Discovery and Exploitation of Inhibitor-resistant Aurora and Polo Kinase Mutants for the Analysis of Mitotic Networks

Paul J. Scott, Matthew L. H. Chu, Dominic A. Sloane, Mike Cherry, Colin R. Bignell, David H. Williams, and Patrick A. Eyers

The Aurora and Polo-like kinases are central components of mitotic signaling pathways, and recent evidence suggests that substantial cross-talk exists between Aurora A and Plk1. In addition to their validation as novel anticancer agents, small molecule kinase inhibitors are increasingly important tools to help dissect clinically relevant protein phosphorylation networks. However, one major problem associated with kinase inhibitors is their promiscuity toward “off-target” members of the kinome, which makes interpretation of data obtained from complex cellular systems challenging. Additionally, the emergence of inhibitor resistance in patients makes it clear that an understanding of resistance mechanisms is essential to inform drug design. In this study, we exploited structural knowledge of the binding modes of VX-680, an Aurora kinase inhibitor, and BI 2536, a Polo-like kinase inhibitor, to design and evaluate drug-resistant kinase mutants. Using inducible stable human cell lines, we authenticated mitotic targets for both compounds and demonstrated that Aurora A mutants exhibit differential cellular sensitivity toward the inhibitors VX-680 and MLN8054. In addition, we validated Aurora B as an important anti-proliferative target for VX-680 in model human cancer cells. Finally, this chemical genetic approach allowed us to prove that Aurora A activation loop phosphorylation is controlled by a Plk1-mediated pathway in human cells.

Protein kinase inhibitors are prime examples of small molecules with the potential to revolutionize the treatment of chronic disease states such as inflammation and cancer (1, 2). For example, the discovery of inhibitors of the BCR-ABL kinase has transformed the survival rates of patients diagnosed with tyrosine kinase-driven leukemias (3). Moreover, inhibitors of many distinct protein kinases have emerged as indispensable biological tools, in part through their rapid and often reversible mode of action, but also because of their widespread availability and utility in a range of research settings. Remarkably, scientific conclusions drawn in many thousands of peer-reviewed research papers every year rely upon experiments conducted with kinase inhibitors, but in only a handful of studies is the important question of inhibitor specificity explicitly addressed (4–7). This is a vital issue because claims for specificity have rarely stood the test of time, yet a detailed knowledge of kinase inhibitor promiscuity would be beneficial in the clinic, where the simultaneous blockade of multiple signaling pathways can be exploited as an anticancer strategy (8).

The vast majority of kinase inhibitors bind in the conserved ATP-binding site located between the N- and C-terminal lobes of the catalytic domain, where they prevent nucleotide binding or lock the kinase into a structurally inactive confirmation. Inhibitor structure-activity relationship trends, which are often gleaned from combined biochemical and structural analysis, can be mechanistically revealing, but often fail to adequately address the interconnected issues of specificity and chemical resistance. Indeed, the emergence of drug resistance in chronic myeloid leukemia patients is testament to the high mutagenic susceptibility of protein kinases either selected for, or induced by, inhibitor exposure in vivo, making the discovery of mechanistically distinct inhibitors as backup therapies vitally important (9, 10).

In human cells, the key mitotic events of centrosome separation, bipolar spindle formation, and chromosome segregation are linked to the physical separation of the genomes during cytokinesis (11). These conserved signaling programs are controlled by dedicated mitotic protein kinases, which include two prominent human gene families, the Aurora kinases (comprising Aurora A, B, and C) and the Polo-like kinases (comprising Plk1–4), whose overexpression in a spectrum of cancers makes them outstanding drug candidates (12). A more detailed knowledge of the substrates and physiological events regulated by Aurora and Polo signaling pathways has been facilitated by the development of potent inhibitors of both enzyme families (13, 14). These include clinical candidates such as the dual Aurora/tyrosine kinase inhibitors VX-680 (15, 16) and AT9283 (17) and the Aurora inhibitors MLN8054 (18) and AZD1152 (19). In addition, the clinically advanced Plk1–3 inhibitor BI 2536 has been well characterized in human cells (20) and cancer models (21).

