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Drug-Resistant Aurora A Mutants for Cellular Target Validation of the Small Molecule Kinase Inhibitors MLN8054 and MLN8237

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ABSTRACT The Aurora kinases regulate multiple aspects of mitotic progression, and their overexpression in diverse tumor types makes them appealing oncology targets. An intensive research effort over the past decade has led to the discovery of chemically distinct families of small molecule Aurora kinase inhibitors, many of which have demonstrated therapeutic potential in model systems. These agents are also important tools to help dissect signaling pathways that are orchestrated by Aurora kinases, and the antiproliferative target of pan-Aurora inhibitors such as VX-680 has been validated using chemical genetic techniques. In many cases the nonspecific nature of Aurora inhibitors toward unrelated kinases is well established, potentially broadening the spectrum of cancers to which these compounds might be applied. However, unambiguously demonstrating the molecular target(s) for clinical kinase inhibitors is an important challenge, one that is absolutely critical for deciphering the molecular basis of compound specificity, resistance, and efficacy. In this paper, we have investigated amino acid requirements for Aurora A sensitivity to the benzazepine-based Aurora inhibitor MLN8054 and the close analogue MLN8237, a second-generation compound that is in phase II clinical trials. A crystallographic analysis facilitated the design and biochemical investigation of a panel of resistant Aurora A mutants, a subset of which were then selected as candidate drug-resistance targets for further evaluation. Using inducible human cell lines, we show that cells expressing near-physiological levels of a functional but partially drug-resistant Aurora A T217D mutant survive in the presence of MLN8054 or MLN8237, authenticating Aurora A as a critical antiproliferative target of these compounds.

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During the past two decades protein kinase inhibitors have emerged as an important family of experimental and clinical small molecules (*1*). The vast majority of these compounds represent late-stage distillates from the drug discovery process, which endows many of them with useful pharmacological properties (*2*). However, because of the ATP-dependent mechanism of most inhibitors and the highly conserved nature of the nucleotide-binding site in all protein kinases, the potential for promiscuity must be taken into account prior to exploitation in cell-based systems. This issue is usually broached indirectly through inter-kinome specificity analyses employing extended subsets of the human kinome (*3, 4*), which are inevitably biased toward well-characterized and/or highly druggable kinase targets. Moreover, because many kinases are assayed at artificially low ATP concentrations in the form of truncated catalytic domains or in isolation from their physiological partners, high-throughput screens cannot accurately mimic the complex environment under which these compounds function *in vivo*. An increasingly important avenue of research in cell signaling therefore seeks to accurately define the mechanism of action of protein kinase inhibitors by identifying and validating the precise intracellular drug target(s) that induce biological effects (*2, 5–7*). One such technique involves the development of *in vitro* and *in vivo* screens for the identification of resistance-conferring mutations in protein kinases.

In cells, the key mitotic events of centrosome separation, bipolar spindle formation, chromosome segregation, and cytokinesis are controlled by highly regulated protein kinases, including the evolutionarily conserved Aurora family, comprising Aurora A, B, and C in humans

(*8*). Aurora A associates with centrosomes and the mitotic spindle and controls entry into mitosis, centrosome separation, and spindle bipolarity (*9*). Human Aurora A is an overexpressed, oncogenic kinase (*10, 11*), and functional cross-talk occurs between Aurora A and the p53 and p73 apoptotic pathways in cancer cells (*12, 13*). The very closely related kinase Aurora B functions in a multiprotein mitotic complex, which monitors and regulates chromosome segregation (*14*). Aurora B kinase activity is also required for cytokinesis, explaining the polyploid phenotype detected after exposure to small molecule Aurora B inhibitors (*15*). The diverse cellular regulation and function of the Aurora kinases can be traced to specific amino acid determinants located both within (*16, 17*) and adjacent to (*18*) the conserved catalytic domain.

Aurora kinase inhibition represents an important intervention strategy for combating proliferative diseases such as cancer, where a central role in mitosis and frequent overexpression make them highly attractive drug targets (*15, 19*). Although no specific Aurora inhibitors have yet reached the market, they have been widely appraised in cancer models and in early stage clinical trials (*19, 20*). Examples of orally bioavailable inhibitors include the dual Aurora A and B inhibitor VX-680/MK-057 (*21, 22*), the more selective Aurora B inhibitor AZD1152 (*23*), and the benzazepine compounds MLN8054 (*24*) and MLN8237 (*25, 26*), which have advanced to early phase clinical trials.

Aurora A and Aurora B exhibit very high catalytic domain identity (*15*), making the development of specific inhibitors a technical challenge. Indeed, one problem associated with interpreting experimental and clinical data obtained with Aurora inhibitors is their largely unknown cellular selectivity (*27*). For example, depending upon the assay employed, MLN8054 exhibits between 7- and 43-fold specificity for Aurora A over Aurora B *in vitro* (*4, 24*), and ATP-binding site displacement assays reveal several additional non-Aurora kinase targets interspersed throughout the human kinome (*4*). These include the centrosomal regulator Polo-like kinase (Plk) 4, the tyrosine kinases Tie2, Abl, Src and Yes, and the poorly characterized kinases BLK and DRAK2, which are inhibited as potently as the Aurora kinases *in vitro*. Accordingly, careful titration of the MLN8054 concentration is needed to induce phenotypic effects that are consistent with exclusive Aurora A inhibition in cultured cells. Moreover, phenotypes more normally associated with Aurora B inhibition, including chromosome alignment defects, spindle assembly checkpoint override, and polyploidy, are apparent when cultured cells are exposed to intermediate concentrations of this drug (*21, 28–30*).

By employing drug-resistant Aurora A and B mutants, it has been established that the pan-Aurora kinase inhibitor VX-680 induces antiproliferative effects in human cells through direct inhibition of Aurora B rather than Aurora A (*29, 31*), although both kinases represent simultaneous intracellular VX-680 targets (*22*). This dominant Aurora B-inhibitory phenotype raises the question of whether discrete inhibition of Aurora A by MLN8054 really represents a critical mechanism for inducing cell death or if another target might be important for its anticancer properties. In this paper, we report the evaluation of a panel of drug-resistant Aurora A mutants and the discovery of specific point mutations that render them partially resistant to MLN8054 and MLN8237 *in vitro*. Using a powerful chemical genetic approach, we go on to analyze several of these mutants in human cells, where we prove unambiguously that Aurora A is a critical antiproliferative target of both drugs. Because these mutational studies pinpoint amino acid side chains required for MLN8054 interaction with Aurora A, they will be useful to evaluate the sensitivity and resistance profiles of chemically distinct classes of Aurora A inhibitors. Moreover, to our knowledge, these findings represent the first validation of Aurora A as a stand-alone antiproliferative target for a small molecule inhibitor.

RESULTS AND DISCUSSION

Analysis of MLN8054 Binding Mode to Aurora A and Plk4. Human Aurora A and Aurora B are thought to have evolved through a complex series of events from a single kinase ancestor (*15, 32*), explaining the 73% amino acid identity and ~85% amino acid homology between catalytic domains. This high level of conservation makes the development of inhibitors with specificity for either enzyme a technical challenge, and evaluating such molecules is currently hindered by a paucity of tools to unequivocally distinguish between long-term Aurora A and/or Aurora B inhibition in model cell-based systems. We therefore sought to extend our chemical genetic analysis of the Aurora inhibitor VX-680 (Figure 1, panel A), an equipotent Aurora A and B inhibitor (*22, 31*), to include the more *in vitro*-selective Aurora A in-

hibitors MLN8054 and MLN8237. These clinical stage compounds, which exhibit subtly different chemistry (Figure 1, panel A), display similar potency toward Aurora A *in vitro* (Figure 1, panel B). One recently reported non-Aurora MLN8054 target is Plk4 (*4*), a potential anticancer target whose regulation by autophosphorylation is important for centrosome biology (*33*). We confirmed by radiometric assay that both MLN8054 and VX-680 inhibit Plk4, with respective IC_{50} values of 3 and <0.3 μM in the presence of 100 μM competing ATP (Figure 1, panels C, D). The small amino acid side chain presented by Gly216 of Aurora A is specifically required for VX-680 inhibition, because mutation to Leu abolishes phenotypes associated with exposure to VX-680 but not MLN8054 (*31*). Remarkably, the inhibition of Plk4 by both compounds requires the equivalent Gly residue (Gly95), because mutation to Arg, the corresponding charged residue in the MLN8054/VX-680-resistant Plk1-3 enzymes (*4, 34*), induces profound resistance (Figure 1, panels C, D). This suggests that the affinity of two Aurora A inhibitors for Plk4 is due to interaction with a determinant adjoining Gly95 in the drug-binding site.

