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Molecular Basis of Drug Resistance in Aurora Kinases

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SUMMARY

Aurora kinases have emerged as potential targets in cancer therapy, and several drugs are currently undergoing preclinical and clinical validation. Whether clinical resistance to these drugs can arise is unclear. We exploited a hypermutagenic cancer cell line to select mutations conferring resistance to a well-studied Aurora inhibitor, ZM447439. All resistant clones contained dominant point mutations in *Aurora B*. Three mutations map to residues in the ATP-binding pocket that are distinct from the "gatekeeper" residue. The mutants retain wild-type catalytic activity and were resistant to all of the Aurora inhibitors tested. Our studies predict that drug-resistant Aurora B mutants are likely to arise during clinical treatment. Furthermore, because the plasticity of the ATP-binding pocket renders Aurora B insensitive to multiple inhibitors, our observations indicate that the drug-resistant Aurora B mutants should be exploited as novel drug targets.

INTRODUCTION

The Auroras are serine/threonine kinases required for multiple aspects of mitosis in eukaryotic cells. Aurora A, the "polar-kinase," promotes centrosome maturation and spindle assembly (Barr and Gergely, 2007). Aurora B, the "equatorial-kinase," is required for Histone H3 phosphorylation, chromosome biorientation, the spindle assembly checkpoint, and cytokinesis (Ruchaud et al., 2007).

After the discovery that they are often deregulated in cancer, the Aurora kinases have attracted considerable attention as potential targets for cancer chemotherapy. Several Aurora inhibitors have been described, including dual Aurora A/B inhibitors such as VX-680 and PHA-680632; selective Aurora B inhibitors such as Hesperadin, ZM447439, and AZD1152; and a selective Aurora A inhibitor, MLN8054 (reviewed by Taylor and Peters [2008]). The emerging picture is that these agents have potent antiproliferative effects, inducing apoptosis in human tumor cell lines. Importantly, VX-680, PHA-680632, AZD1152, and MLN8054 have antitumor activity in rodent xenograft models (Harrington et al., 2004; Soncini et al., 2006; Manfredi et al., 2007; Wilkinson et al., 2007). Phase I and II clinical trails are underway, but results are not yet in the public domain.

The enthusiasm for targeting cell-cycle kinases in cancer has been fuelled by the success of BCR-ABL inhibitors such as imatinib in the treatment of chronic myeloid leukemia (Sherbenou and Druker, 2007). However, a sobering lesson has also emerged: clinical resistance can arise rapidly due to mutations in the Abl kinase domain that prevent inhibitor binding (Gorre et al., 2001; Shah et al., 2002). To circumvent imatinib resistance, second-generation inhibitors with distinct modes of action are being used; dasatinib and nilotinib were selected on the basis that they should inhibit imatinib-resistant BCR-ABL mutants (Shah et al., 2004; Weisberg et al., 2005). Importantly, these inhibitors have been used successfully to treat imatinib-resistant patients (Kantarjian et al., 2006; Talpaz et al., 2006). However, sequential treatment can yield subclones with compound mutations, thus rendering patients resistant to multiple inhibitors (Shah et al., 2007). This finding argues that in order to minimize resistance to selective kinase inhibitors, multiple agents targeting a broad range of mutations will be required, analogous to the use of cocktails to treat HIV (Sawyers, 2005).

The BCR-ABL experience illustrates how a protein kinase can be effectively targeted, how resistance can emerge, and how rational combinatorial strategies can overcome resistance (Daub et al., 2004; Sherbenou and Druker, 2007). Over 50 mutations in the Abl kinase domain have been implicated in imatinib resistance (Daub et al., 2004; Weisberg et al., 2007). The fact that these amino acid substitutions do not impair the kinase's catalytic activity indicates that Abl is a remarkably plastic enzyme. However, receptor tyrosine kinases such as Abl only constitute a fraction of the kinome (Manning et al., 2002). Whether other classes of protein kinase of therapeutic interest have the same extensive capacity for drug resistance is unclear. In this respect, it is important to develop tools to predict the type and the effects of mutations that might arise during clinical evaluation.

Toward this end, we developed what is to our knowledge a new assay to isolate cell lines resistant to Aurora inhibitors and used the ZM447439 inhibitor as our benchmark. Sequencing *Aurora* cDNAs identified four individual point mutations that are sufficient to render Aurora B resistant to ZM447439. Three mutations are in the active site, namely, Y156H, G160E, and G160V, whereas the fourth, H250Y, is near the activation loop. The mutations in the active site also confer resistance to VX-680, MLN8054, and Hesperadin, indicating that these agents share common modes of action. Thus, our observations suggest that

it is mechanistically possible for resistance to emerge against cytotoxic agents targeting serine/threonine kinases. Furthermore, the mutations confer resistance against a broad spectrum of inhibitors representing different chemical classes, thus revealing the challenges lying ahead in drug design. We suggest that in order to tackle drug resistance, the mutants described here should be considered as de novo drug targets. And, finally, we demonstrate that the drug-resistant Aurora B mutants are powerful tools to delineate the on- and off-target effects of Aurora inhibitors, demonstrating an important proof of principle applicable to other protein kinase inhibitors.

RESULTS

An Assay to Identify ZM447439-Resistant Cell Lines: Identification of Aurora B Mutations

To determine whether human cancer cells can develop resistance to Aurora inhibitors, we treated HCT-116 cells with a cytotoxic concentration of ZM447439, a selective Aurora B inhibitor (Girdler et al., 2006). We chose HCT-116 cells because they are hypermutagenic due to a mismatch repair defect (Glaab and Tindall, 1997). We therefore suspected that these cells might contain larger numbers of initial variants for selection under the conditions of our assay. In addition, HCT-116 cells express little or no drug transporters, reducing the possibility of resistance due to induction of drug pumps (Teraishi et al., 2005). At 1 μM, ZM447439 is potently cytotoxic in colony formation assays with ~200 cells (Figure 1A). However, when ~5 × 10^6 cells were continuously exposed for 3 weeks, ~20 colonies appeared, from which we generated 7 cell lines, designated R1–R7. In colony formation and proliferation assays, 1 μM ZM447439 had no effect on these lines, demonstrating that they are indeed drug resistant (Figures 1A and 1B). In control cells, ZM447439 inhibits cell division and suppresses Histone H3(Ser10) phosphorylation (Ditchfield et al., 2003). However, cell division and H3(Ser10) phosphorylation were unaffected by ZM447439 in lines R1 and R2 (Figures 1C and 1D), indicating that Aurora B is still active in the drug-resistant lines despite the presence of ZM447439. After the BCR-ABL experience, we asked whether this might be due to mutations in Aurora B rendering it resistant to the inhibitor. Sequencing Aurora cDNAs from the drug-resistant clones revealed that all seven lines harbored point mutations in *Aurora B*, yielding five amino acid substitutions, namely, Y156H, G160E, G160V, H250Y, and L308P (Figures 1E and 1F). Three lines harbored two mutations, namely, H250Y in combination with either G160V (R3 and R4) or G160E (R6). To determine whether these mutations were in the same allele of *Aurora B*, we subcloned and sequenced individual cDNAs. Each cDNA contained one mutation or the other, but not both, indicating that the two mutations are in separate alleles. Note that these lines were also resistant to other compounds related to ZM447329, namely, ZM2, ZM3 (Girdler et al., 2006), and AZD1152 (Wilkinson et al., 2007) (data not shown).