One of the frustrations associated with interpreting cellular data obtained with compounds such as VX-680 and BI 2536 is their unknown cellular selectivity. No kinome-wide data are
available in public data bases for any kinase inhibitors, and it is likely that these compounds have multiple kinase and non-kinase targets in human cells. For example, VX-680 inhibits both Aurora A and B in human cells and tyrosine kinases such as ABL, Src, and Flt3 in vitro (15, 22), raising the question as to which, if any, of these targets are critical for phenotypes and anti-proliferative effects observed after drug exposure. In addition, Plk1 and Aurora A signaling functions are mutually dependent in proliferating human cells (23–26). This makes interpretation of experiments in which Aurora A or Plk1 inhibitors are employed potentially confusing because phenotypes assigned to one inhibitor target might actually be due to indirect inhibition of the other kinase. To begin to address these issues, we have investigated the cellular plasticity of kinase inhibition by both VX-680 and BI 2536. By evaluating drug-resistant inhibition of the other kinase and exploiting these mutants in stable human cell lines, we demonstrate that drug-resistant forms of these kinases can be used to prove that phenotypes arising from VX-680 exposure are actually due to inhibition of the predicted mitotic targets. We demonstrate that a VX-680-resistant Aurora A mutant remains sensitive to the distinct anti-proliferative agent MLN8054 in human cells and that Aurora B is the critical target of VX-680 that promotes cell death in a cancer cell model. Furthermore, by analyzing a Plk1 mutant with decreased sensitivity to BI 2536, we establish that a mitotic phenotype arising from exposure to this drug is indeed due to Plk1 inhibition and that, during mitosis, Plk1 controls Aurora A phosphorylation at the critical activating residue Thr288.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology and Protein Expression**—cDNA encoding full-length human Aurora A or the T210D Plk1 kinase domain mutant (encoding amino acids 1–364) was inserted into plasmid pET30-Ek/LIC (Novagen) and subjected to PCR to generate the desired point mutants. His-tagged *Xenopus* Aurora B-INcenp2 (or the PCR-generated G176L mutant) and His-tagged Aurora A and Plk1 proteins were produced in *Escherichia coli* BL21(DE3) pLysS (Novagen), affinity-purified, dialyzed, and stored at −80 °C prior to use. Full-length human Aurora A and its G216L and G216S mutants, human Aurora B and its G160L mutant, and human Plk1 and its R136G mutant were cloned as N-terminally Myc-tagged PCR products in the mid pET30-Ek/LIC (Novagen) and subjected to PCR to generate the desired point mutants. His-tagged Aurora A and Plk1 proteins were produced in *Escherichia coli*– Anti-Myc and anti-α-tubulin monoclonal antibodies were obtained from Cancer Research UK. Anti-γ-tubulin antibody (clone GTU-88) was purchased from Sigma. Anti-AIM1 (Aurora B), anti-N-terminal Plk1, anti-phospho-Thr210 Plk1, and anti-CDCK5 antibodies were obtained from Cell Signaling. Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Anti-phospho-Thr288 Aurora A, anti-phospho-Thr210 Aurora B, and anti-phosphoser15 histone H3 antibodies were all generated in-house.

**RESULTS**

**VX-680 Inhibits Human Aurora and ABL Kinases in Vitro**—VX-680 (also termed MK-0457; supplemental Fig. 1) is an equipotent Aurora inhibitor, with IC50 values of between 18 and 25 nm for human Aurora kinases when assessed in our standard radiometric assay (Table 1). To build upon recent reports identifying Aurora inhibitors as dual inhibitors of the ABL (Abelson) tyrosine kinase (10, 28), which is a key oncogenic driver in chronic myeloid leukemia, we evaluated whether VX-680...
inhibits several distinct kinases, including the T315I ABL gatekeeper mutant found in a significant proportion of imatinib-resistant chronic myeloid leukemia patients (9). As shown in Table 1, VX-680 is a potent inhibitor of the activity of ABL kinase activity, being an order of magnitude better at preventing phosphorylation of a peptide substrate than the clinical compound imatinib. In addition, the multigrid-resistant T315I mutant was one of four imatinib-resistant ABL kinases that were still sensitive to submicromolar concentrations of VX-680 in vitro, verifying the dual sensitivity of both Aurora and tyrosine kinase family members to this compound. We also investigated three important mitotic protein kinases, human Plk1, Cdc2/cyclin B (maturation-promoting factor), and Cdk2/cyclin E, and established that they are all resistant to VX-680 in vitro.

Drug-resistant Aurora A Mutants—The amino acid side chains lining the nucleotide-binding site of protein kinases contribute directly to the affinity of ATP-dependent inhibitors for their targets. Some residues are critical for ATP positioning and catalytic activity, but others, such as the gatekeeper residue (Leu210 in Aurora A), control access to regions of the hydrophobic cleft not occupied by ATP; these loci are exploited for fine-tuning of inhibitor potency and can sometimes be diagnostic for inhibitor sensitivity and selectivity (29). The crystal structures of Aurora A in complex with VX-680 (30) and both VX-680 and TPX2 (31) provided a convenient starting point for investigating pertinent amino acid side chains that might confer VX-680 sensitivity toward active Aurora A. To achieve this, we devised a dual activity and inhibitor resistance strategy to analyze a panel of full-length mutant Aurora A proteins. To help eliminate inactive Aurora A point mutants, we examined phosphorylation at Thr288 as a surrogate for autoactivation (32). As shown in Table 2 and supplemental Table 1, amino acid substitutions at position 216 are particularly interesting because they do not abolish Aurora A activity, yet confer differential resistance to VX-680. Gly216 is conserved in all three human Aurora kinases and in the VX-680-sensitive kinases ABL, Flt3, Lck, and Plk4, but is absent in the VX-680-resistant mitotic kinase Plk1 (Fig. 1A). As detailed experimentally in Fig. 1B, the identity of the side chain at this position in Aurora A is important for dictating VX-680 sensitivity because mutation of distinct amino acids conferred different degrees of resistance to purified Aurora A proteins. Encouragingly, an Aurora A mutant containing a Leu side chain at position 216 was highly resistant, being some 250-fold less sensitive to VX-680 than WT Aurora A (IC_{50} ~ 5 μM) (Fig. 1, B and C). A comparative structural analysis of this residue suggested that a hydrophobic Leu side chain is likely to confer resistance by sterically hindering interactions between the VX-680 methylpiperazino moiety and a small pocket near the hinge region guarded by Gly216 (Fig. 1D).