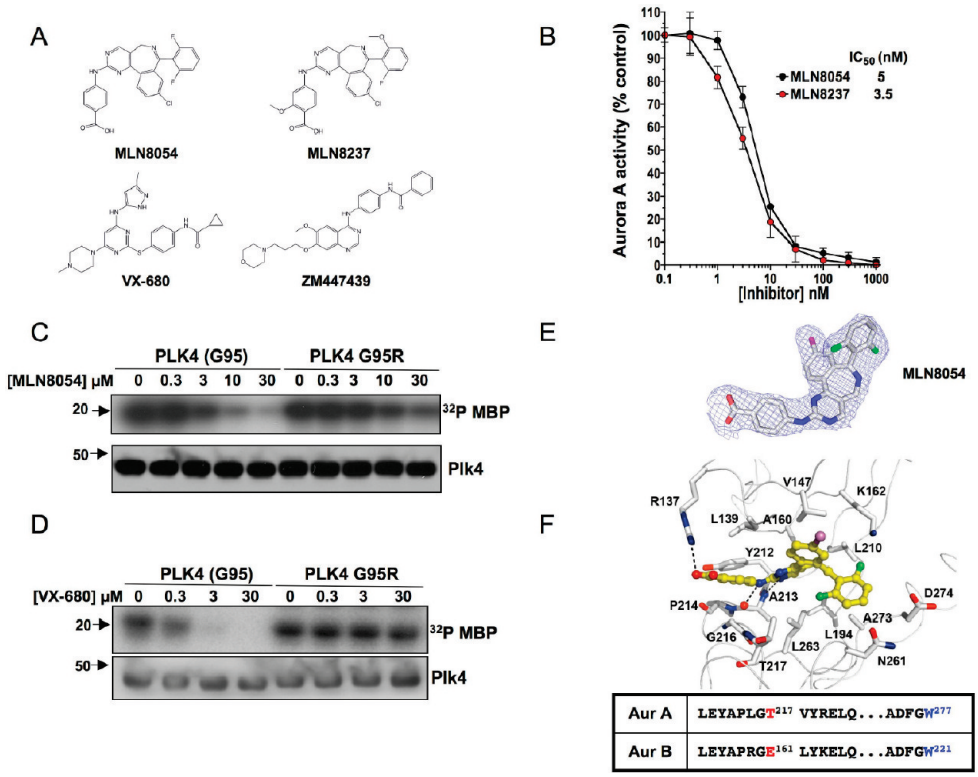

Figure 1. Analysis of Aurora inhibitors and MLN8054 binding mode. A) Chemical structure of Aurora kinase inhibitors evaluated in this study. B) Inhibition of Aurora A (12.5 nM) by MLN8054 or MLN8237 was assessed in duplicate radiometric assays containing 100 μM [γ-^{32}P] ATP and quantified by p81 phosphocellulose assay and scintillation counting. Kinase activity is reported as a percentage of control calculated from duplicate incubations containing 2.5% (v/v) DMSO. IC_{50} values represent the mean ± SEM calculated from two independent experiments. C, D) The kinase activity of a 6His-tagged Plk4 catalytic domain or a G95R Plk4 mutant was assessed by autoradiography in the presence of the indicated concentrations of (C) MLN8054 or (D) VX-680, using the nonspecific substrate myelin basic protein (MBP). Equal His-Plk4 loading was demonstrated with a monoclonal anti-6His antibody. E) The final $2F_o - F_c$ electron density map around MLN8054 (shown in blue mesh) and contoured at 1.0 σ. F) The complex of Aurora A and MLN8054, highlighting the MLN8054-binding site in the nucleotide pocket, with prominent amino acids adjacent to the inhibitor highlighted in black. An amino acid alignment of human Aurora A and B, beginning with the conserved Leu "gatekeeper" residue is also shown. The distinctive amino acid in the hinge region of Aurora kinases (Thr217 in Aurora A, Glu161 in Aurora B) is highlighted in red, and the conserved Trp residue located after the DFG motif in Aurora kinases is depicted in blue.

We previously found that mutation of Gly216 to Leu in Aurora A decreases the IC_{50} value for MLN8054 only 2-fold but increases resistance to VX-680 some 250-fold, suggesting distinctive binding of MLN8054 and VX-680 (*31*). To examine the interaction mode of MLN8054 to Aurora A, we determined the X-ray structure of WT Aurora A in a co-complex with MLN8054 (Figure 1, panel E). As detailed in Table 1, the complex diffracts to 2.9 Å, allowing us to examine the details of the MLN8054 binding site in Aurora A. As shown in Figure 1, panel F, amino acids lining the interaction site are delineated, revealing side chains that are in proximity to the drug, including Arg137, Lys162, Leu210 (the gatekeeper residue), Tyr212, Ala 213, Gly216, and Thr217. With the exception of Thr217 in Aurora A, which is a negatively charged Glu residue (Glu161) in Aurora B, all of these residues are completely conserved in Aurora B (Figure 1, panel F).

Analysis of Aurora A Drug-Resistance Mutations. To assess the potential importance of Thr217 for Aurora A sensitivity to small molecules, we generated a series of

TABLE 1. Data-processing and refinement statistics for the human WT Aurora A:MLN8054 complex (PDB ID 2X81)[a]

Data collection	
Space group	$P6_122$
Unit-cell parameters (Å)	$a = b = 83.21$, $c = 167.24$
Matthew coefficient (Å^3 Da^{-1})	3.12
Solvent content (%)	60.3
No. of molecules per ASU	1
X-ray source	Rigaku Micromax 007HF
Wavelength (Å)	1.5418
Resolution (Å)	24.5− 2.91 (3.0−2.91)
Total observations	12743
Unique observations	8043
Completeness (%)	98.3 (96.2)
R_{merge} (%)	7.0 (48)
$\langle I/s(I)\rangle$	18.2 (2.0)
refinement	
Resolution (Å)	24.5−2.91
R_{factor} (%)	24.4
R_{free} (%)[b]	30.3
Rmsd bond lengths (Å)	0.006
Rmsd bond angles (deg)	0.970
Mean B-factor (Å^2)/no. of atoms:	
Protein non-H atoms	81.7/1877
Ligand non-H atoms	71.3/34
F/Y angles (%):	
Most favored region	92.0
Additionally allowed region	6.6
Generously allowed region	0.9

[a]Values in parentheses are for the highest resolution shell. [b]R_{free} was calculated using 4.6% of data excluded from refinement.

