Binding of TPX2 to Aurora A Alters Substrate and Inhibitor Interactions

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ABSTRACT: The Aurora kinases are a family of serine/threonine kinases involved in mitosis. The expression of AurA is ubiquitous and cell cycle regulated. It is overexpressed in many tumor types, including breast, colon, and ovarian. TPX2 is a binding partner and activator of AurA. A fragment of TPX2 (residues 1−43) has been shown to be sufficient for binding, kinase activation, and protection from dephosphorylation. We have shown that the addition of TPX2(1−43) increases the catalytic efficiency of AurA. While TPX2 binding has no effect on the turnover number of AurA and does not change the reaction mechanism (characterized here to be a rapid equilibrium random mechanism), it increases the binding affinity of both ATP and a peptide substrate. We have also demonstrated differences in the inhibitor structure−activity relationship (SAR) in the presence or absence of TPX2(1−43). To better understand the differential SAR, we carried out computer modeling studies to gain insight into the effect of TPX2 on the binding interactions between AurA and inhibitors. Our working hypothesis is that TPX2 binding decreases the size and accessibility of a hydrophobic pocket, adjacent to the ATP site, to inhibitors.

Aurora kinases (AurA,[1] -B, and -C) are a group of Ser/Thr kinases that play key roles in mitosis and cytokinesis (*1*−*3*). AurA mainly associates with the centrosome and the spindle microtubules during mitosis and functions in centrosome maturation, spindle assembly, and the maintenance of spindle bipolarity. It is also involved in mitotic checkpoint control. AurB has a more "equatorial" localization and is involved in chromosome alignment and segregation during mitosis and the formation of the cleavage furrow during cytokinesis. AurC is strongly expressed in the testis and plays a role in spermatogenesis. Recent findings suggest that AurC can complement AurB functions in mitosis and is also required for cytokinesis (*4*).

Like AurB and -C, AurA is composed of an N-terminal regulatory domain and a C-terminal kinase domain. The kinase activity of AurA is tightly regulated throughout the cell cycle (*5*−*7*). AurA is activated through the phosphorylation of T288 (human sequence) on its activation loop. Likewise, AurA can be inactivated through dephosphorylation of T288 by protein phosphatase 1 (PP1). Beyond phosphorylation and dephosphorylation, AurA activity is also regulated by its expression and degradation. AurA expression peaks at the G2−M transition, which likely contributes to the entry of cells into the M phase. Upon mitotic exit, AurA is rapidly degraded by the anaphase-promoting complex/cyclosome (APC/C).

Cellular functions of AurA are mediated via a number of proteins involved in mitosis. Notably, a cellular component, target protein of XKlp2 (TPX2), has been shown to be an important binding partner and regulator of AurA (*8*−*11*). TPX2 binding not only prevents the inactivation of AurA but also increases the kinase activity of the activated AurA. The interaction between these two proteins was mapped to the N-terminus of TPX2 (residues 1−43 of the human sequence) and the C-terminal kinase domain of AurA (*9*). It has been shown that the activating effect of TPX2 is fully retained in a peptide representing the 43 N-terminal residues of the protein. The recently published cocrystal structure of AurA and TPX2(1−43) revealed conformational changes around the AurA active sites upon TPX2 binding (*9*). The hypothesis proposed on the basis of the structural information is that TPX2 facilitates substrate binding and prevents T288 dephosphorylation by PP1, leading to the stabilization of the active state of AurA and the enhancement of its kinase activity.

Many reports suggest that dysregulation of Aurora kinases contributes to tumorigenesis (*1*, *12*, *13*). AurA is frequently amplified and/or overexpressed in tumor malignancies. The oncogenic potential of AurA has also been demonstrated by the induction of colony formation in cell culture and tumors in nude mice after transfection of NIH3T3 and rodent Rat1 cell lines with AurA (*14*). Inhibition of AurA or AurB activity results in impaired chromosome alignment, abrogation of the mitotic checkpoint, polyploidy, and subsequent cell death in dividing tumor cells. To date, a number of potent

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[1] Abbreviations: AurA, Aurora A kinase; TPX2, target protein for *Xenopus* kinesin-like protein 2; SAR, structure−activity relationship; Thp, tris(hydroxypropyl)phosphine.

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Table 1: Synthetic Peptides Used in This Study[a]

peptide name	peptide sequence
peptide substrate	biotin-Ahx-RARRRLSFFFFAKKK-$CONH_2$
alanine-peptide	biotin-Ahx-RARRRLAFFFFAKKK-COOH
phosphopeptide	biotin-Ahx-RARRRLpSFFFFAKKK-COOH
TPX2(1−43)	acetyl-MSQVKSSYSYDAPSDFINFSSLDD-EGDTQNIDSWFEEKANLEN-OH

[a] Serine (underlined) in the substrate peptide is replaced by alanine and phosphoserine in alanine-peptide and phosphopeptide, respectively.

small molecule inhibitors of Aurora kinases have been reported and clinical trials of multiple Aurora inhibitors are ongoing (*15*−*20*).