Aurora kinases are subject to allosteric control by subunits such as the Aurora A-specific activator TPX2 (33, 34). We therefore investigated the activation of WT Aurora A and mutant G216L by a TPX2 fragment in vitro. Interestingly, the reduced activity of the recombinant G216L mutant was rescued by incubation with TPX2, suggesting that this mutation does not markedly interfere with the ability of Aurora A to be activated by a physiological regulator (Fig. 1E). As reported previously (31), TPX2-bound Aurora A exhibited decreased sensitivity to VX-680 compared with the monomeric enzyme, whereas the activated G216L Aurora A-TPX2 complex was completely resistant.

A useful consequence of the recent explosion in the number of Aurora kinase inhibitors is the ability to evaluate compounds of distinct chemical structure. As demonstrated in Fig. 1F, the G216L Aurora A mutant was also resistant to the quinazoline inhibitor ZM447439, which was originally identified in a screen for Aurora A inhibitors (35). In contrast, this mutation did not prevent inhibition by the unrelated clinical Aurora A inhibitor MLN8054 because G216L Aurora A exhibited only a minor reduction in sensitivity to this compound (Fig. 1G).

Inhibitor-resistant Aurora B Mutant—To extend our findings with Aurora A to include the closely related drug target Aurora B, we investigated drug resistance induced by mutation of the equivalent conserved Gly residue. Human Aurora B exhibits low activity in vitro, so we utilized a highly active Xenopus Aurora B-INCENP complex for our mutagenesis studies (36). As shown in supplemental Fig. 2, the recombinant Aurora B-INCENP complex was inhibited by VX-680 with an IC_{50} value of 72 nM. In close agreement with our findings for Aurora A, replacement of Gly216 with Leu did not markedly affect Aurora B-INCENP activity, but increased the inhibition constant for VX-680 by several orders of magnitude to >10 μM. We next investigated the effects of the Aurora inhibitor

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**Table 1**

Sensitivity of selected protein kinases to imatinib and VX-680 in vitro

| Kinase     | Imatinib IC_{50} (nM) | VX-680 IC_{50} (nM) |
|------------|-----------------------|---------------------|
| Aurora A   | >30,000               | 20                  |
| Aurora B   | >30,000               | 18                  |
| Aurora C   | >30,000               | 25                  |
| ABL        | 1,200                 | 98                  |
| T315I ABL  | >100,000              | 320                 |
| Y225F ABL  | 8,100                 | 243                 |
| E255K ABL  | 8,600                 | 420                 |
| G250E ABL  | 3,100                 | 420                 |
| Plk1       | ND                    | >10,000             |
| Cdk1/cyclin B | ND                   | >10,000             |
| Cdk2/cyclin E | ND                   | >10,000             |

*ND, not determined.

**Table 2**

Analysis of Aurora A kinases mutated at Gly216

| Aurora A | Phospho-Thr288 Activity | VX-680 IC_{50} (nM) |
|----------|-------------------------|---------------------|
| WT       | Yes                     | 100                 |
| G216L    | Yes                     | 6                   |
| G216T    | Yes                     | 3                   |
| G216Q    | Yes                     | 6                   |
| G216E    | Yes                     | 12                  |
| G216D    | Yes                     | 32                  |
| G216H    | Yes                     | 6                   |
| G216S    | Yes                     | 33                  |
| G216N    | Yes                     | 21                  |
| G216A    | Yes                     | 5                   |
| G216P    | Yes                     | <1                  |
| G216F    | Yes                     | <1                  |
| G216R    | Yes                     | <1                  |
| G216l    | Yes                     | <1                  |
| G216V    | Yes                     | <1                  |
| D274A    | No                      | <1                  |

*ND, not determined.*
MLN8054, which induces Aurora A-like inhibitory cellular phenotypes, but also inhibits Aurora B in vitro (18). Interestingly, WT Aurora B-INCENP was readily inhibited by MLN8054, whereas the G176L mutant was desensitized by nearly 2 orders of magnitude (supplemental Fig. 2B), in marked contrast to the potent inhibitory effect of MLN8054 on G216L Aurora A.

**Biochemical Analysis of Plk1 Mutants**—Our success in isolating VX-680-resistant Aurora A and B kinase mutants encouraged us to investigate the recently described Plk1 inhibitor BI 2536, which has been reported to inhibit Plk1–3 in vitro, but which induces phenotypes consistent with Plk1 inhibition in cells (20). As shown in Fig. 2A, BI 2536 inhibited human Plk1 expressed in Sf9 cells, preventing both autophosphorylation and Plk1 activity with similar IC$_{50}$ values of ~20 nM. We next exploited a co-crystallographic analysis of Plk1 and BI 2536 to identify potential BI 2536-interacting residues in Plk1 (37).