Aurora A mutants containing selected point mutations. As depicted in Figure 2, panel A, each mutant was expressed and phosphorylated on Thr288, indicating functional autophosphorylation in bacteria (*31*). For inhibitor assays, we initially screened the activity of purified Aurora A mutants (125 nM) in the presence of an intermediate concentration (100 nM) of VX-680 (VX) or MLN8054 (ML), equivalent to ~5-times the IC_{50} value for each compound under these conditions (*31*). As shown in Figure 2, panels B and C, all but two of the mutants exhibit catalytic activity and the introduction of Asp and, to a very much lesser extent, Glu at position 217 induces detectable resistance to both VX-680 and MLN8054. Interestingly, mutation to other side chains, including Ala, Gly, Ser, or Cys, does not recapitulate this resistance profile, suggesting that introduction of a negative charge at this position might prevent Aurora A inhibition by these drugs. This hypothesis is supported by a comparative analysis in which a chemically uncharged Asn residue replaces Asp. The presence of the Asn amide group in Aurora A does not induce resistance to 100 nM MLN8054 (Figure 2, panel D), although the resistance afforded toward VX-680 (Figure 2, panel D) is further evidence of a distinct Aurora A binding mode. In addition, the observation that both WT and T217D Aurora A display linear kinetics in our assay and a very similar Michaelis−Menten constant (K_m) for ATP (Table 2 and Supplementary Figure S1) implies that partial MLN8054 resistance is unlikely to be imparted by inefficient ATP binding to the Asp mutant and is instead caused by disruption within the inhibitor binding site.

Analysis of Drug-Resistance in the Presence of Targeting Protein for Xenopus Kinesin-Like Protein 2 (TPX2). One of the weaknesses of inhibitor screening approaches using recombinant kinases is the absence of stoichiometric regulatory subunits, which target enzyme activity under physiological conditions. In cells, Aurora A is activated through a series of mechanisms, the best characterized of which involves the TPX2-mediated stabilization of a highly active Aurora A configuration important for mitotic spindle dynamics (*35–37*). Moreover, the activating portion of TPX2, comprising

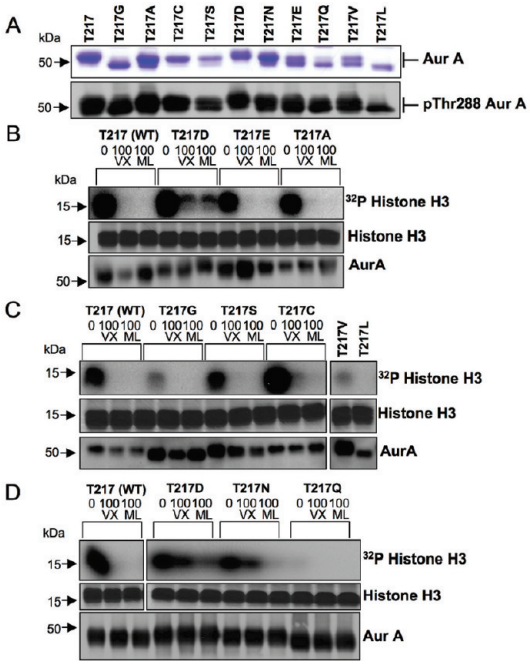

Figure 2. Biochemical and inhibitor analysis of Aurora A Thr217 mutants. A) Recombinant 6His-tagged full length human Aurora A proteins were expressed in *E.coli* and purified by affinity chromatography. Five micrograms of WT Aurora A or the indicated point mutant was denatured in 2% SDS, and proteins were identified by Coomassie Blue staining after SDS-PAGE (top panel). In addition, 200 ng of each Aurora A protein was immunoblotted with a phosphospecific pThr288 Aurora A antibody (bottom panel). B) WT, T217D, T217E, or T217A mutants were assayed in the presence or absence of 100 nM VX-680 or 100 nM MLN8054, using Histone H3 as substrate and 100 μM [γ-^{32}P] ATP. Phosphorylation was detected after SDS-PAGE by autoradiography (top panel). Histone H3 (middle panel) or Aurora A loading was evaluated by Coomassie Blue staining or immuoblotting with Aurora A antibody. C) WT, T217G, T217S, and T217C Aurora A proteins were assayed as described for panel B. In addition, T217 V and T217L Aurora A mutants were also assayed, but in the absence of Aurora inhibitors. D) WT, T217D, T217N and T217Q mutants were evaluated as described for panel B.

amino acids 1−43, markedly decreases the inhibitory potency of VX-680 when bound to Aurora A *in vitro* (*31, 38*). Given the likelihood that this complex represents a physiologically relevant mitotic form of Aurora A, we assessed the effects of TPX2 on Aurora A sensitivity to the compounds MLN8054 (Aurora A > Aurora B), VX-680 (~ equipotent), and ZM447439 (Figure 1, panel A), an experimental quinazoline with a known preference for Aurora B over Aurora A (*39*). In line with a previous analysis (*38*), TPX2 decreases the K_m [ATP] for WT Aurora A by 2.7-fold, and we determined that the drug-resistant T217D mutant exhibits approximately 5-fold higher affinity for ATP in the presence of TPX2 (Table 2). Consistent with this difference, WT Aurora A bound to TPX2 is 2- to 3-fold less sensitive to MLN8054 inhibition when compared to the enzyme alone (Figure 3). Moreover, both T217E and T217D Aurora A mutants are approximately 5-fold less sensitive toward MLN8054 in the presence of TPX2, although both mutants are still considerably more resistant to the drug than WT Aurora A. A similar pattern is observed toward VX-680, with sensitivity of the T217D mutant decreasing some 8-fold in the presence of TPX2 (Supplementary Figure S2), although this mutant is still much more resistant to VX-680 than WT Aurora A. However, a contrasting profile is seen with ZM447439, with the T217D mutant exhibiting resistance to the drug in the presence and absence of TPX2, but a T217E mutant displaying increased sensitivity (Supplementary Figure S3). Indeed, the IC_{50} values decrease 3-fold in the absence of TPX2 and some 6-fold in the presence of TPX2 when compared to WT Aurora A. Taken together, these data establish that TPX2 binding decreases the *in vitro* activity for all of these inhibitors toward Aurora A, in all probability through an increase in the affinity for ATP, as suggested for VX-680 (*31, 38*). To endorse the importance of a negative charge at position 217 for inducing resistance (Figure 2), we also examined the effects of mutating this residue to Asp or Asn in the presence of TPX2. As detailed in Supplementary Figure S4, the T217N Aurora A mutant is as sensitive to MLN8054 as WT Aurora A under conditions where T217D Aurora A exhibits partial resistance. In contrast, both T217N and T217D mutants remain partially resistant to VX-680 when compared to WT Aurora A, confirming data from our initial screening procedure performed in the absence of TPX2.

Recent structural studies have revealed the potential importance of Thr217 for a hydrogen-bonding interaction with pyrazole compounds that exhibit specificity for Aurora A over Aurora B (*40*). Consistently, the introduction of a Glu residue at Thr217 in Aurora A has been demonstrated to decrease the K_i for MLN8054 some 6-fold (*41*), comparable to Aurora B (*4*), and the selectivity of 2,4-bisanilinopyrimidine inhibitors can be switched by exchanging the equivalent Thr and Glu residues in human Aurora A and B (*42*). Our finding that

TABLE 2. Determination of K_m [ATP] for WT, T217D, and T217D/W277E Aurora A kinases[a]

	K_m ATP (μM)	K_m ATP (μM) +TPX2 [1−43]	Change in K_m [ATP] (fold decrease)
WT	14.31 ± 1.6	5.22 ± 0.8	2.7
T217D	9.56 ± 0.64	1.8 ± 0.3	5.3
T217D/W277E	12.24 ± 2.32	1.56 ± 0.51	7.8

[a]The Michaelis−Menten constant for ATP was determined from a kinetic analysis using fixed concentrations of Aurora A and Histone H3 substrate over a range of ATP concentrations. Values were calculated from a nonlinear regression analysis of repeat duplicate assays using Prism software.

