restore proliferation. Specific inhibition of Aurora A induces apoptosis of CML cells and blocks acquisition of BCR-ABL mutation upon treatment with tyrosine kinase inhibitors. Inhibition of Aurora A activity may have implication for preventing CML drug resistance and improving the disease treatment.

Materials and methods

Cell lines and drugs

CML cell lines KCL-22 and K562 were purchased from German Collection of Cell Cultures, Braunschweig, Germany and grown in RPMI 1640 medium with 10% fetal bovine serum (SH30071.03; Hyclone). T315I mutant KCL-22M cells are derived from KCL-22 cells. The identity of these cell lines has recently been verified by cytogenetics as reported in ref. 13. Imatinib (STI-571) was kindly provided by Novartis, Basel, Switzerland or purchased from LC Laboratories (Woburn, MA). Nilotinib, dasatinib and VX-680 were purchased from LC laboratory. Aurora A-specific inhibitor I (cat# S1451) was purchased from Selleck and erythropoietin (EPO, 4000 U/ml) from Procrit.

Cell growth, cell cycle, imatinib resistance and apoptosis assays

These assays were performed as described before (13). In brief, one-half million KCL-22 cells were seeded in 1 ml medium per well in 24-well plates and treated with different concentrations of STI-571. Aliquots of cells at specified time points were removed and counted. Cell viability was assessed by trypan blue exclusion. Mutation frequency assay was performed using a standard two-layer soft agar culture by seeding 1 million cells per well with imatinib in six-well plates, and apoptosis was analyzed by annexin V (BD Pharmingen) staining. Cell growth was measured using XTT [2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] Cell Proliferation kit (Roche).

Antibodies and western blot analysis

The cell lysates of purified bone marrow CD34⁺ cells from CML patients and normal CD34⁺ cells with mock or BCR-ABL transduction were kindly provided by Ravi Bhatia. BCR-ABL and Aurora A expression were analyzed by western blot using anti-c-ABL monoclonal antibody (BD Pharmingen) at 1:250 dilution and anti-Aurora A antibody (Upstate Biotechnology) at 1:3000 dilution. Glyceraldehyde 3-phosphate dehydrogenase was analyzed as a loading control with a rabbit antibody (Trevigen) at 1:10 000 dilution.

Immunofluorescent staining

KCL-22 cells were treated with Aurora A-specific inhibitor S1451 (Selleck) at 1 and 2.5 μ M for 4 h. Cells were mounted to glass slides by cytospin and then

Abbreviations: CML, chronic myelogenous leukemia; EPO, erythropoietin; shRNA, small hairpin RNA.

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fixed with 2% paraformaldehyde for 15 min. After blocking with 2% bovine serum albumin and permeabilized with 0.2% Triton X-100, the cells were stained with anti-Aurora A (phospho-T288) antibody for 2 h at dilution of 1:200, followed by washing and incubation with a secondary antibody of Alexa Fluor 488 labeled goat anti-rabbit IgG antibody (Invitrogen) at the dilution of 1:1000 for 1 h. After three times of wash, the stained cells were visualized with a fluorescent microscope and images were taken.

Quantitative reverse transcription-polymerase chain reaction

Total cellular RNA was extracted with Trizol (Invitrogen) and quantitative real-time polymerase chain reaction was performed with an SYBR Green label kit (Invitrogen) as per the manufacture's instruction on the Bio-Rad machine OPTICON. The primers for Aurora A are: 5'-TCAAATCTTCCAGCGCA-3' (forward) and 5'-TTTGTATGCCAGTTCCTCTC-3' (reverse); the primers for glyceraldehyde 3-phosphate dehydrogenase are: 5'-GGAAGGTGAAGGTCGAGATC-3' (forward) and 5'-TTCCCGTCTCAGC-CTTGAC-3' (reverse). The relative expression is calculated based on the ΔC_t (C_t of Aurora A – C_t of glyceraldehyde 3-phosphate dehydrogenase) at threshold 0.015 of fluorescence intensity.

shRNA lentiviral vectors and gene knockdown

Oligos for ShAuroraA (5'-GAATATCAGC-GGTTGAATT-3') or ShAuroraA2 (5'-ACAAGCCGGTTCAGATCA-3') were synthesized and cloned into the pSicoR vector (Addgene) that contains a selection cassette for puromycin by a standard protocol. A scrambled small hairpin RNA (shRNA) (5'-GCGC-GCTTTGTAGGATTCG-3') was subcloned into the vector as a mock control. The lentiviral packaging for ShAuroraA and ShAuroraA2 was performed as we did for lentiviral ShABL in our previous study (13). Transduction was typically carried out with multiplicity of infection around five.

Results

Alteration of Aurora A kinase expression during acquisition of BCR-ABL mutation

As shown in our recent report (13), one early characteristic of KCL-22 cell relapse on imatinib is the appearance of small clusters of enlarged cells after 8–10 days of drug treatment. The relapsed cells, named KCL-22M, bear homogenous T315I mutation. These newly relapsed cells have significant increase of G₂/M fraction, indicating probably slower progression through mitosis. Such morphological enlargement gradually disappears as the cells are allowed to expand for a couple of more weeks into the fully grown KCL-22M cells. We speculated that genes for mitosis might be altered during the initial process of acquisition of BCR-ABL mutations. Given that Aurora kinases have essential roles in mitosis and VX-680 can inhibit both Aurora kinases and BCR-ABL, we explored the possibility of alteration of Aurora kinases during development of imatinib resistance in KCL-22 cells.