Prominent among these was Arg$_{136}$, which is in close proximity to the phenyl, amide, and piperidine moieties of the inhibitor (Fig. 2B), at an equivalent position to Gly$_{216}$ of Aurora A (Fig. 1A). To test whether the guanidinium side chain at position 136 is important for Plk1 inhibition by BI 2536, we expressed activated mutants of either the WT or R136G Plk1 catalytic domain in bacteria and assessed the inhibitor sensitivity of the purified enzymes. As detailed in Fig. 2C, a recombinant Plk1 catalytic domain was inhibited by BI 2536 with an IC$_{50}$ of ~20 nM, very similar to that of full-length human Plk1. R136G Plk1 was still active, proving that this substitution does not obstruct catalytic function. Significantly, however, the R136G mutant was desensitized to BI 2536 at concentrations that completely abolished WT Plk1 activity, inducing a modest (3–4-fold) increase in the IC$_{50}$ value (Fig. 2C). In contrast, WT and R136G Plk1 were
equally sensitive to the unrelated Plk1 inhibitor GW843682X (Fig. 2D) (38), demonstrating that resistance is inhibitor-specific. Arg136 is replaced with Gly in Plk4, which is resistant to BI 2536 but sensitive to VX-680 in vitro (39). We therefore tested whether a Plk1 mutant containing a Gly residue is sensitized to VX-680. However, both WT and R136G Plk1 were completely resistant to VX-680 and MLN8054 (data not shown), demonstrating that, although this Arg residue is important for optimal inhibition of Plk1 by BI 2536, mutation to Gly is not sufficient to render Plk1 sensitive to these Aurora inhibitors.

Analysis of Drug-resistant Aurora Kinase Mutants in Human Cells—Recent analyses of Aurora kinase inhibitors such as VX-680 and MLN8054 and Plk1 inhibitors such as BI 2536 have corroborated and expanded previous findings obtained using classical genetic and cell biological approaches (40). However, because of their ATP-dependent inhibitory mechanism, cellular data are potentially compromised by assumptions of specificity, given that compounds such as VX-680 are unable to discriminate in cells between distinct but functionally related kinases such as Aurora A and B. To investigate the intracellular targets of these compounds, we therefore devised a chemical genetic strategy in which biochemically validated drug-resistant mutants of mitotic kinases were stably expressed as epitope-tagged transgenes from a defined locus in HeLa cell lines. As a control, similar levels of drug-sensitive tagged WT kinases were expressed from the same Tet-inducible locus (Fig. 3A). The induced kinase was readily resolved from endogenous protein by the decreased electrophoretic mobility imparted by the Myc tag, and the phosphorylation of Aurora A at Thr288 was adopted as a validated marker for Aurora A activity in these cells (22).

FIGURE 2. Inhibition of recombinant Plk1 enzymes. A, shown is the inhibition of SF9-expressed human Plk1 by BI 2536. Full-length His-Plk1 was assayed in duplicate in the presence of 100 μM [γ-32P]ATP and the indicated concentrations of BI 2536 using α-casein (red line) or Plk1 (black line) as substrate. Activity is reported as a percentage of the control calculated from incubations containing 2.5% (v/v) DMSO. B, shown is the complex of Plk1 and BI 2536, highlighting residues adjacent to the drug-binding site, including Arg136 (left), and as modeled with Gly in the R136G mutant (right). C, T210D Plk1 and R136G/T210D Plk1 catalytic domains were assayed in the presence of 100 μM [γ-32P]ATP and the indicated concentrations of BI 2536 (left). Equal Plk1 loading was demonstrated using horseradish peroxidase-conjugated anti-His antibody. Phosphorylated casein bands were quantified, and the data are plotted graphically (right). Activity is reported as a percentage of the control calculated from incubations containing 2.5% (v/v) DMSO. Similar results were seen in two independent experiments. D, conditions were the same as described for C, except that the unrelated Plk inhibitor GW843682X replaced BI 2536.
Aurora A became dephosphorylated (Fig. 3B, right panels, asterisks). In contrast, the phosphorylation of histone H3, a known Aurora B substrate, was sensitive to VX-680 in the same cell extracts, demonstrating that the drug-resistant Aurora A mutant had not adversely affected Aurora B-mediated signaling. In addition, we determined that both the phosphorylation of CDC25C, as judged by its decreased electrophoretic mobility, and the phosphorylation of Plk1 at Thr²¹⁰ were both insensitive to VX-680, indicating that they are not downstream of Aurora A (or Aurora B) under these experimental conditions (Fig. 3B).

The G216L Aurora A mutant exhibited similar sensitivity to MLN8054 compared with WT Aurora A in vitro (Fig. 1F). We therefore assessed the phosphorylation status of Aurora A at Thr²⁸⁸ in stable lines exposed to MLN8054. In contrast to our findings with VX-680, autophosphorylation of both WT and G216L Aurora A at Thr²⁸⁸ was potently inhibited in the presence of MLN8054 (Fig. 3C, asterisks), confirming that resistance had been specifically generated toward VX-680 in these cells. In addition, we determined that phosphorylation of both CDC25C and Plk1 at Thr²¹⁰ was insensitive to MLN8054, providing further evidence that Aurora kinases are not rate-limiting for their phosphorylation (Fig. 3C).