Because of the importance of the Aurora kinases as anticancer targets, a better understanding of their kinetic mechanisms is needed to provide insights into how to develop better inhibitors of these enzymes. Given the role of TPX2 in modulating AurA activity, it is of interest to study the effect of TPX2 on AurA catalysis as well as on the interactions of inhibitors with the enzyme. Here we report data indicating that while TPX2 binding has no significant effect on the turnover number of AurA and does not change its reaction mechanism, it does increase the binding affinity of both ATP and of a peptide substrate. We also report changes in inhibitor affinity upon TPX2 binding. To better understand the differential SAR, we carried out computer modeling studies to gain insight into the effect of TPX2 on the binding interactions of AurA and inhibitors. Our working hypothesis is that TPX2 binding decreases the size and accessibility of a hydrophobic pocket, adjacent to the ATP site, to inhibitors.

MATERIALS AND METHODS

ATP was purchased from Sigma-Aldrich. [γ-^{33}P]ATP (10 mCi/mL in 10 mM Tricine) was purchased from Perkin-Elmer. The peptide substrate and inhibitors (Table 1) were custom synthesized by 21st Century Biochemicals. TPX2-(1−43) was synthesized by California Peptide Research, Inc. All peptides are >95% pure and were identified by mass spectrometry and amino acid analysis. The lyophilized peptide powder was resuspended in deionized water, aliquoted, and stored at −80 °C. Peptide concentrations were determined by amino acid analysis. All other reagents are analytical grade or higher.

Construct Design and Expression of Full-Length His6-Thrombin-Aurora A Kinase 2-403. Full-length Aurora A kinase 2-403 was modified by PCR to remove the methionine and to add a thrombin (Thr) cleavage site to the 5′ end. The plasmid for Aurora A kinase 2-403 (His6-Thr-AurA) was constructed by the Gateway cloning system (Invitrogen, Carlsbad, CA). Protein expression was performed in Sf9 insect cells using the Bac-To-Bac baculovirus expression system (Invitrogen). Okadaic acid (Calbiochem, catalog no. 495604) at a concentration of 5 nM was added to the culture 4 h before it was harvested to retain the kinase activity of AurA by preventing dephosphorylation (*21*).

Purification of Full-Length His6-Thr-AurA Kinase 2-403. Baculovirus Sf9 cells containing expressed His6-Thr-Aurora A kinase 2-403 were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM 2-mercaptoethanol, 50 nM okadaic acid, 1 mM tris(hydroxypropyl)phosphine (Thp), 10 mM NaF, 10 mM glycerol phosphate, 0.1 mM sodium vanadate, protease inhibitor cocktail for polyhistidine-tagged proteins (Sigma catalog no. P8849), and phosphatase inhibitor cocktail (Sigma catalog no. P2850). The cells were lysed by sonication. The lysate was centrifuged at 30000*g* for 30 min at 4 °C. The supernatant was mixed with equilibrated Ni-NTA agarose and incubated at 4 °C for 1 h. The mixture was column packed, and unbound proteins were eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 5 mM 2-mercaptoethanol. Bound protein was then eluted via a stepwise imidazole gradient of 10, 30, and 300 mM in the same buffer. Batches were analyzed by SDS−PAGE, and desired protein batches were pooled and dialyzed against buffer [50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 2 mM DTT] to remove imidazole. N-Terminal amino acid sequencing confirmed protein identity. Western blot analysis and LC−MS indicated that the protein was phosphorylated. The level of T288 phosphorylation was determined by site-specific phosphorylation analysis of partially trypsin digested AurA by LC−MS to be 27%.

Aurora A Kinase Assay. A filter binding assay was used to measure the kinase activity of AurA (with or without TPX2). The assay was typically run in a buffer containing 50 mM HEPES (pH 7.5), 0.01% Tween 20, 4 mM $MgCl_2$, 25 mM KCl, 0.15 mg/mL BSA, and 5 mM DTT. The concentrations of AurA and TPX2 were 2 and 40 nM, respectively, unless otherwise specified. The concentrations of ATP, the peptide substrate, and inhibitors are given in the figure legends. [γ-^{33}P]ATP was included at a specific activity of 500−2000 CPM/pmol. All assays were carried out in a 96-well, half-area non-binding surface (NBS) plate (Corning) in a final volume of 40 μL/well. After a specific reaction time, reactions were quenched by the addition of 40 μL of 1% phosphoric acid, and mixtures were transferred to 96-well MultiScreen-PH filter plates with phosphocellulose cation exchange paper membranes (Millipore) pre-equilibrated with a 0.5% phosphoric acid solution. The transferred reaction mixtures were allowed to incubate in the plate at room temperature for 45 min and then filtered through the membrane using the MultiScreen Vacuum Manifold (96-well, Millipore). Plates were washed five times with 100 μL of a 0.5% phosphoric acid solution per well and dried in a 50 °C oven for 30 min. Then 50 μL of scintillation cocktail (MicroScint 20, Perkin-Elmer) per well was added, and the radioactivity retained on the filter membrane was measured with a 1450 MicroBeta liquid scintillation and luminescence counter (Perkin-Elmer). A small volume of the reaction mixture containing a known amount of ATP was spotted in an empty well to calculate the specific activity of ATP which enabled the conversion of the radioactivity (counts per minute) into picomoles of ATP.