Ectopic Expression of Aurora B Mutants Restores Histone H3(Ser10) Phosphorylation

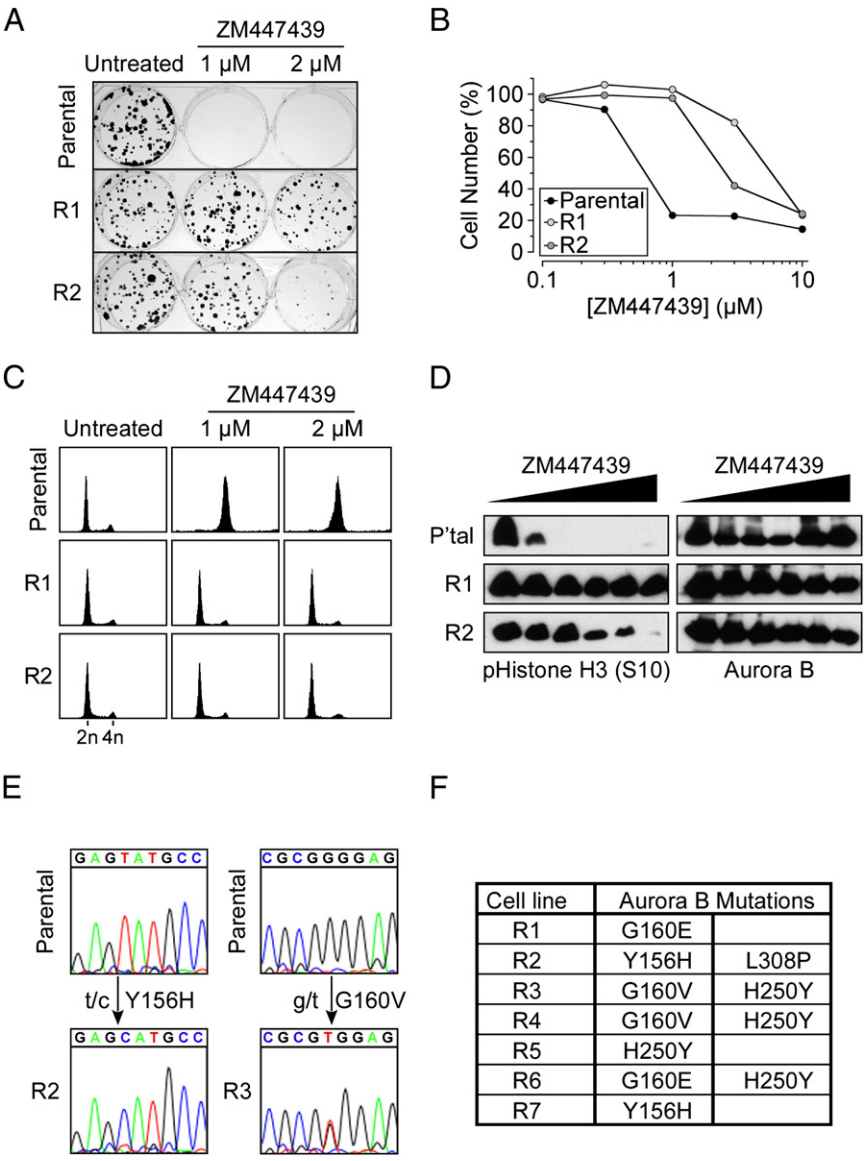
To test whether the Aurora B mutations are sufficient to cause drug resistance, we ectopically expressed the mutants as Myc-tagged fusions in DLD-1 cells (Figure 2A). Importantly, the Myc-tagged Aurora B proteins localized to mitotic centromeres (Figure 2B) and were catalytically active (Figure 2C), demonstrating that they are functional kinases. To determine whether the mutants could restore Aurora B activity upon exposure to ZM447439, we counted the number of mitotic cells positive for H3(Ser10) phosphorylation. Although induction of wild-type Aurora B had no effect, ectopic expression of the Y156H, G160V, and H250Y mutants clearly restored Aurora B activity (Figure 2D). The effect of the G160V mutant was particularly penetrant, with >75% of the cells staining positive at 4 μM ZM447439. The Y156H and H250Y mutants were less effective, restoring the number of phospho-H3(Ser10)-positive cells in 2 μM ZM447439 to 80% and 45%, respectively. For reasons that are not clear, Aurora B G160E expressed poorly and is therefore not characterized further in a cellular context; we do, however, demonstrate that this mutant is drug resistant in vitro (see below). In addition, L308P did not appear to confer resistance and is therefore not discussed further.

Drug-Resistant Mutants Rescue Aurora B's Cell-Cycle Functions

ZM447439 prevents chromosome alignment, compromises the spindle checkpoint, and blocks cell division, yielding a potent cytotoxic effect (Ditchfield et al., 2003). If these phenotypes are due to Aurora B inhibition, as opposed to an off-target effect, then they should be reverted by ectopic expression of the drug-resistant mutants. To test this, we first counted the number of metaphase configurations in MG132-treated cells. Whereas ZM447439 reduced the proportion of metaphases from ~77% to ~24% in controls (Figure 3A), induction of the G160V mutant restored chromosome alignment, with 48% of cells reaching metaphase. Next, we analyzed the spindle checkpoint; whereas overexpressing wild-type Aurora B had no effect on the ability of ZM447439 to override a taxol-induced mitotic arrest, inducing the Y156H and G160V mutants substantially restored spindle-checkpoint function (Figure 3B). Finally, we analyzed cell division; whereas ZM447439 induced cell-division failure and endoreduplication in controls, induction of Aurora B G160V restored a near-normal DNA-content profile (Figure 3C). Quantitating cells with DNA contents > 4n showed that Aurora B G160V limited endoreduplication even at higher concentrations of ZM447439 (Figure 3C). Induction of Aurora B Y156H and H250Y also reduced endoreduplication in the presence of ZM447439. These observations therefore provide compelling evidence that the cell-cycle defects induced by ZM447439 are indeed due to inhibition of Aurora B. To determine whether ZM447439's cytotoxicity is also due to Aurora B inhibition, we performed colony formation assays. A total of 2 μM ZM447439 typically reduces the number of DLD-1 colonies to <10%. Whereas induction of wild-type Aurora B had no effect, induction of the G160V, Y156H, and H250Y mutants restored colony numbers to 70%, 50%, and 40% respectively (Figure 3D), indicating that the Aurora B mutants do indeed confer cytoprotection against ZM447439.

In Vitro Activity of Aurora B Mutants

To determine the effects of the mutations on Aurora B's enzymatic activity, we purified to homogeneity from bacteria a complex of human Aurora B bound to an activating fragment of



Cell line	Aurora B Mutations	
R1	G160E	
R2	Y156H	L308P
R3	G160V	H250Y
R4	G160V	H250Y
R5	H250Y	
R6	G160E	H250Y
R7	Y156H	

Figure 1. Isolation of ZM447439-Resistant Cancer Cells

(A) Crystal violet-stained colonies of parental HCT-116 cells and two drug-resistant lines after 14 days of exposure to ZM447439.

(B) Proliferation assay showing cell number after exposure to increasing concentrations of ZM447439, plotted as a percentage of untreated cells.

(C) DNA-content profiles 24 hr after drug exposure.