**Analysis of Mitotic Cells Expressing Drug-resistant Aurora Kinase Mutants**—To corroborate these findings in a distinct cell type, we generated a complementary colon carcinoma (DLD-1) stable cell line expressing identical Myc-tagged WT or G216L Aurora A transgenes. We first investigated spindle monopolarity induced by VX-680 in individual WT or G216L Aurora A-expressing DLD-1 cells to confirm that this was indeed due to inhibition of Aurora A (22). Both WT and G216L Aurora A transgenes were expressed at similar levels and localized normally to the centrosomes when stained with an anti-Myc antibody (Fig. 4, A and B). Tet-mediated expression of WT Aurora A did not interfere with VX-680-induced monopolarity (Fig. 4A), but expression of G216L Aurora A markedly decreased the number of monopolar spindles present in cells exposed to VX-680 (Fig. 4B). By quantifying immunofluorescence data from multiple cells, we validated that...
VX-680 had induced mitotic monopolarity through direct inhibition of Aurora A because bipolarity was restored in cells expressing the drug-resistant kinase (Fig. 4C). We further characterized this drug-resistant phenotype using a stable cell line harboring a partially drug-resistant G216S Aurora A gene. We found that this mutant could also suppress spindle monopolarity in the presence of VX-680 (supplemental Fig. 3, A and B), although its ability to revert this phenotype was decreased compared with the highly resistant G216L mutant, consistent with the increased sensitivity of G216S Aurora A to VX-680 in vitro (Fig. 1C). To investigate the response of stable cells to a separate biological phenomenon, we determined that the number of mitotic cells containing unaligned metaphase chromosomes, which are a likely consequence of VX-680-mediated Aurora B inhibition, was not significantly altered by expression of the G216L Aurora A mutant (Fig. 4D).

Aurora B Is an Important Target of VX-680 in Human Cancer Cells—VX-680 is a simultaneous dual inhibitor of Aurora A and B in cells (22). We next investigated human Aurora B sensitivity to VX-680 by generating stable HeLa cell lines inducibly expressing similar levels of WT or G160L Aurora B transgenics (Fig. 5A). Interestingly, we found that phosphorylation of histone H3 at Ser\(^{10}\), a known Aurora B phosphorylation site (35), was sensitive to VX-680 in WT but not G160L cell lines exposed to nocodazole (Fig. 5B). In marked contrast, no difference in Aurora A autophosphorylation at Thr\(^{288}\) was detected in WT or G160L cells exposed to the drug, emphasizing the desensitization of Aurora B-mediated VX-680-sensitive signaling by the G160L mutant (Fig. 5B). When assessed by immunofluorescence, both WT and mutant Aurora B proteins correctly co-localized with active (Thr\(^{232}\), phosphorylated) Aurora B on centromeres (Fig. 5C). As quantified in Fig. 5D, stable mitotic cells expressing WT but not G160L Aurora B contained misaligned chromosomes in the presence of VX-680, suggesting that Aurora B is the intracellular target that induced this phenomenon. Moreover, the percentage of monopolar spindles identified in identically treated cells expressing WT or G160L Aurora B was not markedly different in the presence of VX-680 (Fig. 5E), consistent with our finding that defects in centrosome separation are due to Aurora A-mediated inhibition (Fig. 4).

To investigate the long-term phenotypic effects of VX-680 exposure in cells expressing drug-resistant mutants of either Aurora A or B, we investigated the DNA content of the stable cell populations by fluorescence-activated cell sorting analysis 2 days after exposure to VX-680. As shown in Fig. 6A, 50 nM VX-680 induced a multiploid (>4n) phenotype in both WT and G216L Aurora A stable cell lines, and this was not rescued in the presence of Tet (Fig. 6A). In contrast, G160L Aurora B-expressing cells were refractory toward VX-680-induced polyploidy, as demonstrated by the loss of the >4n peak in Tet-exposed cells (Fig. 6B).

Defining the intracellular target of VX-680 that induces cell death in transformed cell populations would be extremely useful because this information could be used to tailor drug discovery efforts. Therefore, we investigated the effects of sustained expression of Aurora A and B mutants using a clonogenic assay in which both WT and drug-resistant Aurora A and B kinases were expressed for the duration of the experiment (Fig. 6C). Remarkably, cells expressing G160L Aurora B were still able to proliferate in the presence of VX-680, in contrast to cells overexpressing WT Aurora B, WT Aurora A, or VX-680-resistant G216L Aurora A, which were eliminated in a dose-dependent manner (Fig. 6D). Quantification of cellular data revealed that clonal expansion reached 51% of the DMSO-treated control population in cells expressing G160L Aurora B after prolonged exposure to 25 nM VX-680, compared with just 4.4% in WT Aurora B control cells. Increasing the concentration of VX-680 to 50 nM prevented cell proliferation completely in WT Aurora B-expressing cells, but G160L-expressing cells grew to 8.8% of control levels (Fig. 6D). We next investigated the effects of MLN8054, which inhibits WT and G216L Aurora A with similar
ilar potency but has a markedly reduced effect on VX-680-resistant Aurora B in vitro. We found that MLN8054 induced dose-dependent cell death in a similar manner in all four stable cell lines (Fig. 6E), suggesting that its anti-proliferative target is unlikely to be Aurora B.