We first examined if the morphological change seen with the nascent KCL-22M cells could be recapitulated with Aurora kinase inhibition in the fully grown KCL-22M cells. Indeed, we found that VX-680 at 0.1–5 μ M induced dramatic morphological enlargement and irregularity in KCL-22M cells, whereas imatinib has no effect (Figure 1A). Interestingly, VX-680 at the low concentration (0.1 μ M) inhibited phosphorylation of histone H3 serine 10, a substrate of Aurora kinases A and B (25) but had only minimal effect on BCR-ABL tyrosine kinase activity (Figure 1B), indicating that inhibition of Aurora kinases but not BCR-ABL activity might contribute to the morphological changes. Similar effect of VX-680 was seen in KCL-22 cells (Supplementary Figure S1 is available at *Carcinogenesis* Online). We found that VX-680 treatment for 12 h resulted in markedly increased G₂/M cell fraction, and aneuploidy was evident after 24 h (Figure 1C), consistent with the important roles of Aurora kinases for mitotic progression.

We next examined the changes of Aurora kinase expression in CML cells upon imatinib treatment. Imatinib significantly reduced the protein levels of Aurora A and, to a lesser extent, Aurora B, in KCL-22 cells; in contrast, imatinib did not change either Aurora A or B expression in KCL-22M cells (Figure 1D). The reduction of Aurora A protein level was imatinib dose dependent in KCL-22 cells, with the lowest Aurora A levels observed under the imatinib concentrations (2.5–10 μ M) that resulted in acquisition of BCR-ABL mutations (13). In contrast, no imatinib dose-dependent change of Aurora A was seen

in KCL-22M cells or prostate cancer cell line PC3 (Figure 1E and data not shown).

To further confirm that Aurora A expression is indeed altered in the newly relapsed KCL-22M cells, we sorted these cells into two pools: enlarged/irregularly shaped cells and normal-sized cells by flow cytometry (Figure 1F). Real-time polymerase chain reaction analysis showed that Aurora A RNA level was reduced in both populations of cells as compared with parental KCL-22 cells, with the enlarged newly relapsed cells having the lowest level of Aurora A (Figure 1F). Together with the above data showing VX-680 but not imatinib induced morphological enlargement of KCL-22M cells, these results indicate that inhibition of BCR-ABL by imatinib reduces Aurora A expression in KCL-22 cells before or while T315I mutation is acquired but not after the mutation acquisition is completed. As the emerging mutant cells pass initial mitosis, they suffer mitotic crisis due to reduced Aurora A level and become abnormally enlarged. Once these cells pass initial mitosis, they gradually regain Aurora A expression and proliferation and eventually restore normal morphology. These findings indicate potential roles of Aurora A in CML acquired resistance.

BCR-ABL transformation increased Aurora A expression in CML cells

Although Aurora A is overexpressed in many types of solid tumors, its expression and functions in CML are less characterized. To examine relation of BCR-ABL and Aurora A expression, we knocked down BCR-ABL using lentiviral shRNA in CML cells (13) and found Aurora A expression was dramatically reduced in imatinib-responsive KCL-22 and K562 cells and in imatinib-resistant KCL-22M cells (Figure 2A). Intriguingly, reduction of Aurora A in KCL-22M cells after BCR-ABL knockdown was slower than in KCL-22 and K562 cells. Aurora A is destroyed after mitotic exit through ubiquitin ligases, which is regulated by two conserved short amino acid sequences, an N-terminal A box and a C-terminal D box, and mutations of these sequences stabilize Aurora A (26,27). To determine if KCL-22M cells might also acquire Aurora A mutations to stabilize the protein, we sequenced the entire Aurora A complementary DNA from KCL-22M and KCL-22 cells. We found a T/A polymorphism at nucleotide 91 encoding amino acid 31 of phenylalanine or isoleucine, but no mutations, and this T/A polymorphism was randomly distributed between KCL-22 and KCL-22M cells (data not shown). These data suggest slower reduction of Aurora A in KCL-22M cells is not due to a stabilizing mutation. Instead, it is probably because KCL-22M cells gain additional survival advantage and can stay for a longer time in cell cycle upon BCR-ABL knockdown as we described before (13).

We next examined Aurora A expression in primary CD34⁺ cells after ectopic expression of BCR-ABL using retroviral vector MIG210 (28). We found that BCR-ABL expression significantly increased Aurora A protein levels in normal umbilical cord-derived CD34⁺ cells from two independent donors but not in cell lines K562, HL-60 and WI38 (Figure 2B). This is consistent with the functions of BCR-ABL to stimulate proliferation of CD34⁺ cells, whereas cell lines are either already proliferative or not appropriate cell type (WI38) for BCR-ABL transformation. In line with this notion, Aurora A levels were higher in more proliferative K562 and HL-60 cancer cells than in less proliferative non-transformed WI38 cells (Figure 2B). Consistent with the increased Aurora A protein levels, we found that Aurora A RNA level was also increased in the BCR-ABL transduced CD34⁺ cells, and conversely BCR-ABL knockdown reduced Aurora A RNA in KCL-22 cells (Figure 2C), in agreement with the above observation of reduced Aurora A RNA in the newly relapsed KCL-22M cells after imatinib treatment. Furthermore, we found that Aurora A protein was readily detected in all 11 cases of primary CML CD34⁺ cells from chronic to blast crisis phases of the disease (Figure 2D). Together, these data suggest that Aurora A gene expression may increase as a consequence of BCR-ABL transformation-mediated cell cycling and proliferation.