(D) Western blots probed to detect phospho-histone H3 (Ser10) and Aurora B 24 hr after exposure to 0–9 μM ZM447439.

(E) DNA sequences of Aurora B cDNAs in parental and two drug-resistant lines.

(F) Amino acid substitutions identified in Aurora B cDNAs.

human INCENP (Figure 4A). In vitro kinase assays in which Histone H3 was used as a substrate demonstrated that the mutants were as active as the wild-type complex (Figure 4B). In response to increasing concentrations of ZM447439, wild-type Aurora B was inhibited to background levels at ZM447439 concentrations in the 1–3 μM range (Figure 4C). Whereas the H250Y mutation only had a mild effect, the Y156H mutation had a pronounced effect, with an ~10-fold reduction of drug efficacy. Strikingly, the G160V and G160E mutations generated an enzyme completely insensitive to ZM447439, even at concentrations up to 500 μM (Figure 4D).

Next, we asked if the Aurora B mutants conferred resistance toward other Aurora inhibitors. The Y156H mutant conferred very strong resistance to VX-680, with an ~50-fold reduction in drug efficacy (Figure 4E). The effects on Hesperadin were slightly weaker than those observed with ZM447439 (Figure 4F). As with ZM447439, the H250Y mutant had a much milder effect on VX-680 and Hesperadin, whereas both the G160V and G160E mutants were strongly resistant to VX-680 and Hesperadin (Figures 4E and 4F).

Mechanisms of Drug Resistance

To determine how the various mutations render Aurora B drug resistant, we soaked crystals of the *Xenopus laevis* (Xl) Aurora B:INCENP complex (Sessa et al., 2005) with ZM447439 and collected diffraction data to 1.85 Å resolution (Table 1 and see Protein Data Bank [PDB] code: 2VRX). ZM447439 occupies the deep ATP-binding cleft at the interface between the small and the large lobes of the kinase (Figure 5B), and its binding does not result in significant conformational changes relative to the unbound kinase, which crystallizes in a partially active state (Sessa et al., 2005).

Y156 (F172 in *Xenopus*) maps to the hinge loop connecting the small and large lobes and is located in the proximity of prominent aromatic moieties in ZM447439 (Figure 5C). Altering this residue may weaken van der Waals contacts with the inhibitor. The most effective resistance-conferring mutations are those substituting G160, which also maps to the hinge loop, with bulkier residues (Figures 2–4). The structural basis for this is immediately evident from the structure: the morpholino-propoxy moiety of ZM447439 extends over the hinge loop (Figure 5D), and the substitution of G160 (G176 in *Xenopus*) is expected to create direct steric hindrance (Figure 5E), without interfering with ATP binding (Figure 5F). Y156 and G160 are also implicated in the binding of VX-680 and Hesperadin (Figure 4). Although they represent different chemical classes, these inhibitors have chemical groups that are equivalent to the morpholino-propoxy moiety of ZM447439 and that interact with the same region of Aurora B (Sessa et al., 2005; Cheetham et al., 2007). Thus, the similar modes of binding explain why all three inhibitors are affected by the G160V/E mutations.

The third residue, H250 (equivalent to H266 in *Xenopus*), is located just under the activation loop. Although this mutation may

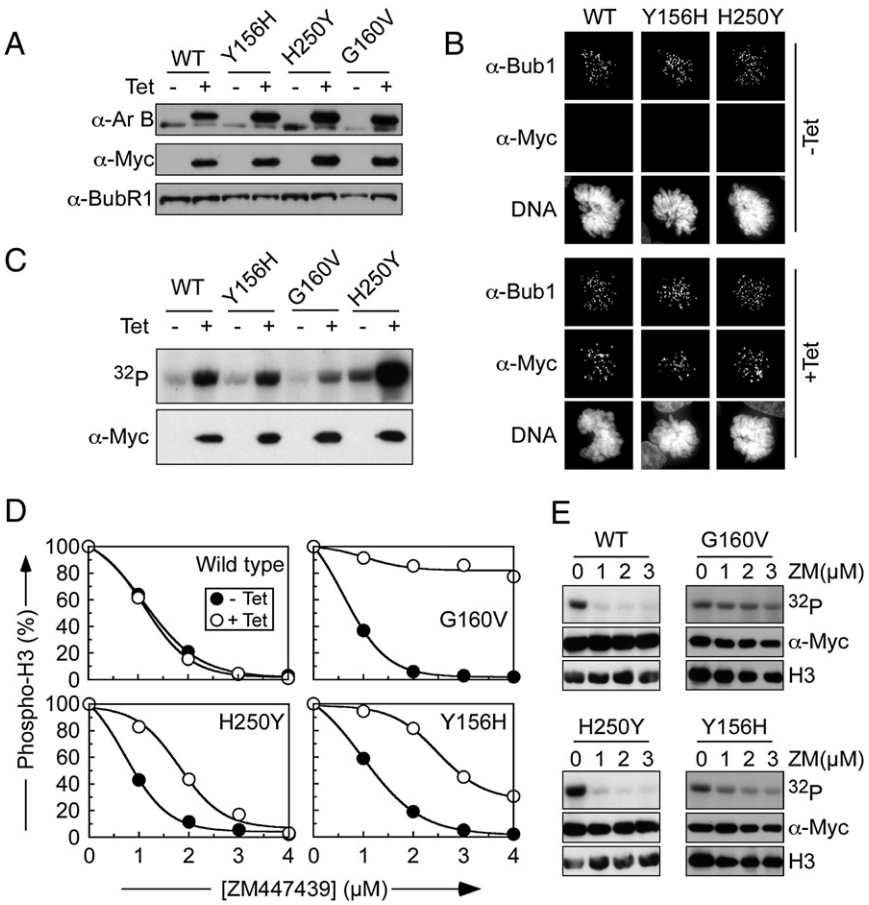


Figure 2. Aurora B Mutants Restore Histone H3(Ser10) Phosphorylation

DLD-1 transgenic lines were induced with tetracycline, then analyzed.

(A) Immunoblot showing induction of Myc-tagged Aurora B proteins.

(B) Immunofluorescence images showing centromeric localization of exogenous Aurora B proteins.

(C) Immunoprecipitation kinase assays showing that the Aurora B mutants are catalytically active.

(D) Line graphs quantitating the percentage of mitotic cells positive for phospho-histone H3 (Ser10) in the presence of ZM447449.

(E) Kinase assays showing that the G160V and Y165H mutants are active in the presence of up to 3 μM ZM447439.

affect the conformation of the enzyme, and thus indirectly affect drug binding in the active site, the H250Y protein demonstrated only marginal resistance toward the Aurora inhibitors in vitro (Figure 4). However, when we assayed the kinase activity of the Aurora B mutants immunoprecipitated from cells, Aurora B H250Y appeared to be hyperactive; even in the uninduced sample, the small amounts of protein due to leaky expression resulted in substantial activity (Figure 2C). Thus, whereas the Y156H and G160V mutants appear to be genuinely drug resistant, the H250Y mutant may confer cellular resistance by hyperactivating the catalytic activity of the kinase.