**Analysis of a Drug-resistant Plk1 Mutant in Human Cells—**
We next turned our attention to the Plk inhibitor BI 2536, which has been demonstrated to induce multiple phenotypes in human cells, including the induction of a monopolar spindle phenotype and the inhibition of cytokinesis (20). Both these phenotypes are similar to those obtained by ablating Plk1 function using other approaches (41–43), although Aurora A and B are also required for these processes in human cells (44, 45). To validate the target of BI 2536 in human cells, we first demonstrated that both WT and R136G Plk1 mutants were inducibly expressed at similar levels in stable cells and that both became phosphorylated at the activation loop residue Thr210 (Fig. 7A). Exposure of mitotic cells expressing WT Aurora B to BI 2536 and MG132 led to a dose-dependent increase in the mobility of the dual-specificity phosphatase CDC25C and a concomitant loss of Aurora A Thr288 phosphorylation without significant effects on the total levels of Aurora A protein (Fig. 7B, left panels). In contrast, the CDC25C band shift in cells expressing the R136G mutant of Plk1 was refractory to BI 2536 inhibition, and the phosphorylation of Aurora A at Thr288 was sensitive to BI 2536 only at the highest concentrations tested, demonstrating that both CDC25C phosphorylation and Aurora A Thr288 auto-phosphorylation were under the control of Plk1 (Fig. 7B, right panels). To rule out direct inhibition of Aurora A by BI 2536, we confirmed that it does not inhibit Aurora A activity in vitro (supplemental Fig. 4).

**Cellular Plk1 Resistance to BI 2536 Reverts Spindle Monopolarity and Restores Centrosome Aurora A Thr288 Phosphorylation—**To assess the effects of the drug-resistant Plk1 mutant in mitotic cells that had not been exposed to nocodazole, we examined individual cells expressing WT or R136G Plk1. Both ectopic WT and R136G Plk1 mutants co-localized with endogenous Plk1 at the midbody in telophase (Fig. 8, A and B). WT

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**FIGURE 5. Analysis of HeLa cells harboring inducible Aurora B genes.** A, WT or G160L human Aurora B-encoding Myc-tagged plasmids were stably integrated in HeLa cells. Cell extracts were analyzed by Western blotting with antibodies recognizing Myc (upper) or Aurora B (AurB; lower). B, Tet-exposed asynchronous (AS) and mitotic cells expressing WT or G160L Aurora B were cultured in the presence or absence of the indicated concentrations of VX-680. Cell lysates were probed with the indicated antibodies. Similar results were seen in two independent experiments. C, asynchronous WT Aurora B HeLa cells (upper) or G160L Aurora B cells (lower) were incubated with or without Tet, fixed in methanol, and stained with the indicated antibodies. Note that the anti-phospho-Thr232 Aurora B antibody (pT232 AurB) also cross-reacted with phospho-Thr288 Aurora A on the centrosome. D, spindle morphology was scored in cell lines exposed to VX-680 in the presence and absence of Tet. E, chromosome alignment defects were scored in cells exposed to VX-680 in the presence and absence of Tet. Graphs show the mean ± S.E. (95% confidence interval) from two experiments.

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**Drug-resistant Mitotic Kinases**

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and R136G Plk1 were also detected on and around centrosomes at metaphase after exposure of cells to Tet, confirming efficient subcellular targeting of both kinases (Fig. 8, C and D). In the presence of Tet, all mitotic cells expressing WT Plk1 were monopolar in the presence of 25 nM BI 2536 and contained lower levels of Thr288-phosphorylated Aurora A. In contrast, Tet-mediated expression of R136G Plk1 induced a marked decrease in the number of monopolar spindles, with approximately half reverting to bipolar structures in the presence of the inhibitor compared with WTPlk1-expressing cells (Fig. 8, C–E). In addition, both Aurora A localization and phosphorylation at Thr288 were rescued in the presence of BI 2536 (Fig. 8C).

**DISCUSSION**

**Discovery and Properties of Drug-resistant Aurora Kinases**

In this study, we have exploited structural data to modify the architecture of the ATP-binding site and to evaluate inhibitor resistance toward Aurora A, Aurora B, and Plk1, all of which represent important anticancer drug targets. VX-680 binds efficiently to both inactive (30) and TPX2-bound (31) Aurora A, consistent with its ability to inhibit both the activation and activity of Aurora A kinase (34). Using an ABL kinase activity assay, we demonstrated that VX-680 is also a nanomolar inhibitor of catalytically active ABL kinases, including the T315I mutant found in imatinib-resistant cancer patients (Table 1). In contrast, we found that, under the same conditions, imatinib was a relatively poor ABL inhibitor (Table 1), in line with its increased affinity for a structurally inactive variant of ABL kinase (46). These data help strengthen the assertion that VX-680, or a similar molecule, will be useful therapeutically for multiple kinase targets, including drug-resistant tyrosine kinase mutants (47).

The primary goal of this work was to dissect VX-680 resistance toward the oncogenic Ser/Thr kinase Aurora A. Using a biochemical screen, we identified mutations at a single locus inside the ATP-binding cavity that induced resistance to VX-680 and ZM447439 but remained sensitive to the chemically unrelated clinical compound MLN8054 (Fig. 1). The conserved Gly residue at this site localizes to kinase subdomain V, which links the N- and C-terminal lobes in protein kinases,
forming a hinge region adjacent to hydrophobic pockets containing critical amino acid side chains that are known to act as selectivity filters (2, 29). The equivalent residue is absent entirely in several well characterized kinases such as Cdk2 and Erk2, but amino acids other than Gly are often found in other members of the human kinome, in line with our finding that Aurora A can tolerate a wide range of amino acid substitutions at this position and maintain activity (Table 2). Not surprisingly, introduction of several foreign residues within the Aurora A hydrophobic cleft completely abolished activity; these mutations are likely to change the affinity for ATP or induce structural changes that reposition critical catalytic residues elsewhere. They include non-conservative mutation of amino acids close to and at the gatekeeper residue Leu210, which eliminated Aurora A activity (supplemental Table 1). In addition, not all mutations that preserved activity changed the sensitivity of Aurora A to VX-680, and these include some well tolerated mutations at Thr217, a potential mediator of Aurora kinase inhibition by unrelated compounds (48).