To further examine the relationship of Aurora A expression and cell proliferation, we analyzed K562 cells that were cultured with EPO in the presence of imatinib. It is shown that EPO at 4 U/ml or higher partially restores K562 cell growth in the presence of 1 μ M imatinib

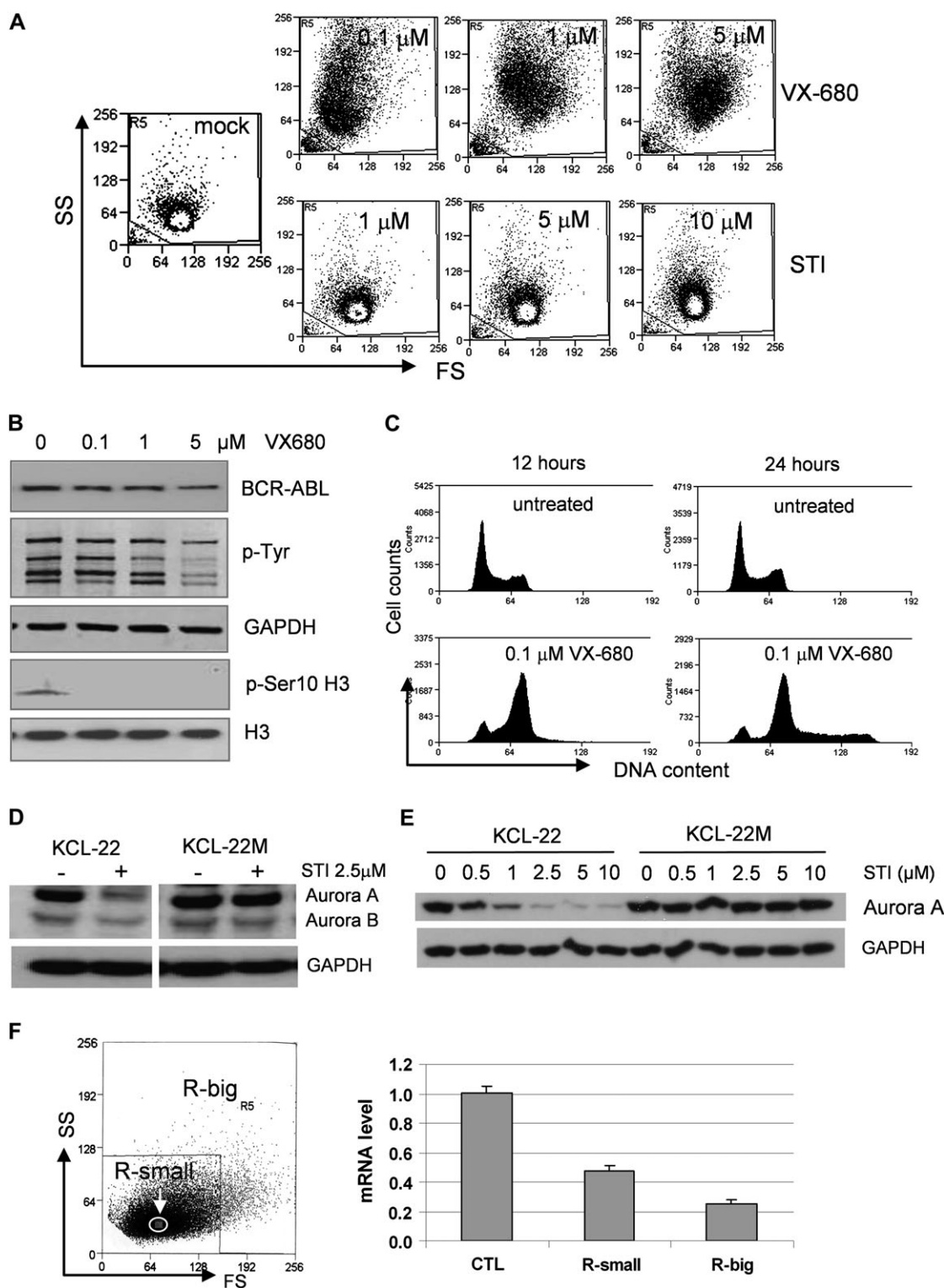


Fig. 1. Alteration of Aurora A expression during development of CML acquired resistance. **(A)** Effect of VX-680 and imatinib (STI) on morphological change of KCL-22M cells. Cells were treated with the drugs at indicated concentrations for 24 h. **(B)** Changes of tyrosine phosphorylation and histone H3 serine 10 phosphorylation after 24 h of VX-680 treatment in KCL-22M cells. **(C)** Effects of VX-680 on cell cycle of KCL-22 cells. **(D)** Changes of Aurora kinase expression in KCL-22 and KCL-22M cells after 2.5 μ M imatinib treatment for 48 h. **(E)** Imatinib dose-dependent change of Aurora A expression in KCL-22 cells after treatment with the drug for 48 h. **(F)** Left, the newly relapsed KCL-22 cells after 8 days of imatinib treatment were stained with 4',6-diamidino-2-phenylindole; 4',6-diamidino-2-phenylindole-negative cells were then sorted according to the side and forward scatters as shown. R-big, relapsed big cells; R-small, relapsed normal-sized cells (arrow-pointed circle was used for cell collection). Right, real-time reverse transcription-polymerase chain reaction results of Aurora A for sorted, relapsed cells versus parental KCL-22 cells as control (CTL).