Aurora B Active Site Mutants Confer Resistance to Multiple Aurora Inhibitors

Having established that the Y156H and G160V mutants also render Aurora B resistant to VX-680 and Hesperadin in vitro (Figure 4), we asked whether these mutants could be used to delineate on- and off-target effects induced by Aurora inhibitors. It has been suggested that VX-680 is a dual Aurora inhibitor (Harrington et al., 2004; Tyler et al., 2007). Thus, the cell-cycle and cytotoxic effects of VX-680 could be due to inhibition of Aurora A, Aurora B, or both. Induction of Aurora B Y156H restored a normal cell-cycle profile in VX-680-treated cells (Figure 6A). This demonstrates that the cell-division failure caused by VX-680 must be predominantly due to Aurora B inhibition. Furthermore, induction of the Y156H mutant restored colony formation, confirming that the cytotoxic effects of VX-680 are also due predominantly to Aurora B inhibition (Figure 6B). Thus, our studies provide very strong evidence that Aurora B is the relevant in vivo target for the cytotoxic effects of VX-680, and these findings illustrate the formidable potential of drug-resistant mutants in the validation of the mechanisms of drug toxicity.

Based on the in vitro kinase assays (Figure 4), we anticipated that the Y156H and G160V/E mutants would render cells resistant to Hesperadin. Surprisingly, under conditions in which induction of Aurora B G160V conferred penetrant resistance to 2 μM ZM447439, each mutant only conferred limited resistance to 50 nM Hesperadin, as judged by the ability to rescue the drug-induced cell-division failure (Figure 6C). These observations strongly suggest that, in addition to Aurora B, Hesperadin may have another cellular target that is required for cell division.

Finally, we asked whether the mutants conferred resistance to MLN8054. Although MLN8054 is selective for Aurora A at submicromolar doses, above 2 μM it inhibits Aurora B (Manfredi et al., 2007). Consistently, 5 μM MLN8054 prevented DLD-1 cells from dividing, as judged by the loss of cells with 2n DNA contents (Figure 6D). However, induction of Aurora B Y156H restored the 2n population, demonstrating the restoration of Aurora B activity (Figure 6D). Similarly, the G160V mutant also reverted the high-dose effects of MLN8054. Thus, the two mutations in the catalytic cleft, Y156H and G160V, render Aurora B resistant to four classes of inhibitor, namely, ZM447439, VX680, Hesperadin, and MLN8054.

Interestingly, although ectopic expression of Aurora B Y156H in DLD-1 cells reverted the high-dose cell-cycle effects of MLN8045 (Figure 6D), it did not restore cloning potential (data not shown). Consistently, MLN8054 was equally efficient at inhibiting colony formation in parental HCT116 and R2, the line that harbors the Y156H mutation (Figure 6E). This suggests, therefore, that the cytotoxicity of MLN8054 is not mediated via Aurora B, but rather via another kinase, most likely Aurora A. However, if selective inhibition of Aurora A is cytotoxic, then why does VX-680 not kill cells expressing the drug-resistant

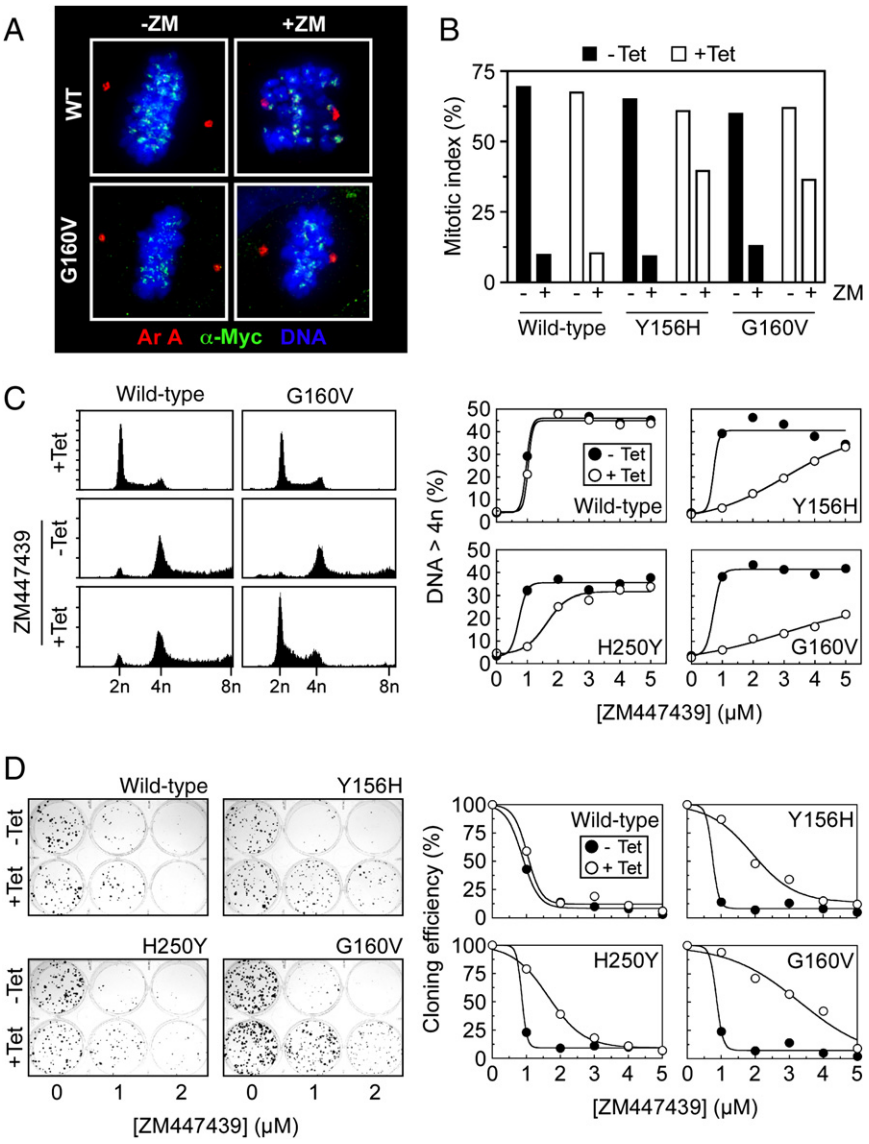


Figure 3. Drug-Resistant Mutants Rescue Aurora B Function

DLD-1 transgenic lines were induced, exposed to ZM447439, then analyzed.

(A) Immunofluorescence images showing that induction of Aurora B G160V facilitates chromosome alignment despite the presence of ZM447439.

(B) Bar graph quantitating the mitotic index of taxol-treated populations showing that Y156H and G160V restore spindle-checkpoint function in the presence of ZM447439.

(C) DNA-content histograms after 24 hr drug exposures showing that Aurora B G160V suppresses endoreduplication. Line graphs quantitate the percentage of cells with DNA contents > 4n.

(D) Crystal violet-stained colonies and line graphs quantitating colony number after 14 days of exposure to ZM447439.

Aurora B mutants (Figure 6B)? We suspect that this is because the extent of Aurora A inhibition at 200 nM VX-680 is not sufficient to cause cell death, whereas at 1 μM, MLN8054 does inhibit Aurora A enough to suppress viability.