By target hopping, we observed that a similar Gly-to-Leu mutation in Aurora B induced chemical resistance to both VX-680 and MLN8054 (supplemental Fig. 2). Like Aurora A, the Aurora B mutant was active, and the G160L Aurora B mutant also localized correctly to centromeres in metaphase cells. Together, our biochemical and cellular data demonstrate that Aurora A and B drug-resistant mutants are catalytically active when expressed in cells. Resistance is therefore likely to be conferred by precluding inhibitor binding, as originally intended, rather than through changes in subcellular localization, enzyme activity, or substrate preference.

**Cellular Expression of Drug-resistant Aurora Kinases Reverts Phenotype**—We have exploited drug-resistant Aurora A mutants to prove unequivocally that Aurora A is required for centrosome separation and spindle bipolarity in human cells. Moreover, we found that Thr288 phosphorylation is mediated by Aurora A itself rather than a distinct protein kinase because expression of a drug-resistant Aurora A mutant rescued phosphorylation at this site in the presence of the drug. Using a VX-680-resistant Aurora B mutant, we went on to validate that Aurora B is a physiological Ser10 histone H3 kinase because expression of a drug-resistant Aurora B mutant rescued mitotic phosphorylation at this site in the presence of VX-680. Using a similar approach, we proved that chromosome alignment defects induced by VX-680 are also due to Aurora B inhibition and that Aurora B is the critical inhibitory target of VX-680 that prevents proliferation in these cells. These findings verify that VX-680 induces these mitotic phenotypes through the sole inhibition of Aurora kinases and validate Aurora B as the critical target for the anti-proliferative effects of this compound in these human cancer cells. However, additional experiments in distinct cell lines will be needed to demonstrate whether VX-680 inhibition of Aurora B, Aurora A, or both is fundamental for efficacy in cancer cells, given the multifaceted cell cycle and apoptotic signaling networks present in distinct cancer cell types, which might make the prediction of inhibitor responses more complex than in the situation described here. However, in broad agreement with our findings with VX-680-resistant Aurora kinase mutants, selective Aurora B inhibitors such as AZD1152 also exhibit impressive anti-mitotic properties in distinct cancer cell populations (19).
Our findings with Aurora B are corroborated by recent work in which distinct Aurora B mutants were identified in a cellular screen for resistance to the research compound ZM447439. Several of these mutants also conferred partial resistance to the unrelated Aurora inhibitors Hesperadin and VX-680 (49). Taken together, these studies point toward a requirement for specific residues in the hinge region of the ATP-binding site to impart Aurora B sensitivity to chemically unrelated compounds, including VX-680, ZM447439, Hesperadin, and MLN8054. Our data also imply that MLN8054 interacts with Aurora B (but not Aurora A) through a mechanism that relies on the presence of a specific Gly residue because the VX-680-resistant G216L Aurora A mutant was still potently inhibited by MLN8054 (Figs. 1G and 3C). A co-crystal structure of an Aurora kinase with MLN8054 will be needed to explain the molecular basis for this finding, although we have already demonstrated that chemically distinct inhibitors such as MLN8054 can be exploited to target the activity of VX-680-resistant Aurora A in cells. In the future, it will also be important to assess the sensitivity of the clinical compound AZD1152 (which is chemically related to ZM447439) toward drug-resistant Aurora B mutants. Our study also raises the interesting possibility that mutation of Gly126 in human Aurora C will also induce resistance to Aurora kinase inhibitors. In the absence of specific Aurora C inhibitors, chemical genetic approaches such as those described here might permit mechanistically validated compounds to be used as surrogates for investigating the cellular roles of this kinase.

Analysis of a BI 2536-resistant Plk1 Mutant—By exploiting a recent co-crystal structure of Plk1 and BI 2536, we found that mutation of Arg136 to Gly did not abolish Plk1 activity, but altered the sensitivity of the kinase in a compound-specific
manner, proving that this residue is important for the affinity of BI 2536 in the Plk1 ATP-binding pocket. It will be highly informative to investigate whether the introduction of an Arg residue at the equivalent position in Plk4 (Gly596) confers BI 2536 sensitivity and/or creates VX-680 resistance. However, the complementary introduction of an Arg side chain at this position in Aurora A led to enzyme inactivation in vitro (Table 2), suggesting that the ability to tolerate particular amino acid side chains at this position will depend on other kinase-specific amino acids elsewhere in the ATP-binding site. Whether this residue is diagnostic for sensitivity or resistance to these compounds or whether this is restricted to the cases discussed in this work can only be reconciled when all members of the kinase have been assayed in the presence of these inhibitors.