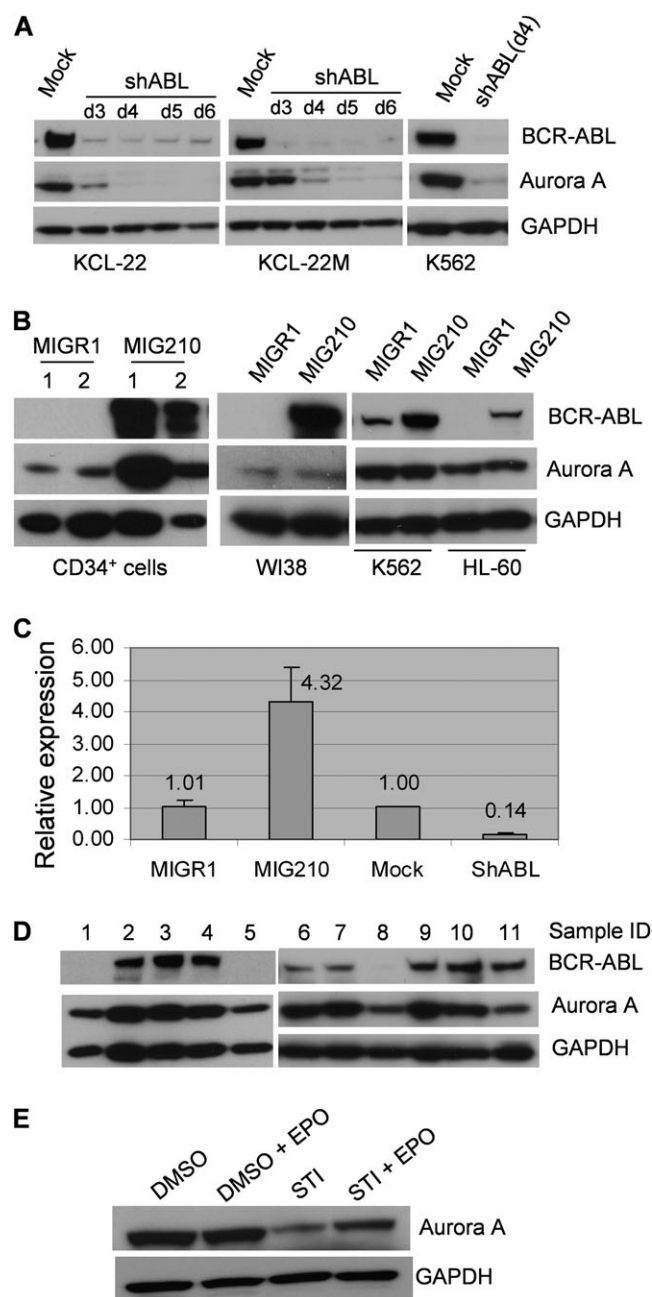


Fig. 2. Aurora A expression in CML cells. (A) Aurora A expression in KCL-22, KCL-22M and K562 cells after BCR-ABL knockdown. Cells were analyzed at days indicated after ShABL or scrambled shRNA (mock) infection. (B) Aurora A expression in umbilical cord-derived normal CD34⁺ cells, WI38, K562 and HL-60 before and after ectopical expression of BCR-ABL by a retroviral vector MIG210. The empty vector MIGR1 was used as a mock control. (C) Real time reverse transcription–polymerase chain reaction analysis of relative Aurora A messenger RNA level in umbilical cord-derived CD34⁺ cells after BCR-ABL expression (MIG210) and in KCL-22 cells after BCR-ABL knockdown (ShABL). MIGR1 and scrambled shRNA were used as mock controls. (D) Aurora A protein expression in primary CML CD34⁺ cells derived from CML patients. BCR-ABL levels in some CML samples were low and not visible with short film exposure. (E) Aurora A expression in K562 cells upon treatment with 10 U/ml EPO in the presence or absence of 0.5 μ M imatinib for 48 h.

when imatinib concentration was reduced to 0.5 μ M, partial restoration of cell growth by EPO became more appreciable (Supplementary Figure S2D is available at *Carcinogenesis* Online), which was accompanied by partial restoration of Aurora A protein level (Figure 2E). These results provide additional support that Aurora A expression is correlated with the state of cellular proliferation to a certain degree, and BCR-ABL inhibition reduces cell proliferation and results in Aurora A reduction.

Aurora A knockdown suppressed CML cell growth and transformation

To determine the functional significance of increased Aurora A expression, we carried out gene knockdown using lentiviral shRNA in CML cell lines KCL-22, KCL-22M and K562. Aurora A expression was significantly decreased in Aurora A shRNA-transduced cells as compared with the mock knockdown by a scrambled shRNA (Figure 3A). Aurora A knockdown increased G₂/M fraction in KCL-22 cells similar to newly relapsed KCL-22M cells (Supplementary Figure S3 is available at *Carcinogenesis* Online). Aurora A knockdown significantly increased apoptosis of CML cells analyzed by Annexin V staining (Figure 3B). Cell growth, assessed by plating equivalent number of cells 3 days after transduction, was markedly delayed (Figure 3C). The effect of Aurora A knockdown on clonogenic growth was assessed by standard two-layer soft agar assay. Three days after transduction, cells were seeded in soft agar for 3 weeks and significantly fewer colonies were apparent in Aurora A knockdown cells (Figure 3D). To rule out the possibility of off target effect of shRNA, another set of shRNA against Aurora A (ShAuroraA2) was designed. We found that ShAuroraA2 similarly inhibited Aurora A expression and induced apoptosis in KCL-22 cells (Supplementary Figure S4 is available at *Carcinogenesis* Online).

Aurora A knockdown sensitizes CML cell lines to imatinib treatment and suppresses acquired resistance

To examine if Aurora A knockdown promotes cell death and blocks CML cell relapse after exposure to imatinib, KCL-22 and K562 cells were infected with scrambled or Aurora A shRNA lentiviral vectors. Three days after transduction, cells were treated with imatinib and viable cells were counted. We found that Aurora A knockdown caused significantly more cell death than control knockdown after 2.5 μ M imatinib treatment in both KCL-22 and K562 cells (Figure 4A), and the increased apoptosis with Aurora A knockdown was confirmed by Annexin V analysis (Figure 4B). Aurora A knockdown delayed KCL-22 cell relapse on 2.5 μ M imatinib and completely blocked the relapse on 5 μ M imatinib in the prolonged culture (Figure 4C). The inhibition of acquired resistance was further confirmed by soft agar colony assay showing dramatic suppression of imatinib-resistant colony formation (Figure 4D). Our data indicate that Aurora A inhibition sensitizes CML cells to imatinib and suppresses development of acquired resistance in KCL-22 cells upon imatinib treatment.