DISCUSSION

A sobering lesson to emerge after implementation of the new-generation targeted chemotherapeutics is the rapid emergence of clinical resistance (Daub et al., 2004; Sherbenou and Druker, 2007). Because drug-resistant subclones are likely to exist prior to treatment (Roche-Lestienne et al., 2002), combination therapy with multiple agents with differing modes of action will therefore be required to circumvent resistance (Carter et al., 2005). The rational design of appropriate combinatorial approaches will however require an a priori understanding of a drug's mode of action and the mechanisms by which resistance may occur. Here, we show that point mutations that result in single amino acid substitutions in the Aurora B kinase domain can render cancer cells resistant to multiple Aurora kinase inhibitors. As the same mutations render Aurora B resistant to ZM compounds, VX-680, Hesperadin, and MLN-8054, our data indicate that these agents share common modes of action and would therefore not make suitable combinations. Therefore, in order to fully exploit the Aurora kinases as oncology targets, second-generation inhibitors with differing modes of action will be required. Specifically, drugs that inhibit the Aurora B mutants described here will be necessary.

A Genetic Screen to Identify Drug-Resistant Aurora B Alleles

The genetic screen described here yielded seven HCT-116 subclones resistant to the ZM series of Aurora inhibitors, all of which harbored Y156H, G160V/E, or H250Y mutations in *Aurora B*. The fact that transgenic expression of these alleles in DLD-1 cells is sufficient to render them resistant to ZM447439 provides compelling evidence that the drug resistance in the HCT-116 cells is due to these mutations. Thus, the genetic screen itself demonstrates that cancer cells can acquire resistance to an Aurora kinase inhibitor, at least one that is relatively selective for Aurora B. If selective Aurora B inhibitors prove to have antitumor activity in patients when used as monotherapy agents, these observations therefore demonstrate that the emergence of clinical resistance is mechanistically possible and thus highly likely.

Although we identified each drug-resistant allele more than once, whether the screen was saturating remains to be seen; it may be possible to identify additional ZM447439-resistant Aurora B mutants. Would similar screens be useful to identify *Aurora* alleles resistant to other inhibitors? The fact that all of the revertants harbored drug-resistant Aurora B alleles provides compelling evidence that the cytoxicity induced by 1 μM

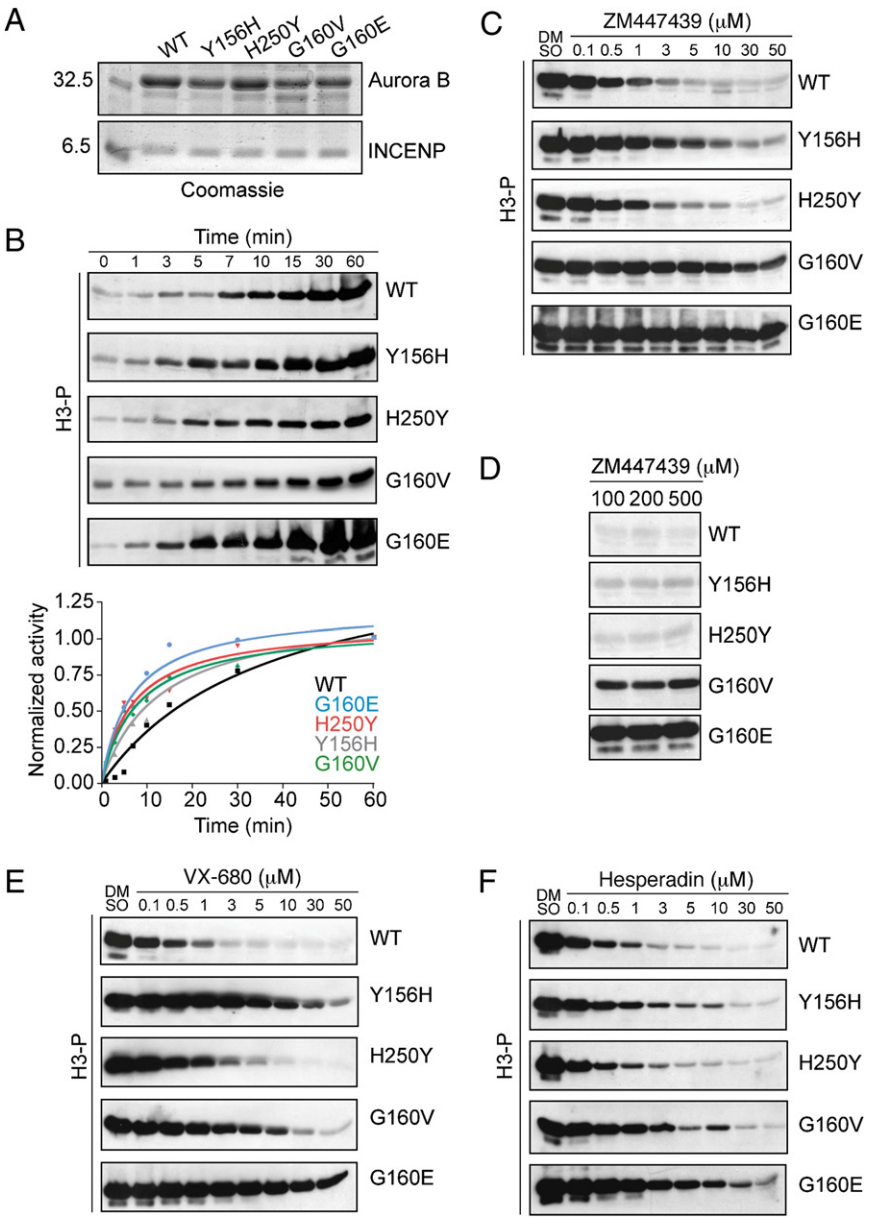


Figure 4. In Vitro Activity of Aurora B Mutants

(A) Wild-type human Aurora B$^{45–344}$:INCENP$^{835–903}$ and mutants utilized for the kinase assays.

(B) Time course experiment comparing the activity of the wild-type and mutant kinases, plus quantification.

(C) Wild-type and mutant kinases were incubated with ZM447439 as indicated, and the reaction was protracted for 15 min. The DMSO solvent control (3% v/v) is indicated.

(D) ZM447439 concentrations as high as 500 μM failed to inhibit the G160V and G160E mutants.

(E and F) (E) The same experiment as described in (C) was carried out with VX-680 as an inhibitor or (F) with Hesperadin.

ZM447439 is mediated exclusively via Aurora B inhibition. Indeed, we suspect that this screen was fruitful because Aurora B is the only significant target of ZM447439 at this particular concentration. If the cytotoxicity was mediated via two or more targets, survival would require drug-resistant mutations in two or more genes, the probability of which is much less likely. To test this, our selection strategy could be adapted to investigate the efficacy of combination therapy with multiple inhibitors, for instance by testing whether mutants are selected when multiple drugs are combined. Experiments in this direction are underway.

Crossresistance Demonstrates Common Modes of Action

Based on the BCR-ABL paradigm, drug resistance can result from substitutions of a given residue in the catalytic cleft with a bulkier one that occludes inhibitor access (Gorre et al., 2001). Conversely, resistance can arise from mutations that change a bulky residue in direct contact with the inhibitor into a smaller one, thereby resulting in the loss of essential van der Waals contacts (Burgess et al., 2005). In other cases, the mutated residue may not be in direct contact with the inhibitor but may change the conformation of the kinase, thus affecting inhibitor binding indirectly. Examining the crystal structure of *Xenopus* Aurora B bound to ZM447439 indicates that substituting glycine 160 with bulkier valine or glutamic acid probably occludes inhibitor binding. The Y156H substitution, also affecting a residue in the catalytic cleft, confers resistance to high drug concentrations both in vitro and in cells, albeit not to the same extent as G160V/E. Our structural analysis suggests that resistance caused by mutations at this site might arise due to the loss of productive van der Waals contacts with the inhibitor, although we cannot exclude that steric hindrance also plays a role.