**Cellular Effects of BI 2536-resistant Plk1 Expression**—In this study, we found that the 3–4-fold loss of in vitro sensitivity to BI 2536 identified in the R136G Plk1 mutant was sufficient to generate a window of resistance in human cells expressing this protein. This allowed us to demonstrate unequivocally that Plk1 is the "trigger kinase" targeted by BI 2536 that mediates the phosphorylation and decreased electrophoretic mobility of mitotic CDC25C, as suggested using other techniques (50). Plk1 activity has recently been demonstrated to be important for targeting Aurora A to centrosomes (23, 42, 43). Using chemical genetics, we have now proven that Aurora A activation on the centrosome occurs through an Aurora A-dependent autophosphorylation mechanism (Figs. 3 and 4), which does indeed lie downstream of Plk1 (Figs. 7 and 8). Interestingly, BI 2536 was a more comprehensive inhibitor of Aurora A Thr288 phosphorylation than either VX-680 or MLN8054 as assessed by immunoblotting (compare Figs. 3 and 7) and immunofluorescence (compare Figs. 4 and 8), even though it exhibited no direct effect on Aurora A activity (supplemental Fig. 4). This finding highlights the fundamental role of Plk1 in regulating both Aurora A localization and activity. It will be important to evaluate whether regulation is due to Aurora A targeting by a phosphorylated Plk1 substrate or whether it might involve direct Plk1-mediated regulation of Aurora A autoactivation, perhaps through the common intermediary protein Bora (26). Plk1 and Aurora kinases have been independently validated as drug targets, but given the importance of Plk1 in controlling Aurora A in mitotic cells (Figs. 7 and 8), we suggest that the efficacy of Plk1 inhibitors might therefore actually be due to dual pharmacological intervention in both the Polo and Aurora A signaling pathways.

The monopolar phenotype induced by BI 2536 was only partially rescued by R136G Plk1 overexpression (Fig. 8), suggesting only partial Plk1 dependence of this phenotype in these cells. It is possible that expression of a Plk1 mutant exhibiting improved BI 2536 resistance might lead to more complete reversion of the phenotype in the presence of the drug; alternatively, it is feasible that other BI 2536 targets such as Plk2/3 might contribute to this process. In contrast, our finding that Aurora A is the sole target of VX-680 that induces spindle monopolarity (Fig. 4) suggests that other putative VX-680 targets that might be important for centrosome biogenesis such as Plk4 (51, 52) are unlikely to be rate-limiting for this process in human cells.

It is remarkable that drug resistance toward VX-680 and especially BI 2536 was achieved in stable cell lines expressing low levels of desensitized, but not completely resistant, Aurora or Polo kinases in the presence of the endogenous enzymes. This demonstrates that even small decreases in drug sensitivity might be sufficient for cells to survive in the presence of antimitotic kinase inhibitors. It is likely that an enhanced ability to progress through mitosis presents these cells with a significant growth advantage in the presence of these agents, perhaps mirroring the situation in inhibitor-exposed cancer patients. It will therefore be important to assess the long-term effects of drug-resistant kinase expression in model systems and to determine whether cell survival is influenced by other factors in addition to kinase resistance, such as changes in p53 status or induction of multidrug resistance genes (53, 54).

**Drug-resistant Mutants as Pharmacological Tools**—We now understand the basic mechanistic requirements for generating drug resistance or sensitivity toward prototypic members of the human kinome (29). These include oncogenic tyrosine kinases (55), MAPK homologs (56, 57), and the oncogenic phosphatidylinositols 3-kinase α-isomers (58). In most (but not all) of these cases, inhibitor resistance can be readily generated (or sensitivity imparted) by introduction of specific side chains at or near the gatekeeper residue, thus controlling access to molecular space in the hydrophobic pocket. This knowledge has recently been exploited to generate knock-in mice harboring a drug-resistant variant of p38α (59) or an inhibitor-sensitized version of JNK1/2 (60), allowing a thorough phenotypic analysis of these enzymes and their inhibitors to be performed in living organisms.

Recent efforts at understanding clinical drug resistance mechanisms have been dominated by studies with tyrosine kinases, including targets of the anticancer agent imatinib (2, 3). By applying selective pressure upon cell populations with kinase inhibitors, a broad spectrum of drug resistance alleles have been identified in cell culture models, many of which have also subsequently been found in drug-resistant patient populations (61, 62). Interestingly, mutations highly analogous to the VX-680-desensitizing Gly mutations described in this study have already been characterized in the ATP-binding site of imatinib-resistant BCR-ABL proteins (62), and it will be important to assess the effects of VX-680 on this and other tyrosine kinases such as mutants of Flt3.

By further expanding and exploiting our panel of Aurora A, Aurora B, and Plk1 drug-resistant mutants and cell lines, it should be trivial to test the inhibitory effects of many kinase inhibitors to identify chemical prototypes whose binding mode does not rely on the presence of Gly or Arg in the hinge region. Simple transfection of drug-resistant Aurora A, Aurora B, or Plk1 mutants should also allow substrates of Aurora or Polo kinases discovered using VX-680 or BI 2536 to be validated, as is routine with putative p38 MAPK targets identified with pyrrolidinylimidazole inhibitors (63). Our novel cell lines will also be useful for investigating the reverse regulation of Plk1 by Aurora A, which has recently been demonstrated during the G2/M transition in cultured human cells (24, 25).

The next logical next step to help understand cellular drug resistance toward VX-680 and BI-2536 is the transfer of bio-
chemical knowledge to whole animal model systems. This should be readily achievable by exploiting the specific mutations described here alongside knock-in strategies in mice. By investigating specificity and resistance mechanisms toward VX-680, BI 2536, and their homologs in cancer models, we will be in a strengthened position to deploy second-line inhibitor therapies should clinical resistance occur in the future.

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