For the determination of IC_{50} values, inhibitor dilution was carried out in polypropylene plates (Corning Costar) in dimethyl sulfoxide (DMSO). The dilution mix (1 μL) was then spotted onto the NBS plates, followed by the addition of the reaction mix containing ATP and the peptide substrate. AurA (with or without TPX2) was added to initiate the reaction (final volume of 40 L). The reactions were quenched after 15 min, and the product was quantified as described above.

Computer Modeling of Binding of Inhibitors to AurA. Binding mode predictions for GW801372X, VX-680, GSK623906A, and AstraZeneca compound 13 (*17*) were generated using the protein docking program Flo+ (version 0802) (*22*). The crystal structure of AurA with the T288D (specified as T287D in ref *17*) activating mutation and a 5-aminopyrimidinyl quinazoline inhibitor (termed AZ compound 13 in the rest of this paper, PDB entry 2c6e) reported by AstraZeneca (*17*) was used for docking after removal of the inhibitor from the active site. All docking calculations performed 1000 Monte Carlo search cycles, allowing full perturbation of the ligand with constraints placed on the atoms in the hinge binding region (residues 209−214), and relaxation of residues Lys 162, Leu 194, and Leu 210 during the minimization steps. The top 25 poses for each calculation were put into a MOE database, viewed in the presence of the protein, and further minimized in MOE using the OPLS-AA force field with the Generalized-Born solvation model. During minimization, the small molecule and the complete side chains of residues within 3.5 Å of it were allowed to move. The lowest-energy poses for GSK623906A and VX-680 were compared to the AurA−AZ compound 13 [PDB entry 2c6e (*17*)] and AurA−VX-680 (*23*) crystal structures.

The superposition of the different AurA crystal structures was achieved by superimposing on the hinge loop and two β-strands at the bottom of the ATP pocket (residues 209−214, 262−264, and 270−272).

Data Analysis. For the TPX2 titration experiment, the initial rates at various TPX2 concentrations were fitted to eq 1 describing one-site binding (*24*)

$$V = (V_b - V_a)K_d/(K_d + L) + V_a \quad (1)$$

where V is the measured initial rate, V_a and V_b represent the rates of AurA and AurA−TPX2, respectively, and K_d and L represent the dissociation constant for AurA and TPX2 and the concentration of TPX2, respectively.

For the substrate double-titration experiment, the initial rates of AurA reaction at various ATP and peptide substrate concentrations were measured and fitted to eq 2 (*24*, *25*)

$$V/[E_0] = k_{cat}[A][B]/(K_{ia}K_b + K_a[B] + K_b[A] + [A][B]) \quad (2)$$

where $[E_0]$, [A], and [B] represent the enzyme, ATP, and peptide substrate concentrations, respectively, k_{cat} is the turnover number, K_a and K_b are the Michaelis constant for ATP and the peptide substrate, respectively, and K_{ia} is the dissociation constant for ATP.

For the inhibition studies, initial rates of the AurA reactions were measured at varying fixed concentrations of product and dead-end inhibitors. The data were fitted to eq 3 (*24*, *25*)

$$V/[E_0] = k_{cat}[S]/[K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})] \quad (3)$$

where K_{is} and K_{ii} are the dissociation constants for inhibitors from the free enzyme and the enzyme−substrate complex, respectively, and [I] is the inhibitor concentration. An α value, representing the K_{ii}/K_{is} ratio, is generated from this fitting. The inhibitor is considered competitive, noncompetitive, or uncompetitive when $\alpha > 10$, $\alpha = 0.1-10$, or $\alpha < 0.1$, respectively.