The third amino acid identified, histidine 250, is distal to the inhibitor-binding site, adjacent to the T loop of Aurora B. H250Y might alter Aurora B's conformation, thereby altering inhibitor binding. Alternatively, in light of its proximity to the T loop, this mutation might enhance the activity of the kinase such that elevated inhibitor concentrations are required to suppress Aurora B function. Whereas the in vitro assays indicate that H250Y does not appear to hyperactivate the recombinant Aurora B:INCENP complex, the activity associated with H250Y immunoprecipitates is higher than with the wild-type enzyme. Thus, the activating effect of H250Y may require some element of Aurora B's cellular context that is not recapitulated by the binding to the INCENP IN-box in vitro.

General Insights into Kinase-Inhibitor Interactions

The rather surprising picture emerging from our studies and from previous studies on Abl and other tyrosine kinases is that the

Table 1. Data Collection and Refinement Statistics

	AuroraB$^{60–361}$:INCENP$^{790–847}$: ZM447439
Data Collection	
Space group	$P2_1$
Beamline	ID14-3
Unit cell dimensions (Å)	45.735, 67.004, 116.583
Unit cell angles (°)	96.93
Resolution (Å)	30.0–1.85 (1.92–1.85)
Total observations	523,418
Unique reflections	59,143
Data completeness (%)	92.7
R_{symm} (%)[a]	4.2 (33.3)[b]
I/σI	19.19 (2.68)[b]
Refinement	
Resolution range (Å)	30.0–1.85
R_{conv}[c]/R_{free}[d]	18.81/24.32
Number of atoms in refinement	5,911
Rmsd bond lengths (Å)	0.008
Rmsd bond angles (°)	1.160
Mean B factor (Å^2)	26.25

[a] $R_{symm} = \sum|I - \langle I\rangle|/\sum I$, where I is the observed intensity of a reflection and <I> is the average intensity obtained from multiple observations of symmetry-related reflections.
[b] Values in parentheses refer to the outer-resolution shell.
[c] $R_{conv} = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.
[d] R_{free} is equivalent to R_{conv} for a 5% subset of reflections not used in the refinement.

kinase scaffold is very tolerant of mutations in the hinge loop that lines the ATP-binding site. A discouraging consequence of this fact is that these mutations are likely to affect a wide range of ATP-competitive inhibitors—even ones from distinct chemical classes—as most ATP competitors are sensitive to the active site's architecture, to which the mutated residues contribute considerably. Further studies with a wider collection of inhibitors will be required to analyze this problem in greater detail. However, we suspect that mutations in residues such as Y156 and G160 of Aurora B could have more generalized effects than those caused by mutations in the "gatekeeper" residue. The latter lies at the periphery of the ATP-binding site and only contributes to the binding of a subset of extended compounds, such as imatinib in the case of Abl. Mutations in residues such as Y156 and G160, which literally line the ATP-binding pocket, are likely to affect the binding of any ATP-competitive inhibitor that targets Aurora B's active site. Indeed, a G321E mutation, which is equivalent to the G160E mutation we report, was initially predicted by an in vitro screen on the Abl kinase, and it was later found to be present in patients who relapsed after imatinib treatment of chronic myelogenous leukemia (CML) (Chu et al., 2005). Abl mutations at F317, which is equivalent to Y156 in Aurora B, to L or I (F317L/I) occur with good frequency during treatment with imatinib, although not at the same frequency of mutations in the gatekeeper residue (Melo and Chuah, 2007; Weisberg et al., 2007).

Prospects for Combination Therapy

Positive responses have been reported in imatinib-resistant CML patients treated with the second-generation BCR-ABL inhibitors dasatinib and nilotinib (Kantarjian et al., 2006; Talpaz et al., 2006). However, sequential treatment can result in the expansion of subclones with compound mutations rendering the patient resistant to multiple inhibitors (Shah et al., 2007). This finding argues that in order to minimize resistance, a cocktail of agents that together inhibit a broad range of mutations will be required, which is analogous to the use of cocktails to treat HIV (Sawyers, 2005). What are the prospects for identifying second-generation Aurora inhibitors suitable for simultaneous combinatorial treatments? We screened several Aurora inhibitors, and all were resisted by the mutations described here, consistent with the notion outlined above—that these mutations are likely to affect a wide range of ATP-competitive inhibitors. Therefore, rather than screening existing inhibitors for ones that block the mutants described here, we suggest that these Aurora B variants are considered as de novo drug targets. Identifying novel chemical scaffolds that inhibit Aurora B Y156H and Aurora B G160E/V may yield drugs suitable for combinations with existing inhibitors.

Drug-Resistant Alleles as Target Validation Tools

A limitation when characterizing small molecules during the drug-discovery process and as research tools is determining whether the inhibitor-induced phenotypes are due to inhibition of the desired target, as opposed to off-target effects. Consequently, other approaches, such as RNA interference, chemical genetics, and the expression of dominant negatives, are often used to validate small-molecule phenotypes (Weiss et al., 2007). However, such approaches can only ascertain whether small-molecule-derived phenotypes are consistent with inhibition of the presumptive target; they do not demonstrate that the effects are actually due to inhibition of the target. Here, by expressing drug-resistant Aurora B alleles, we have unambiguously demonstrated that the cytoxicity of ZM447439 is mediated by inhibition of Aurora B. Furthermore, because the chromosome-alignment, spindle-checkpoint, and cell-division defects induced by ZM447439 are all rescued by expression of the same mutants, we have been able to demonstrate that these phenotypes are also due to Aurora B inhibition.

VX-680 targets both Aurora A and Aurora B in cells (Harrington et al., 2004; Tyler et al., 2007); however, we show that its cytotoxicity is mediated largely via Aurora B. Similarly, because the cell-division failure induced by 5 μM MLN8045 can be reverted by the drug-resistant mutants, this phenotype must also be due to Aurora B inhibition. However, the mutants could not restore cloning potential in 1–2 μM MLN8054, demonstrating that the cytotoxicity of this drug is not mediated via Aurora B, but most likely represent Aurora A inhibition. The Y156H and G160V/E mutations render Aurora B resistant to Hesperadin in vitro, but they offer little protection against Hesperadin-induced cell-division failure. Although many of the phenotypes induced by Hesperadin appear to be due to Aurora B dysfunction (Hauf et al., 2003; Lipp et al., 2007), the cytokinesis failure may therefore reflect inhibition of another target in addition to Aurora B.