Asn is not an effective replacement for Asp for inducement of MLN8054 resistance (Supplementary Figure S4) supports a mechanism in which a negatively charged Asp (or Glu) side chain decreases the ability of MLN8054 to bind to Aurora A. In contrast, the mutation of Thr217 to Glu (but not Asp) sensitizes Aurora A to ZM447439 (Supplementary Figure S3). The presence of a Glu side chain in Aurora B, its preferential target (*38*),

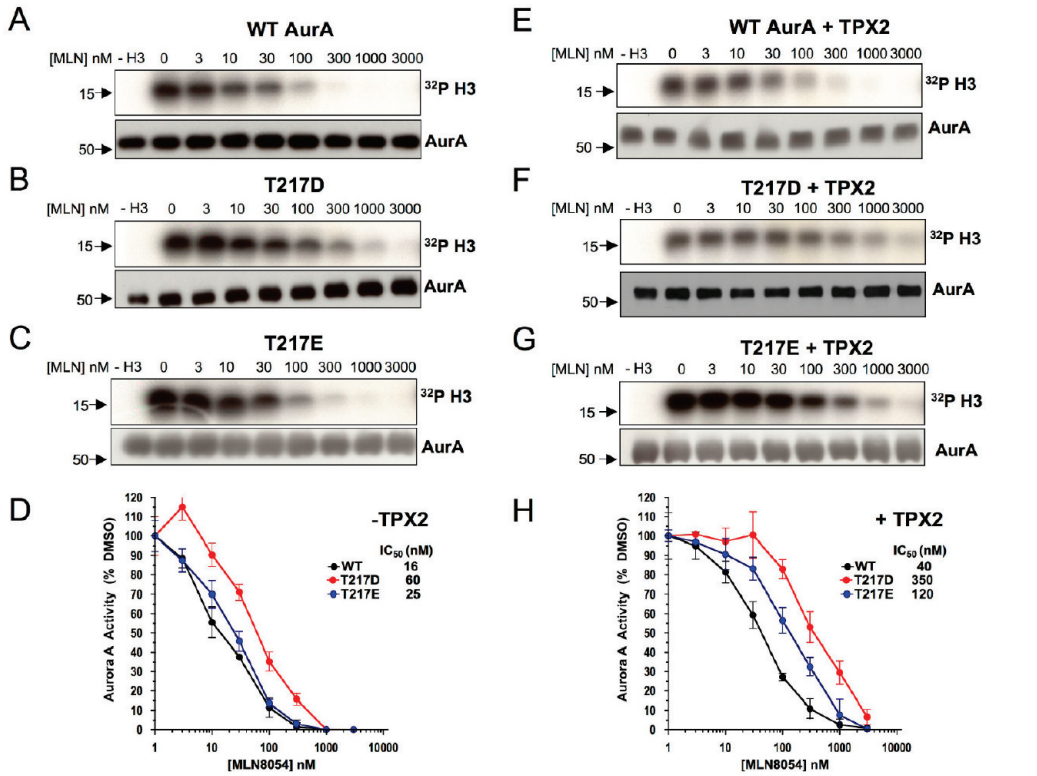

Figure 3. MLN8054 resistance toward charged Thr217 point mutants. Aurora A proteins were assayed side-by-side with Histone H3, in the absence (A−C) or presence (E−G) of TPX2 [1−43] peptide and the indicated concentration of MLN8054 and a final concentration of 100 μM [γ-^{32}P] ATP. After SDS-PAGE, ^{32}P-phosphorylated Histone H3 was revealed by autoradiography (top panels) and total Aurora A amounts were analyzed by immunoblotting with an Aurora A antibody (bottom panels). (D, H) Phosphorylated Histone H3 bands were quantified on a phosphorimager and the data plotted graphically to obtain log−dose response curves. Activity is reported as a percentage of control calculated from incubations containing 2.5% (v/v) DMSO solvent. Calculated IC_{50} values represent the mean ± error (95% CI) determined from two experiments. Similar results were seen in multiple independent experiments.

is entirely consistent with this observation. Together, these data reveal the diverse effects of a negatively charged side chain at this position for regulating affinity toward distinct inhibitor classes.

A Second Drug-Resistance Locus in Aurora A. The unique tryptophan residue found immediately downstream of the Mg^{2+}-binding DFG motif in Aurora kinases (Figure 1, panel E) is a molecular determinant for Aurora A inhibition by quinazoline inhibitors such as ZM3 (*39*), and we hypothesized that it might also be important for interaction with chemically unrelated Aurora inhibitors. As a result of the high mobility of the flexible activation segment, Trp277 is not visible in our Aurora A:MLN8054 co-crystal structure, so to investigate the effects of mutating this Aurora-defining residue (*41*), we evaluated Aurora A mutants in which the indole ring of the Trp residue is replaced by diverse amino acid side chains (Figure 4, panel A). All mutants were approximately twice as active as WT Aurora A (Figure 4, panel B), although W277A and especially W277E Aurora A demonstrate partial resistance toward both VX-680 (VX) and MLN8054 (ML) in the absence of TPX2 (Figure 4, panel B). Remarkably, all mutants exhibit complete resistance to ZM447439 (Figure 4, panel C), illustrating the critical role for this Trp residue in dictating sensitivity to the quinazoline class of Aurora inhibitors (*39*).

To extend our screen, we combined the most prominent resistance mutations identified among the Thr217 and Trp277 series. Interestingly, a T217D/W277E Aurora A double mutation leads to synergistic resistance toward MLN8054 in both the presence and absence of TPX2, the IC_{50} value increasing between 24- and 36-fold, from 25 to 900 nM in the absence of TPX2 and from 50 to 1200 nM in the presence of TPX2 (Figure 5). We completed our screen by analyzing the clinical Aurora A inhibitor MLN8237 and found that T217D, but not T217N, Aurora A is also partially resistant to this drug in the presence of TPX2, the I_{90} value increasing from 300 nM to 1 μM for the T217D Aurora A mutant (Supplementary Figure S5). Consistently, we also found that a T217D/W277E double mutant exhibits superior levels of resistance to MLN8237, with the I_{50} value increasing approximately 20-fold from 30 to 650 nM in the presence of TPX2 (Supplementary Figure S5). In a similar vein to both WT and T217D Aurora A, the decreased activity of MLN8054/MLN8237 for the T217D/W277E Aurora A/TPX2 complex might reflect the increased affinity for ATP induced by cofactor binding to Aurora A (Table 2).

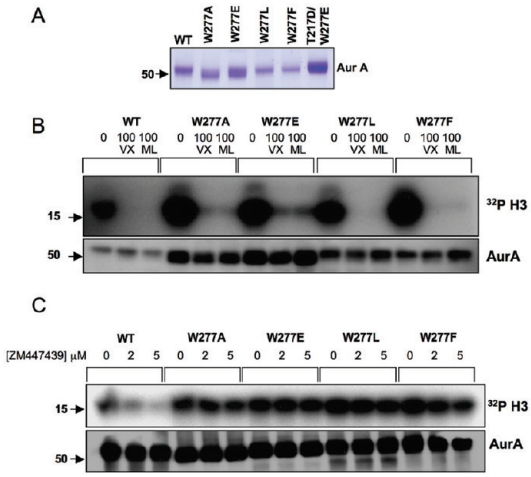

Figure 4. Inhibitor screen for resistant Aurora A Trp277 point mutants. A) Recombinant 6His-tagged full length human Aurora A proteins were expressed in *E.coli* and purified by IMAC. Five micrograms of WT Aurora A or the indicated mutant was denatured in 2% SDS, and Aurora A proteins were analyzed by Coomassie Blue staining after SDS-PAGE. B) The indicated Aurora A proteins were assayed side-by-side using Histone H3, in the presence of 100 nM VX-680 or 100 nM MLN8054 and 100 μM [γ-^{32}P] ATP. After SDS-PAGE, phosphorylated Histone H3 was revealed by autoradiography. Aurora A protein loading was evaluated by immuoblotting with an Aurora A antibody. (C) The indicated Aurora A proteins were assayed side-by-side using Histone H3, in the presence of the indicated concentration of ZM447439 and 100 μM [γ-^{32}P] ATP. After SDS-PAGE, phosphorylated Histone H3 was revealed by autoradiography. Equal Aurora A protein loading was demonstrated by immuoblotting with Aurora A antibody.