Pharmacological inhibition of Aurora A prevented the development of acquired resistance of KCL-22 cells

We examined the effects of Aurora A inhibition using small molecule inhibitors. We found that VX-680 was similarly effective for apoptosis induction from 0.1 to 5 μ M in the absence or presence of imatinib in KCL-22 cells (Supplementary Figure S5A is available at *Carcinogenesis* Online). Administered as a single agent with one single dose, VX-680 at 1 μ M killed KCL-22 without relapse in 2 months continuous culture, whereas cells treated with a single BCR-ABL inhibitor imatinib, nilotinib or dasatinib all relapsed in 2 weeks and developed T315I mutation (Supplementary Figure S5B is available at *Carcinogenesis* Online). When combined with imatinib, a low dose of VX-680 at 50 nM effectively blocked BCR-ABL mutations and KCL-22 cell relapse from imatinib treatment (Supplementary Figure S5C is available at *Carcinogenesis* Online). However, this effect was likely an outcome of VX-680 itself that eliminated both KCL-22 and KCL-22M cells at 50 nM (Supplementary Figure S5C and D is available at *Carcinogenesis* Online). Similarly, VX-680 also suppressed growth of

(29). In our hands, EPO at 4 and 20 U/ml only moderately reduced 1 μ M imatinib-induced apoptosis and slightly improved cell growth, and it did not significantly change Aurora A expression (Supplementary Figure S2A–C is available at *Carcinogenesis* Online). However,

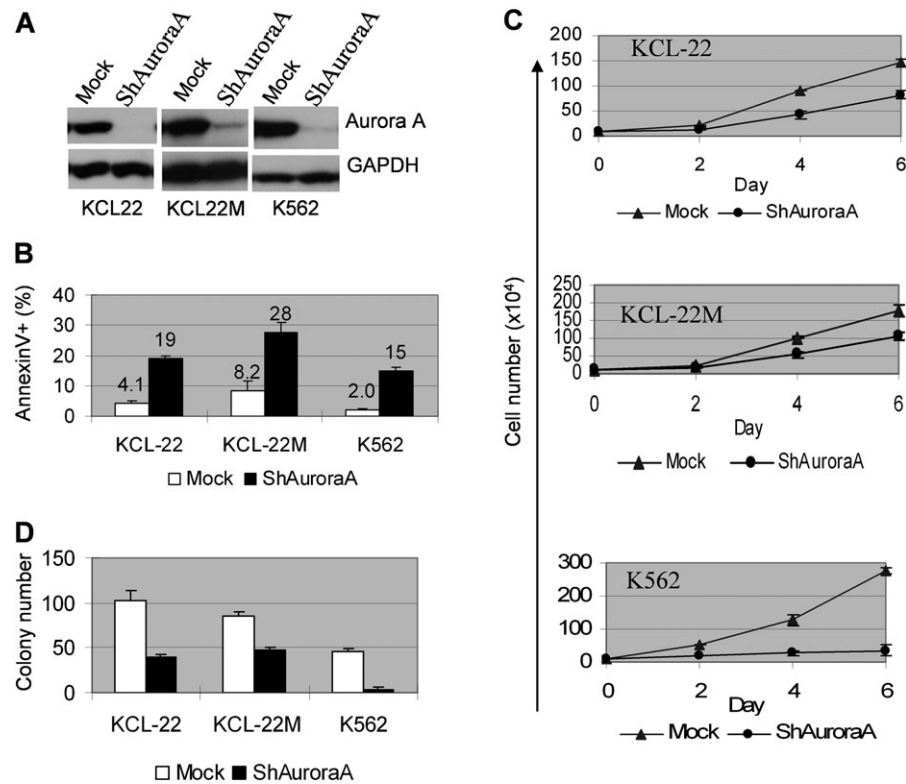


Fig. 3. Effects of Aurora A knockdown on *in vitro* cell growth and apoptosis. (A) Aurora A knockdown in KCL-22, KCL-22M and K562 cells. Cells were analyzed 4 days after scrambled (mock) or Aurora A shRNA infection. (B) Aurora A knockdown induced apoptosis in KCL-22, KCL-22M and K562 cells. Cells were analyzed by annexin V staining 5 days after transduction. (C) Aurora A knockdown suppressed cell growth. Three days after mock or ShAuroraA infection, 1×10^5 cells were cultured in 1 ml media in triplicate in 24-well plate, and viable cells were counted at days indicated. (D) Aurora A knockdown inhibited clonogenic growth of CML cells. Three days after mock or ShAuroraA infection, 500 cells were cultured in standard two-layer soft agar. The numbers of colonies were counted with aid of microscope after 21 days.