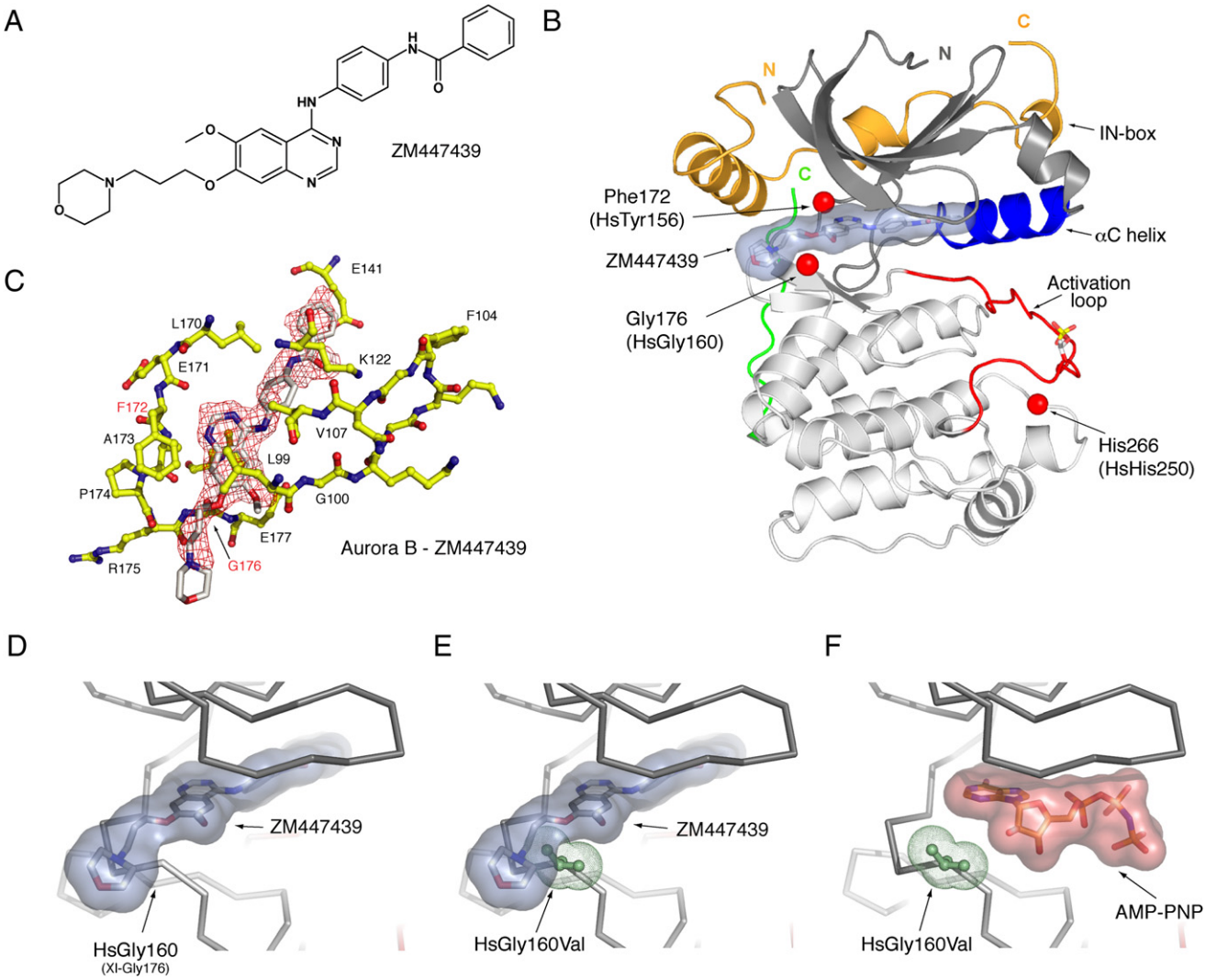


Figure 5. Structure of the Aurora B:INCENP-ZM447439 Complex

(A) Chemical structure of ZM447439.

(B) Aurora B$^{60–361}$, from *Xenopus laevis*, consists of the N-terminal small lobe (dark gray), which contains the catalytically important αC helix (blue); a C-terminal helical large lobe (light gray), which contains the activation loop (red); and a short C-terminal extension (green). In the structure, the activation loop is phosphorylated on a conserved threonine residue as part of the activation mechanism and adopts an extended, active conformation; and a short C-terminal extension (green). INCENP$^{790–847}$ (orange) crowns the small lobe of Aurora B, stabilizing an active conformation of the kinase (Sessa et al., 2005). ZM447439, shown in ball-and-stick representation and surrounded by a semitransparent-light blue surface, occupies the ATP-binding pocket at the interface between the small and large lobes.

(C) Ball-and-stick representation of the interaction of ZM447439 with selected residues of Aurora B. Oxygen and nitrogen are shown in red and blue, respectively. Carbon atoms in ZM447439 and Aurora B are white and yellow, respectively. A semitransparent molecular surface of ZM447439 is shown. Hydrogen bonds are shown as dashed lines. An unbiased $|F_o| - |F_c|$ electron density (red) map, contoured at 2.5 σ, of ZM447439 is shown.

(D) A close-up of the interaction of ZM447439 with the hinge loop.

(E) Model of the G160V mutation (note that the equivalent residue in Xl Aurora B is named V176) showing a collision of the side chain with ZM447439.

(F) A structure of Aurora B:INCENP with bound AMP-PNP (F.S., F.V., and A.M., unpublished data) shows that the same substitution does not create a collision with ATP.

Together, these observations illustrate how drug-resistant mutants provide powerful tools to delineate on- and off-target effects. In addition to teasing apart the effects of Aurora inhibitors in cultured cells, generation of mice harboring the drug-resistant Aurora B mutants should determine whether the antitumor effects and toxicity profiles of these drugs are mediated via inhibition of Aurora B or via an off-target effect.

SIGNIFICANCE

In the quest for novel anticancer drugs, considerable effort continues to be focused on generating highly selective protein kinase inhibitors. The downside of selective inhibitors is the emergence of subclones harboring mutations in the target kinase rendering them drug resistant. To date, mutations