Analysis of Drug-Resistant Aurora A Mutants in Human Cells. In cultured cells, acute exposure to VX-680 inhibits Aurora A and B simultaneously (*22*), but longer-term treatment promotes cell death through an Aurora B-dependent pathway (*29, 31*). Depending upon the concentration applied to the cell culture medium, MLN8054 can inhibit Aurora A and Aurora B, although Aurora B-like inhibitory phenotypes are most evident at higher (μM) concentrations, when Aurora B inhibition does not correlate with cytotoxicity (*29, 31*). However, whether inhibition of Aurora A or an unknown "off-target" kinase represents the *bona fide* cellular antiproliferative target for MLN8054 remains unknown. One approach to validate small molecule specificity involves the employment of cellular drug-resistant mutants. If specific to a given target, the phenotypic effects of a

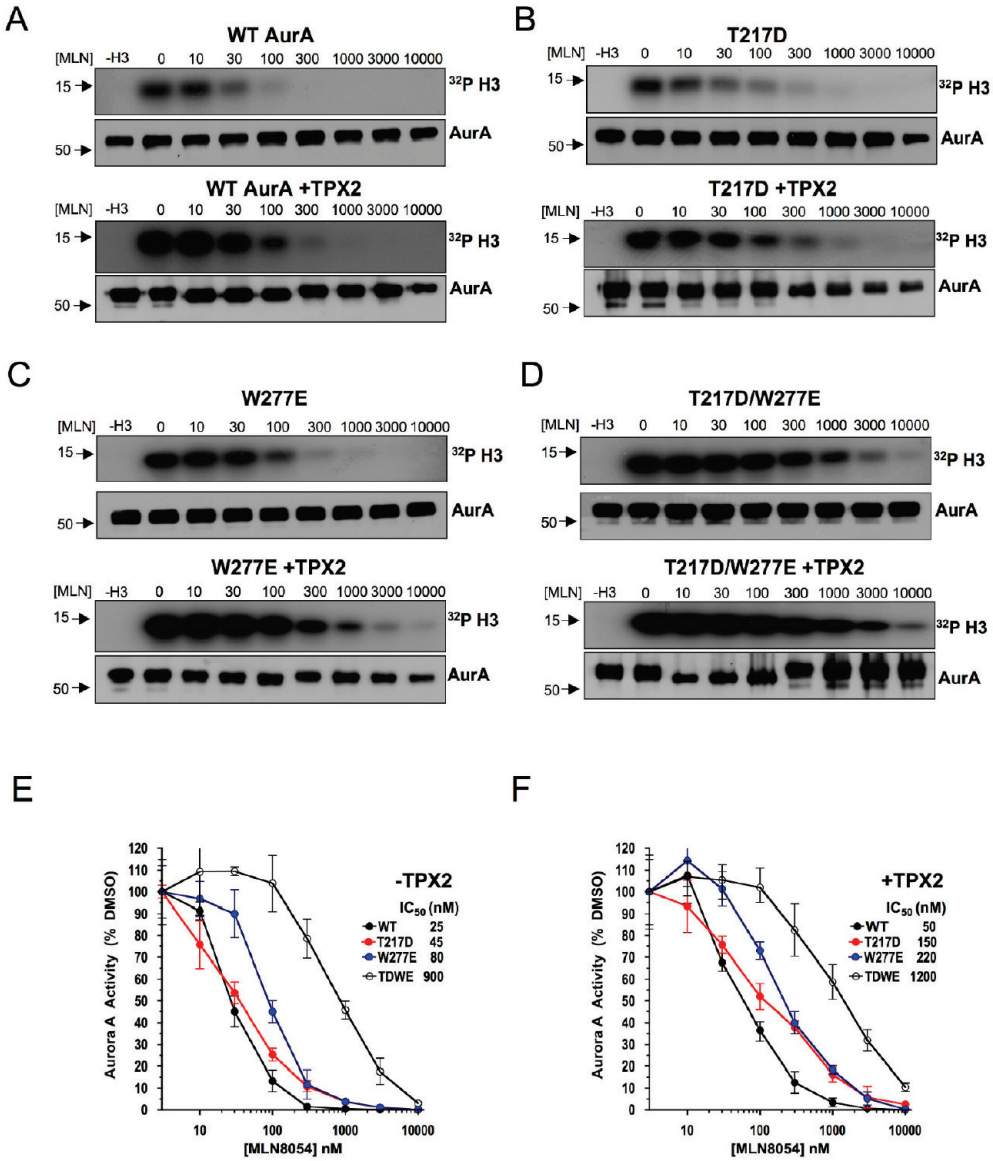

Figure 5. Comparative analysis of Aurora A mutant resistance to MLN8054. A–D) The indicated Aurora A proteins were assayed side-by-side in the absence (top panels) or presence (lower panels) of TPX2 [1–43] peptide, using Histone H3, in the presence of the indicated concentration of MLN8054 and a final concentration of 100 μM [γ-^{32}P] ATP. After SDS-PAGE, phosphorylated proteins were revealed by autoradiography. Phosphorylated bands were quantified on a phosphorimager and the data plotted graphically (E, F) to obtain log–dose response curves. Activity is reported as a percentage of control calculated from incubations containing 2.5% (v/v) DMSO solvent. Calculated IC_{50} values represent the mean ± error (95% CI) determined from two experiments. Similar results were seen in several independent experiments.

compound should not be observed in the presence of a drug-resistant mutant (*6*). To investigate cellular Aurora A resistance to MLN8054, we employed a chemical genetic strategy in human cells, exploiting the partially drug-resistant Aurora A mutants identified from our biochemical screen.

We constructed novel cell lines in which epitope-tagged Aurora A transgenes were stably integrated into Tet-responsive isogenic HeLa cell lines (*31*). As shown in Figure 6, panel A, immunoblotting of cell extracts with a Myc antibody demonstrates that WT, T217D, T217E, and T217D/W277E Aurora A proteins are all expressed at a similar level after exposure to Tet for 24 h (top panel), and relative expression levels are similar to the previously validated VX-680-resistant G160L Aurora B (*31*). The dual Myc-tagged Aurora A proteins exhibit decreased electrophoretic mobility compared to that of endogenous Aurora A, and comparative immunoblotting demonstrates that expression levels are very similar (Figure 6, panel A). The stability of Aurora kinase transgene expression in these cells is readily demonstrated by immunoblotting of cell extracts after 10 days of continuous exposure to Tet (Figure 6, panel A), an attribute that permits the effects of long-term expression to be analyzed in parallel (see below).