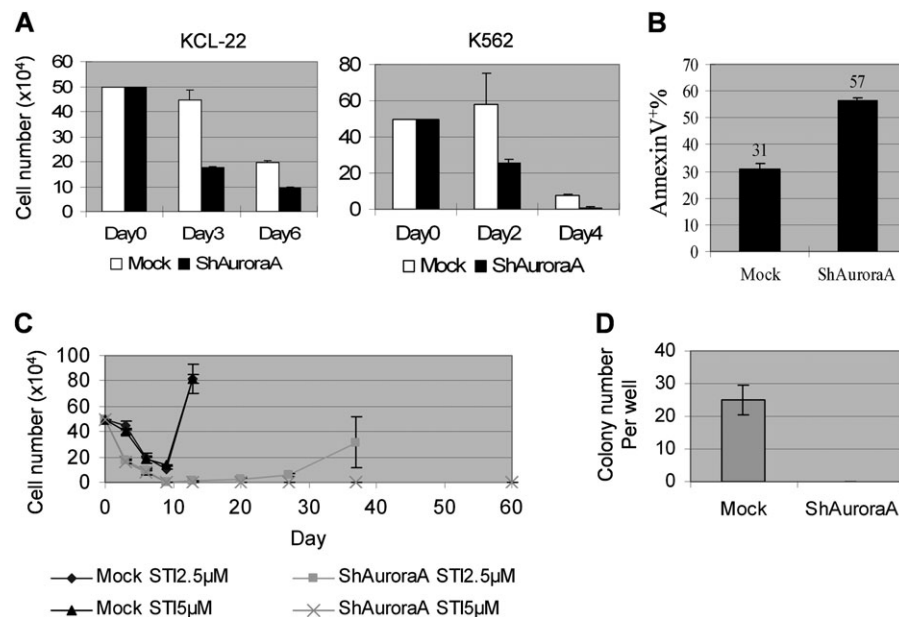


Fig. 4. Aurora A knockdown sensitized CML cells to imatinib treatment. (A) Three days after mock or ShAuroraA infection, 5×10^5 KCL-22 or K562 cells per well were treated with 2.5 or 0.5 μ M imatinib (STI), respectively, in 1 ml media in 24-well plate in triplicate and viable cell numbers were counted at days indicated after the drug treatment. (B) Apoptosis analysis of K562 cells. The cells were transduced by mock or ShAuroraA and treated with 0.5 μ M imatinib as in (A). Apoptosis of K562 cells was analyzed by annexin V staining after 48 h drug treatment. (C) Relapse assay in liquid culture. Three days after mock or ShAuroraA infection, 5×10^5 KCL-22 cells per well were treated with 2.5 or 5 μ M imatinib in 1 ml media in 24-well plate in triplicate and viable cell numbers were counted at indicated days for the prolonged culture. (D) Clonogenic assay for BCR-ABL mutation. Three days after mock or ShAuroraA infection, 1×10^6 KCL-22 cells per well were cultured in standard two-layer soft agar with 5 μ M imatinib in triplicate in six-well plate. The numbers of imatinib resistant colonies were scored after 21 days.

KCL-22 cells on soft agar culture (Supplementary Figure S5E is available at *Carcinogenesis* Online). We found that such effect of VX-680 was associated with its ability to induce polyploidy in these cells, as VX-680 at ≤ 25 nM that was unable to induce polyploidy also failed to elicit the above effects (Supplementary Figure S5F is available at *Carcinogenesis* Online).

Since VX-680 is a pan-Aurora inhibitor, we next examined the effect of a novel and highly specific Aurora A inhibitor I (S1451 hereafter). S1451 exhibits 1000-fold higher selectivity for Aurora A over Aurora B (30). In contrast to VX-680, S1451 increased G₂/M cell fraction of KCL-22 cells without significant induction of polyploidy (Figure 5A). S1451 treatment still induced histone H3 serine 10

phosphorylation (Figure 5B), suggesting whereas Aurora A was inhibited, Aurora B was spared and phosphorylated H3 in response to cell cycle arrest. Further supporting the specificity of Aurora A inhibition, S1451 suppressed mitotic Aurora A autophosphorylation on threonine 288 (Figure 5C). The above inhibition profile of S1451 in CML cells is consistent with that in the initial report using HCT-116 cells (30).

We found that S1451 exhibited dose-dependent suppression of CML cell proliferation from 0.5 to 5 μ M (Figure 6A). S1451 induced moderate apoptosis of KCL-22 cells, which significantly increased when the concentration reached 5 μ M, and it sensitized KCL-22 cells to imatinib-induced apoptosis (Figure 6B). Effects of S1451 on cell