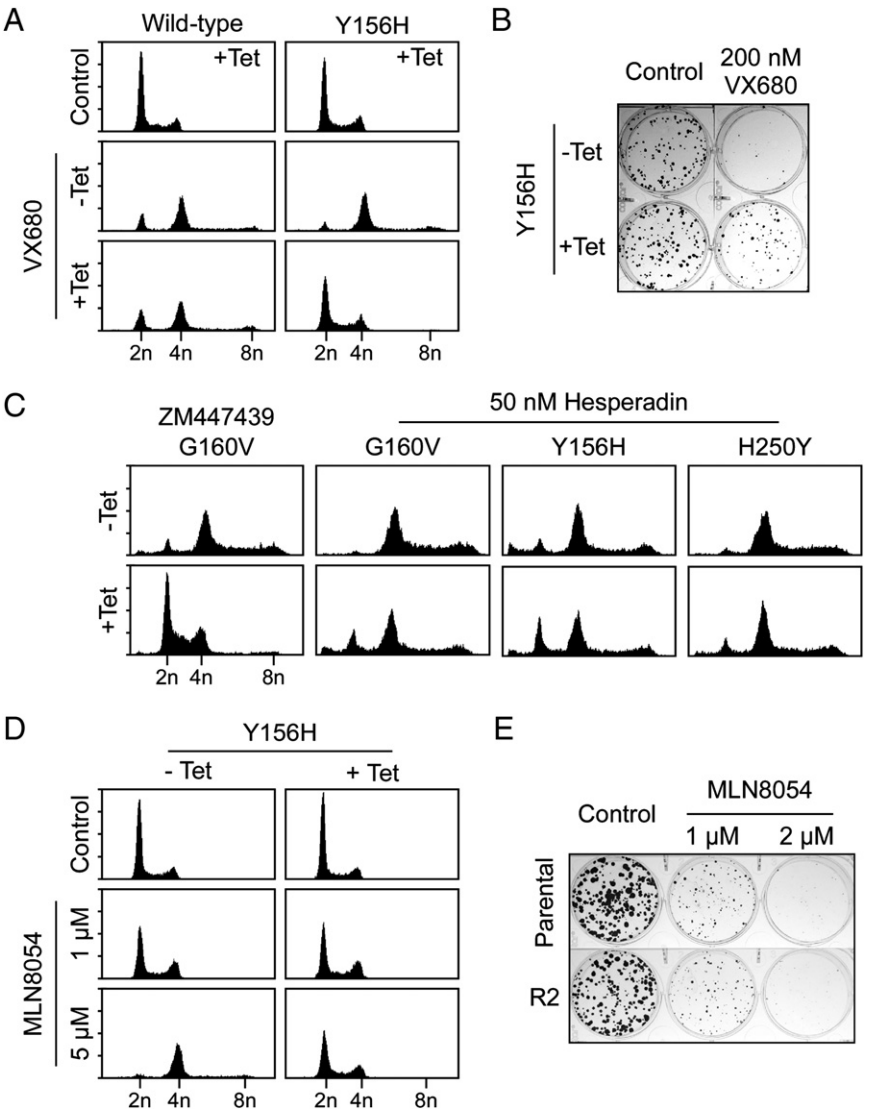


Figure 6. Aurora B Mutants Confer Resistance to VX-680 and MLN8054

Transgenic DLD-1 lines were induced with tetracycline, exposed to Aurora inhibitors, then analyzed.

(A) DNA-content histograms showing that Aurora B Y156H reverts cell-division failure induced by VX680.

(B) Colony formation assay showing that Aurora B Y156H confers cell survival in 200 nM VX680.

(C) DNA-content histograms showing that Aurora B G160V, Y156H, and H250Y confer limited resistance to a Hesperadin.

(D) DNA-content histograms showing that Aurora B Y156H reverts the cell-division failure induced by 5 μM MLN8054, but not the cell-cycle delay observed at 1 μM.

(E) Colony formation assay showing that Aurora B Y156H is not cytoprotective at 1 μM MLN8054. Flow cytometry analysis in (A), (C), and (D) was performed after 24 hr drug exposures.

causing resistance to tyrosine kinase inhibitors have arisen in several targets, most notably in the receptor tyrosine kinases BCR-ABL and EGFR. Here, we show that it is mechanistically possible for resistance to emerge against cytotoxic agents that target serine/threonine kinases required for cell-cycle control, specifically Aurora B. Indeed, this is, to our knowledge, the first demonstration of drug resistance arising in a cell-cycle kinase that is not an oncogene. One strategy aimed at limiting clinical resistance is to use combinations of inhibitors that have distinct modes of action, either in sequence or in parallel. However, the observation that the same mutations render Aurora B resistant to three structurally diverse inhibitors indicates that these agents share common modes of action, and, thus, they are not suitable for combination therapies. Therefore, if the Aurora kinases are to be effective oncology targets, the identification of novel chemical scaffolds that inhibit the mutants described here will be essential for limiting the emergence of clinical drug resistance. And, finally, the isolation and use of drug-resistant mutants to delineate on- and off-target effects demonstrates an important proof of principle that should be applicable to other protein kinase inhibitors.

EXPERIMENTAL PROCEDURES

Molecular Cell Biology and Small-Molecule Inhibitors

HCT-116 and Flp-In TRex DLD-1 cells were as described (Tighe et al., 2004; Girdler et al., 2006). Total mRNA was isolated by using Trizol (Invitrogen), and Aurora cDNAs were amplified by using Superscript One-Step RT-PCR (Invitrogen), then sequenced. Site-directed mutagenesis was performed by using QuickChange (Stratagene) with pcDNA-FRT-TO Myc-Aurora B as templates (Girdler et al., 2006). Stably transfected isogenic DLD-1 cell lines were generated by using Flp/FRT-mediated recombination; transgene expression was induced with 1 μg/ml tetracycline (Girdler et al., 2006). Taxol was used at a final concentration of 10 μM. ZM447439, VX-680, Hesperadin, and MLN8054 were as described (Ditchfield et al., 2003; Hauf et al., 2003; Harrington et al., 2004; Manfredi et al., 2007). Colony formation assays, proliferation assays, flow cytometry, immunofluorescence, immunoblotting, and immunoprecipitation kinase assays were performed essentially as described (Girdler et al., 2006).

Expression and Purification of Recombinant Proteins

For bacterial expression, cDNA segments encoding human Aurora $B^{45–344}$ and human $INCENP^{835–903}$ were subcloned in a bicistronic pGEX-6P vector (Amersham Biotech). Expression of Aurora $B^{45–344}$:$INCENP^{835–903}$ in Rosetta DE3 *E. coli* cells at OD_{600} = 0.45–0.7 was induced for 12–16 hr at 18°C with 0.3 mM IPTG. Bacterial pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.6], 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, Roche Complete Protease Inhibitor Cocktail Tablets) and lysed by sonication, and the lysates were clarified by centrifugation. The supernatants were incubated in lysis buffer with 300 μl GST Sepharose Fast Flow (Amersham Biosciences) per liter of bacterial culture for 4–5 hr at 4°C. Beads were then washed with 30 volumes of lysis buffer, equilibrated in cleavage buffer (50 mM Tris-HCl [pH 7.6], 300 mM NaCl, 1 mM DTT, 1 mM EDTA), then incubated with PreScission Protease (Amersham Biosciences; 10 U/mg substrate) for 16 hr at 4°C to liberate the Aurora B:INCENP complex for subsequent analyses.

In Vitro Kinase Assays

Reactions were carried out in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1 mM DTT. Aurora $B^{45–344}$:$INCENP^{835–903}$ at 50 nM was incubated at 30°C for 15 min in the presence of 200 μM ATP and 5.5 μM Histone H3 (Roche). Reactions were initiated by the addition of ATP and $MgCl_2$, terminated by adding SDS-PAGE loading buffer, then separated by 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween (TBS-Tween), and 5% (w/v) milk for 1 hr, then incubated for 16 hr at 4°C with a monoclonal antibody against phospho-Ser10 Histone H3 (Upstate Biotechnology, Inc.). Detection was performed by using an HRP-conjugated secondary antibody and enhanced chemiluminescence at 30°C (ECL, GE Healthcare, Inc.).

Crystallization and Structure Determination

Expression, purification, and structure determination of *Xl* Aurora $B^{60–361}$:$INCENP^{790–847}$ have been described (Sessa et al., 2005). Crystals obtained by microseeding were gradually transferred in cryobuffer (19% PEG400, 100 mM Bis-Tris-Propane [pH 6.5], 2 mM TCEP), then incubated with 1/100 (v/v) of a 10 mM solution of ZM447439 dissolved in DMSO. After a 16 hr incubation with the inhibitor, crystals were flash frozen. X-ray diffraction data from single crystals were collected at beamline ID14-3 at the European Synchroton Radiation Facility (Grenoble, France). Data processing was carried out by using DENZO/SCALEPACK (Otwinowski and Minor, 1997). For subsequent calculations, we used the CCP4 suite (CCP4, 1994). Molecular replacement was carried out with MOLREP (Vagin and Teplyakov, 1997) by using the Aurora B coordinates as a search model (PDB code: 2BFX). Iterative model building was carried out with Coot (Emsley and Cowtan, 2004) and Refmac (Murshudov et al., 1997), resulting in a model with good stereochemical parameters (Table 1).

ACCESSION NUMBERS

Coordinates have been deposited in the Protein Data Bank with accession code 2VRX.

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