Subcellular Targeting and Activation of Drug-Resistant Aurora A Mutants. In order to execute the signaling functions of Aurora A in cells, drug-resistant Aurora A mutations must be silent with respect to normal protein function, localizing to the same specific mitotic structures

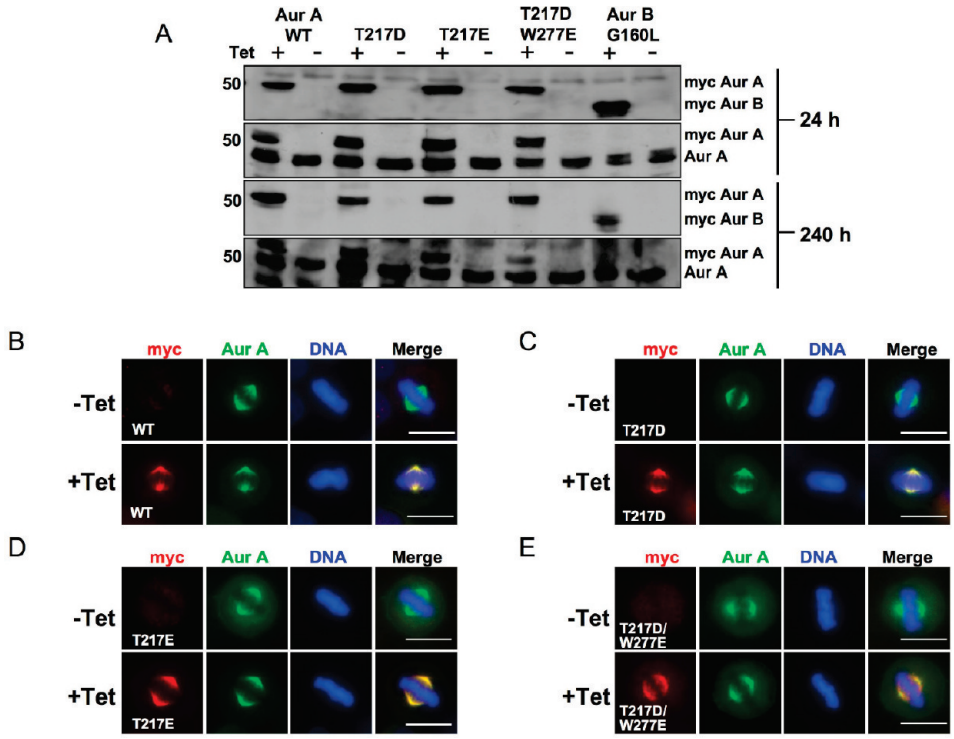

Figure 6. Analysis of Aurora A expression in stable HeLa cells. A) WT or T217D, T217E, T217D/W277E Aurora A or G160L Aurora B-encoding N-terminally Myc-tagged plasmids were stably integrated in HeLa cells, with expression being dependent on the addition of Tet to the culture medium. Cell extracts were analyzed by Western blotting with antibodies recognizing Myc (top panels) or Aurora A (bottom panels) after exposure to Tet for 24 or 240 h. B–E) The indicated asynchronous stable Aurora A HeLa cells were incubated overnight in the presence or absence of Tet and subsequently fixed in methanol prior to processing for immunofluorescence. Cells were costained with antibodies to Myc (red), total Aur A (green) and DAPI (blue). Merged color plots demonstrate co-localization of Myc-tagged Aurora A and endogenous A kinase in a typical metaphase cell. Similar results were seen in multiple random mitotic cells. Scale bar =10 μm.

populated by the endogenous enzymes (*5, 31*). As depicted in Figure 6, panel B, overexpressed WT Aurora A was readily detected by immunofluorescence using a Myc antibody in Tet-exposed cells, where it localizes to both spindle poles and proximal microtubules, the same subcellular localization pattern detected by antibodies recognizing endogenous Aurora A. As detailed in merged images, Myc-tagged Aurora A co-localizes with endogenous Aurora A in metaphase cells. Consistently, we found that T217D, T217E, and T217D/W277E Aurora A mutants also exhibit co-localization with endogenous Aurora A on both mitotic spindle and centrosomes (Figure 6, panels C–E).

The autophosphorylation of Thr288 is a validated Aurora A activity biomarker in human cells (*24, 30, 31*). To investigate Aurora A phosphorylation in intact mitotic cells, we fixed an asynchronous cell population expressing either WT or T217D Aurora A. As detailed in Supplementary Figure S6, Tet exposure induces co-localization of both Myc-WT and Myc-T217D Aurora A staining with that of pThr288 Aurora A, thus defining a correctly activated pool of exogenous WT and T217D Aurora A at the centrosome. We next assessed the effects of MLN8054 exposure on an Aurora A activity biomarker in WT and T217D Aurora A cell lines. As shown in Supplementary Figure S6, the nocodazole-induced phosphorylation of both Myc-WT Aurora A (asterisks) and endogenous Aurora A at Thr288 is sensitive to MLN8054 (top panel), with Tet-induced Myc-Aurora A clearly resolved from the endogenous protein (middle panels). MLN8054 does not decrease the phosphorylation of the Aurora B substrate Histone H3 on Ser10, suggesting it does not detectably inhibit Aurora B at these concentrations. In contrast, in Tet-exposed cells expressing Myc-T217D Aurora A, the nocodazole-induced phosphorylation of overexpressed Aurora A at Thr288 is partially stabilized in a dose-dependent manner (asterisks), proving that drug-resistance to MLN8054 is rendered by expression of Asp at this position in cells. Finally, we investigated the phenomenon of MLN8054-induced changes in mitotic spindle morphology and integrity, which include an increase in monopolarity, a well-characterized Aurora A-dependent phenotype caused by a failure in centrosome separation (*22, 24, 31, 39*). As detailed in Supplementary Figure S7, we found that induction of the partially drug-resistant T217D mutant confers some resistance to the defective spindle phenotypes induced by 500 nM MLN8054. For example, in the presence of Tet, the percentage of monopolar phenotypes decreases from 35% in WT Aurora A cells to 26% in T217D cells