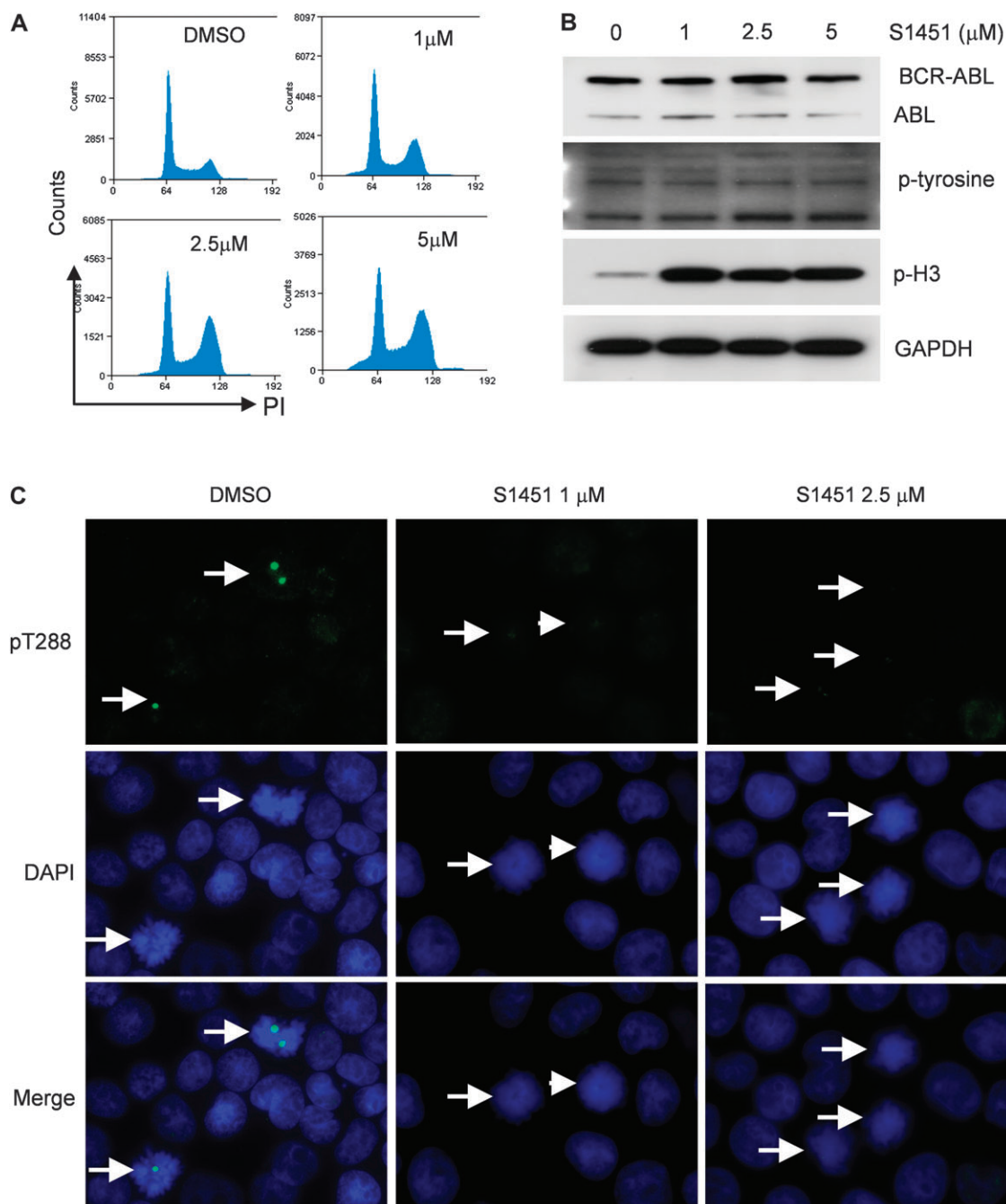


Fig. 5. Inhibition of Aurora A by a specific small molecule inhibitor. (A) Cell cycle analysis of KCL-22 cells treated with Aurora A-specific inhibitor S1451 (1–5 μ M) for 24 h. Similar results were observed after 72 h treatment and data not shown. (B) S1451 treatment increased histone H3 serine 10 phosphorylation but did not change BCR-ABL expression and global tyrosine phosphorylation. (C) Immunofluorescent analysis of Aurora A autophosphorylation on threonine 288 in KCL-22 cells after S1451 treatment for 4 h. Arrows indicate mitotic cells.

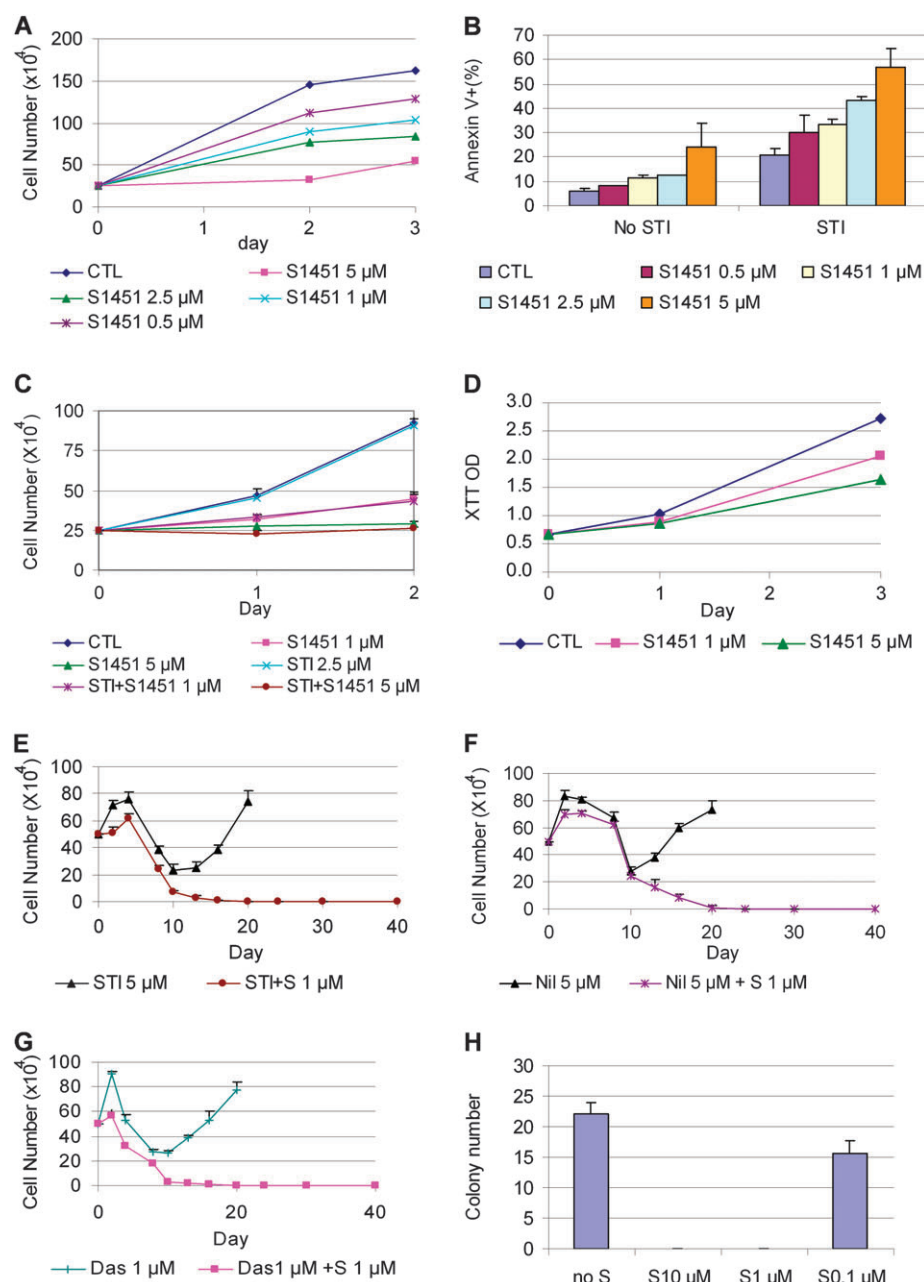


Fig. 6. Effects of Aurora A-specific inhibitor on CML cell survival and acquired resistance. **(A)** Effect of Aurora A-specific inhibitor S1451 on KCL-22 cell growth. **(B)** Effect of S1451 on apoptosis of KCL-22 cells in the absence or presence of 2.5 μ M STI. **(C)** Effect of S1451 on the growth of BCR-ABL-negative leukemia cell line KG-1 in the absence or presence of 2.5 μ M STI. **(D)** Effect of S1451 on the growth of normal cell line WI38. Growth was measured by XTT [2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] assay. **(E–G)** Effect of 1 μ M S1451 on KCL-22 cell relapse assay with imatinib (E), nilotinib (F) and dasatinib (G). **(H)** Clonogenic assay of effects of 0.1–10 μ M S1451 on imatinib (5 μ M)-mediated BCR-ABL mutant colony formation. KCL-22 cells without previous drug treatment were seeded in medium with drugs at the time of the assay setup.

growth was not restricted to KCL-22 cells, as growth of BCR-ABL-negative leukemia cell lines KG-1 and HL-60 were similarly inhibited (Figure 6C; Supplementary Figure S6A is available at *Carcinogenesis* Online). However, S1451 did not sensitize KG-1 and HL-60 cells to imatinib (Supplementary Figure S6B is available at *Carcinogenesis* Online). Compared with leukemia cells, growth of normal cell line WI38 was also suppressed by S1451, but to a lesser extent (Figure 6D). Strikingly, S1451 at 1 μ M blocked acquisition of BCR-ABL mutations and KCL-22 relapse on imatinib, nilotinib or dasatinib treatment (Figure 6E–G) as well as mutant colony formation on soft agar assay (Figure 6H), albeit KCL-22 cells retained the capability to grow at a slower rate with 1 μ M S1451 (Figure 6A). These results suggest that

specific inhibition of Aurora A may prevent development of acquired resistance of blast crisis CML cells to tyrosine kinase inhibitors.

Discussion

In this report, we demonstrate that Aurora A is downregulated in KCL-22 cells by imatinib and causes cell morphological enlargement as the newly emerging mutant cells pass initial mitosis. Aurora A inhibition by gene knockdown or a specific small molecule inhibitor further promotes cell apoptosis mediated by imatinib and prevents the emergence of mutant cells for relapse. Although Aurora A is known to be overexpressed in many solid tumors and pan-Aurora kinase

inhibitors are used for several cancer treatments, our study for the first time demonstrates that Aurora A is critically involved in acquisition of resistant mutation and eventual relapse of CML cells on tyrosine kinase inhibitors. Our results suggest that Aurora A inhibition may provide an alternative approach to improve the treatment of CML, in particular for advanced phases of the disease.

Pharmacological inhibition of Aurora kinases has been explored for cancer treatment (15,22), which inhibits growth of proliferating cells but has minimal impact on non-proliferating cells (14). VX-680 is a potent pan-Aurora kinase inhibitor and also inhibits T315I mutant BCR-ABL. CML patients bearing T315I BCR-ABL respond to VX-680 treatment, but it typically requires high doses (>20 mg/m²/h) of the drug so that the plasma concentration can reach 1 μ M to inhibit mutant BCR-ABL activity (31). Our study shows that low doses of VX-680 can induce cell cycle and morphology change of KCL-22 cells without significantly blocking BCR-ABL kinase activity. The biological effects of low doses of VX-680 to inhibit CML cells are associated with its ability to induce polyploidy that may lead to cell cycle checkpoint failure and induction of apoptosis. This is in agreement with previous reports of low doses of VX-680 to inhibit growth of other CML cell lines K562 and BV-173 without affecting BCR-ABL activity (32,33). However, pan Aurora kinase inhibitors typically bear significant toxicity and clinical development of VX-680 is currently suspended due to cardiac toxicity found during clinical trial (34).

Recently, more Aurora A-selective inhibitors have been developed including MLN8054 and the compound used in this study (30,35). MLN8054 has 43-fold selectivity over Aurora B, but in cellular assays, it has only 8-fold selectivity and still induces polyploidy (35). The compound S1451 used in this study is highly Aurora A specific and does not induce polyploidy. Compared with pan Aurora inhibitors, the Aurora A-selective inhibitors have milder antitumor effects by themselves; however, this also reduces their toxicity profile (36). Our study now show that combination of an Aurora A-specific inhibitor with a tyrosine kinase inhibitor may provide a powerful yet less toxic approach for treatment of CML to prevent acquired resistance and relapse.

It is worth noting that Kelly *et al.* (37) show that another Aurora A selective inhibitor MLN8237 inhibits growth of CML cells bearing wild-type or T315I mutant BCR-ABL. Our results of growth suppression of CML and BCR-ABL-negative leukemia cell lines after Aurora A-specific inhibition are in line with findings by Kelly *et al.* However, our study is distinct from theirs in several ways: (i) MLN8237 is a potent inducer of polyploidy in both K562 and LAMA84 CML cell lines with as low as 30 nM, reminiscent of MLN8054, indicating possible inhibition of Aurora B as previously suggested (35). In contrast, Aurora A-specific gene knockdown or treatment with S1451 in this study did not significantly elicit polyploidy. (ii) Aurora A inhibition by S1451 blocked Aurora A autophosphorylation but spared histone H3 serine 10 phosphorylation, supporting that the drug targets Aurora A specifically. However, this is not shown for MLN8237. (iii) Kelly *et al.* show that MLN8237 inhibits growth of CML cells already acquired T315I BCR-ABL mutation or Ba/F3 cells artificially introduced with T315I BCR-ABL. In contrast, our study shows a role of Aurora A during the early stage of BCR-ABL mutation acquisition, and that Aurora A inhibition can block the emergence of mutant cells on tyrosine kinase inhibitors. Therefore, our study provides a novel insight of development of CML drug resistance.

Intriguingly, mouse genetic studies show that Aurora A germ line heterozygous knockout results in aneuploidy in embryonic fibroblasts and the heterozygous mice are tumor prone (38). Although additional studies are needed to further clarify the tumorigenic outcome of Aurora A inhibition in somatic cells in animals, caution should be exercised when Aurora A inhibitors are used in human. However, given the challenge to treat blast crisis and accelerated phase CML, the benefit of using a specific Aurora A inhibitor in combination with a BCR-ABL inhibitor may outweigh the risk and improve CML treatment.

Supplementary material

Supplementary Figures S1–S6 can be found at <http://carcin.oxfordjournals.org/>

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