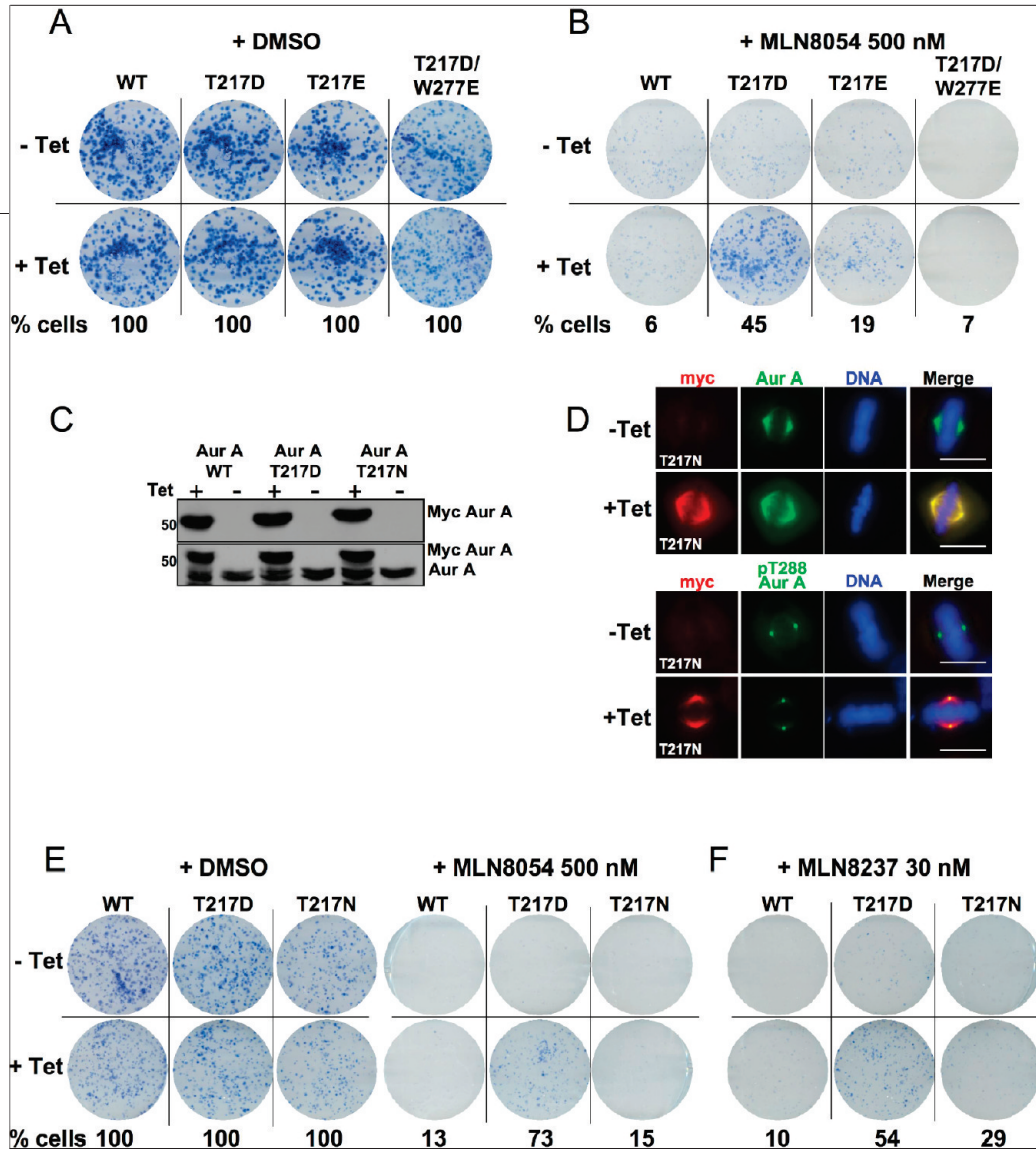

Figure 7. MLN8054/MLN8237-resistant Aurora A expressing cell lines. Stable HeLa cell lines expressing equal levels of WT, T217D, T217E, or T217D/W277E Aurora A were plated at low density, as described in Methods. Tet (+Tet) or solvent (−Tet) was added overnight prior to the addition of compound and was reapplied on the third day. Each well was treated with either DMSO (A) or 500 nM MLN8054 (B) on day 1 of the experiment, and cells were cultured under identical conditions for 10 days, at which point they were fixed and stained with methylene blue to reveal colonies, which were quantified as described in the experimental procedures. C, D) Aurora A induction. Mock or Tet-exposed cell extracts were blotted with Myc or Aurora A antibodies (C), and T217N cells were processed for immunofluorescence (D), by staining with Myc, pan-Aurora A, or pThr288 Aurora A antibodies. E, F) The effects of T217D and T217N Aurora A mutations were directly compared to WT Aurora A-expressing cells. Each well was treated with either DMSO or 500 nM MLN8054 (E) or 30 nM MLN8237 (F) on day 1 of the experiment and cells were cultured for 8 days, at which point they were fixed. For all colony assays, an area encompassing >90% of the colonies per dish is shown. Similar results were seen in two independent duplicate experiments. Bars in panel D = 10 μm.

and the percentage of bipolar (normal) spindles increases from 12% in WT Aurora A cells to 29% in T217D cells exposed to the drug. Together with our pT288 Aurora A biomarker analysis, these data demonstrate that T217D cells are partially resistant to MLN8054 when Aurora A-dependent phenotypes induced after acute exposure to the compound are quantified.

Aurora A Is the Antiproliferative Target of MLN8054 in a Human Cell Line. We next evaluated the drug-sensitivity of our panel of Aurora A-expressing cancer cell lines using a validated cell viability assay (*31*), in which the ability of cells to proliferate and form colonies after extended exposure to MLN8054 is assessed. As shown in Figure 7, panel A, Tet-exposed cells individually expressing the four Aurora A transgenes all form colonies in the presence of DMSO (100% growth). However, after incubation with 500 nM MLN8054 for 10 days, cells that had not been exposed to Tet were largely eliminated (Figure 7, panel B). In contrast, Tet-exposed cells expressing the T217D mutant proliferated

to 45% of control levels, whereas T217E Aurora A mutants grew to 19% of controls in the presence of 500 nM MLN8054; under these conditions WT or T217D/W227E expressing colonies were largely absent (Figure 7, panel B). To investigate the ability of MLN8054 to discriminate between Thr217 side chains of similar size, but differing chemistry, we generated new cell lines stably expressing Thr (WT), Asp or Asn Aurora A (Figure 7, panel C). After confirming that a T217N mutant was correctly localized and activated on centrosomes (compare Figure 7, panel D and Supplementary Figure S6), we analyzed side-by-side cell proliferation. Interestingly, only an Aurora A mutant bearing a negative charge can support growth, because Tet-exposed T217N Aurora A cells, like WT Aurora A cells, are unable to grow in the presence of MLN8054 (Figure 7, panel E). Remarkably, we found a similar pattern of resistance toward the clinically advanced compound MLN8237, with cell growth evident after exposure to 30 nM MLN8237 in T217D but not WT or T217N-expressing cells (Figure 7, panel F). The increased potency of MLN8237 compared to MLN8054 in cells contrasts with similar *in vitro* effects on Aurora A (Figure 1, panel B) and might signify an improved cellular permeability or stability of this inhibitor.

T217D and T217N Aurora A mutations induce partial resistance to VX-680 *in vitro* (Supplementary Figure S2), although the effects of this compound in cells expressing these mutations are unknown, and the critical target of VX-680 in HeLa cells is thought to be Aurora B rather than Aurora A (*31*). We therefore investigated the effects of VX-680 on cells expressing WT, T217N, or MLN8054-resistant T217D Aurora A, by comparing proliferation to an expression level-matched drug-resistant G160L Aurora B mutant (Figure 6, panel A). Interestingly, exposure to 50 nM VX-680 for 10 days kills WT, T217N, and T217D Aurora A-expressing cells (Supplementary Figures S8A and S9). In contrast, Tet-exposed G160L Aurora B control cells grow in the presence of the drug (Supplementary Figure S8B,C) confirming that Aurora B rather than Aurora A is the antiproliferative target of VX-680.

Biological Implications. Aurora A and B are overexpressed in a variety of human tumors (*11, 15*), making both enzymes potential anticancer targets. However, predominant Aurora B inhibitory cell cycle phenotypes are observed upon exposure to dual Aurora A and B inhibitors such as VX-680 (*21, 22, 29, 31, 43*), and cycling human cells are surprisingly insensitive to low levels of exogenous kinase-inactive Aurora A, in marked contrast to Aurora B (*39*). In addition, the validation of Aurora B as the critical target for both ZM447439 (*29*) and VX-680 (*31*) has fueled controversy surrounding the relative merits of Aurora A versus B inhibition and the cytotoxic target of MLN8054 (*15, 44*). Significantly, by employing a drug-resistance strategy, we now prove unequivocally that the critical target of MLN8054 and MLN8237 is Aurora A, rather than Aurora B or a separate "off-target" protein kinase. These findings validate our *in vitro* drug-resistance inhibitor screen and establish a common cellular mechanism and kinase target for both these compounds. Moreover, they endorse the potential significance of validating mechanistically diverse Aurora A inhibitor classes as potential anticancer agents.

In this study we identify two well-tolerated, partially drug-resistant mutants, T217D and T217E, which dominantly induce drug resistance even in the presence of endogenous (drug-sensitive) Aurora A (Figure 7). This strengthens the assertion that subtle changes in drug sensitivity are sufficient for cultured cells to signal, survive, and proliferate in the presence of Aurora kinase inhibitors, as previously demonstrated for more highly drug-resistant Aurora A and B mutants (*29, 31, 39*). We were surprised to find that a T217D/W277E double mutant does not induce cellular resistance to MLN8054 or MLN8237 in cells (Figure 7), given its high levels of drug resistance and enzyme activity *in vitro* (Figure 5). We speculate that despite normal subcellular targeting (Figure 6), unknown factors, perhaps triggered by the significantly lower K_m [ATP] for this mutant in the presence of TPX2 (Table 2), might create a nonfunctional cellular mutant. This finding reiterates the need for a careful evaluation of resistance mutations, to predict signaling viability in intact cells (*5*). In a slightly different sense, this has recently been shown in dramatic fashion for the epidermal growth factor receptor (EGFR) kinase, whereby a T790 M mutation that substantially increases the affinity for ATP *in vitro* also appears to account for the potent drug resistance of an oncogenic L858R EGFR mutant in cells (*45*).

In the future, it will be important to assess whether resistance to Aurora A inhibitors such as MLN8237 can also originate in inhibitor-exposed human subjects, in an analogous manner to compounds targeting tyrosine kinases (*46*). However, the sustained sensitivity of MLN8237-resistant cells to Aurora B inhibitors such as VX-680 (Supplementary Figures S8 and S9), demonstrates that resistance-determining mutations in Aurora

A might still be overridden effectively by inhibition of Aurora B, potentially advocating multitargeted inhibitor strategies to counter drug resistance. In any case, our validation of Aurora A as a principle antiproliferative target for MLN8054 and MLN8237 goes some way toward proving that this kinase does indeed represent a suitable target for pharmacological intervention in its own right.

METHODS

Crystallographic Procedures. cDNA encoding residues 125−391 of human Aurora A was ligated into the polyhedron promoter baculovirus expression vector pSARBac[TEV] (Sareum plc, Cambridge, U.K.) encoding a tobacco etch virus (TEV) cleavable N-terminal 6-His affinity tag. Recombinant baculovirus was generated by recombination in Sf9 cells. After amplification, expression was performed for 3 days at 27 °C. Aurora A was purified by NiNTA affinity (Qiagen) followed by size exclusion chromatography and tag removal with TEV protease (Invitrogen). Aurora A was exchanged into 0.1 M Bicine (pH 9.0), 20% PEG MME 550, and 0.1 M NaCl and then concentrated to 10 mg mL^{-1}. After addition of 1 mM adenosine, crystallization was performed using the hanging drop vapor diffusion method, by placing 1 μL of protein solution + 1 μL of reservoir solution over 0.5 mL of reservoir solution (0.2 M K_2HPO_4/20% (w/v) PEG3350/1 mM Tris(hydroxypropyl)phosphine) in 24-well plates at 9 °C. After 7 days, Aurora A crystals were soaked in reservoir solution containing 20% glycerol and 1 mM MLN8054 for 8 h at 9 °C. To improve resolution, crystals were briefly transferred to crystallization solution containing 40% glycerol and 1 mM MLN8054 for 30 min prior to flash freezing in liquid nitrogen. X-ray data were collected at 100 K on a Rigaku Saturn 944 CCD area detector mounted on a 007HF generator with an XStream 2000 cryosystem and VariMax optics. Crystals belonged to the space group $P6_122$, with unit cell parameters $a = 83.21$, $b = 83.21$, and $c = 167.24$ and routinely diffracted to 2.91 Å. Data were integrated, scaled, and merged with MOSFLM and SCALA (*47*). The structures were solved by molecular replacement using Aurora A (PDB 1MQ4) as the model. The final models were produced by iterative cycles of model building and refinement with COOT (*48*) and REFMAC5 (*49*), respectively. TLS parameters used in refinement were generated with the TLSMD server and ligand geometry and restraints were generated with the Dundee PRODRG server. Model quality was assessed with Molprobity (*50*) and Procheck (*51*). All structural figures were prepared with PyMOL (http://www.pymol.org). Data-processing and refinement statistics are summarized in Table 1. Atomic coordinates and structure factors have been deposited in the PDB (ID 2X81).

Molecular Biology and *E. coli* Protein Expression. Full length, N-terminal 6His-tagged human Aurora A or plasmids encoding the appropriate mutations were generated by standard PCR mutagenesis in the vector pET28a. Recombinant kinases were produced in *Escherichia coli* strain BL21 (DE3) pLysS (Novagen), affinity purified with Talon beads, dialyzed, and stored at −80 °C. cDNA encoding doubly Myc-tagged full-length human Aurora A was cloned into the BamH1/Not1 sites of the Tet-responsive vector pcDNA5-FRT-TO (*31*), and T217D, T217N, T217E, and T217D/W277E mutants were obtained by PCR mutagenesis. All DNA mutations were confirmed by sequencing of the entire coding region.

Protein Kinase Assays and Inhibitors. VX-680 and MLN8054 were synthesized according to published procedures. ZM447439 was purchased from Tocris and MLN8237 was purchased from Selleck. Chemical structures of these compounds are presented in Figure 1, panel A. To measure Aurora A activity, 25 ng (12.5 nM final concentration, Figure 1, panel B and Supplementary Figure S1) or 250 ng (125 nM final concentration, all other assays) of purified bacterially expressed Aurora A was assayed in the presence of the appropriate inhibitors, using Histone H3 as substrate for 20 min at 30 °C in the presence of 100 μM [γ-^{32}P] ATP. For Aurora A/TPX2 assays, 50 ng of a TPX2 [1−43] peptide, representing a 2-fold molar excess over Aurora A, was included. The Aurora A/TPX2 complex was preformed in kinase reactions prior to subsequent addition of inhibitors and ATP. For Plk4 assays, 250 ng of bacterially expressed, purified His-tagged human catalytic domain (amino acids 1−269) was assayed in the presence of the appropriate inhibitors, using myelin basic protein (MBP) as substrate for 20 min at 30 °C in the presence of 100 μM [γ-^{32}P] ATP. To assess Histone H3 and MBP phosphorylation, radiolabel incorporation was quantified by Cerenkov counting of phosphorylated substrates on p81 phosphocellulose paper, or by phosphorimager after SDS-PAGE. Each experiment was repeated at least three times, with similar results seen on each occasion. To determine the K_m [ATP] value for Aurora A and mutants, nonlinear regression analysis was performed on data collated over a range of 1 and 200 μM of [γ-^{32}P] ATP (specific activity 500 cpm $pmol^{-1}$). Data analysis was performed using Prism software.

Antibodies. Sheep pan-Aurora A antibodies were kindly provided by Professor Stephen Taylor (University of Manchester). Aurora B antibodies were from Bethyl. Monoclonal Myc antibodies were from Cancer Research UK. Fluorescent secondary antibodies were from Jackson Immunochemicals. pThr288 Aurora A and pSer10 Histone H3 phosphospecific antibodies have been described previously (*22*).

Cell Culture, Transfection, Phenotypic, and Cellular Proliferation Assays. pCDNA5 FRT/TO encoding Myc-tagged Aurora A transgenes and pOG44 plasmid were transfected with Lipofectamine 2000 (Invitrogen) into parental Zeocin-maintained HeLa cells (*31*). Stable lines were generated *via* Flp/FRT-mediated recombination and selected using blasticidin. Aurora A transgene expression was induced by the addition of 1 μg mL^{-1} Tet to the culture medium. To induce Aurora A activation, cells were incubated with nocodazole for 12 h prior to lysis. DMSO or MLN8054 was added at the indicated concentrations in the presence of 20 μM MG132 (Sigma) for 2 h. Cleared HeLa cell supernatants were prepared for Western blotting with pThr288 Aurora A, Aurora A, or pS10 Histone H3 antibodies as previously described (*22*). To visualize proteins, HeLa cells were fixed in methanol at −20 °C and stained using standard immunofluorescence protocols. For spindle analysis, Tet-exposed HeLa cells were incubated with 500 nM MLN8054 for 2 h prior to fixation. Spindle polarity (100 cells per analysis) was quantified as described in the legend to Supplementary Figure S7. For colony-formation assays, ~1,000 HeLa cells were plated in a dish and induced with Tet overnight, prior to mock (DMSO) or drug treatment (MLN8054, MLN8237, or VX-680) in the continued presence of Tet, with fresh medium and supplements at the experimental half way stage. After 8−10 days, cells were fixed and stained with 1% (w/v) Methylene Blue in 80% (v/v) methanol to permit visualization of cell colonies, and cells were quantified by dissolving in 0.1 M NaOH prior to analysis at 600 nm.

Accession Codes: The coordinates for the Aurora A:MLN8054 complex have been deposited in the PDB with accession number 2X81.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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