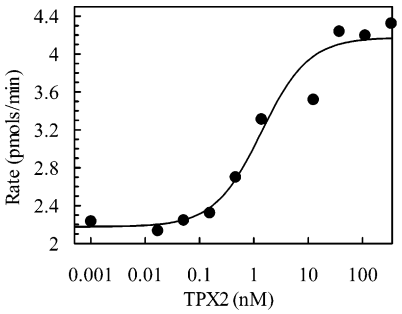


FIGURE 1: Effect of synthetic TPX2(1−43) on the kinase activity of AurA. TPX2 was titrated over a concentration range of 0−1000 nM into reaction mixtures with 20 μM ATP, 20 μM peptide, and 5 nM AurA. The initial velocities of the AurA kinase reactions are plotted vs TPX2 concentration. The data were fitted to eq 1 describing a one-site binding model.

The dose−response curves of AurA inhibitors were fitted to eq 4 to generate the IC_{50} values (*24*).

$$y = 100\%/[1 + ([I]/IC_{50})^h] \quad (4)$$

In this equation, y is the percent activity and h is the Hill coefficient.

The inhibition constant K_i was determined using eq 5 on the basis of the measured IC_{50} and the competitive mode of inhibition.

$$IC_{50} = K_i(1 + [S]/K_m) + E/2 \quad (5)$$

In this equation, [S] is the substrate concentration and E the AurA concentration.

RESULTS

Effect of TPX2 on AurA Kinase Activity. To determine the effect of TPX2 on AurA kinase activity and to measure the constant of binding of these two proteins to each other, the activity of human AurA, expressed as an active, phosphorylated form, was determined at various concentrations of the TPX2 peptide containing residues 1−43 of the TPX2 protein. It has been shown previously that truncated TPX2-(1−43) is necessary and sufficient for interaction with AurA and stimulation of its kinase activity. To detect possible changes in substrate binding affinity induced by the formation of the AurA−TPX2 complex, the assay was run with both substrate concentrations (ATP and peptide) close to their K_m values measured in the absence of TPX2. The peptide substrate was selected from a peptide library screening against AurA. It contains the AurA consensus recognition sequence R/K/N-R-X-S/T-B, where B denotes any hydrophobic residue except Pro (*26*). The initial velocity of the kinase reaction was measured and plotted against TPX2 concentrations (Figure 1). An approximately 2-fold activation of AurA was observed with a saturating amount of TPX2 (1 μM). The association between AurA and TPX2 is rapid since no significant change in the data was observed when a 30 min preincubation of these two proteins was included before the addition of the substrates (data not shown).

Our data were fitted to eq 1 describing one-site binding, yielding a K_d of 2.3 ± 0.8 nM (Figure 1). This value suggests a fairly tight binding between the two proteins. However, the TPX2 titration curve shows some upward curvature at

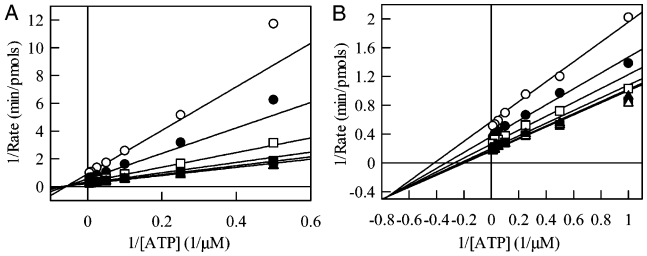


FIGURE 2: ATP and peptide substrate double titrations for AurA and the AurA−TPX2 complex. (A) Double-reciprocal plot of 1/rate vs 1/[ATP] for AurA at six fixed peptide concentrations: (○) 4, (●) 8, (□) 20, (■) 50, (△) 100, and (▲) 200 μM. (B) Double-reciprocal plot of 1/rate vs 1/[ATP] for the AurA−TPX2 complex at six fixed peptide concentrations: (○) 2, (●) 4, (□) 8, (■) 20, (△) 50, and (▲) 100 μM. The untransformed data were fitted to eq 2 and these fits were used in construction of the double-reciprocal plots.

Table 2: Kinetic Constants of AurA-Catalyzed Reactions Using ATP and the Peptide Substrate in the Presence or Absence of TPX2[a]

substrate	TPX2	K_m (μM)	K_d[b] (μM)	k_{cat} (min^{-1})
ATP	no	13.3 ± 1.1	18.3 ± 4.0	66.7 ± 1.3
peptide	no	14.2 ± 1.2	19.5 ± 4.3	
ATP	yes	5.3 ± 0.4	1.3 ± 0.7	85.3 ± 1.7
peptide	yes	5.4 ± 0.4	1.3 ± 0.7	

[a] Experiments were carried out under conditions described in Materials and Methods and in the legend of Figure 2. Kinetic constants were generated by fitting data to eq 2. [b] K_d values represent the K_{ia} values fitted from eq 2.

higher TPX2 concentrations (hundreds of nanomolar), suggesting that there may be some nonspecific interactions between TPX2 and AurA under these conditions. The quality of our data does not justify fitting to a two-site binding model; therefore, we consider the K_d value of 2.3 nM to be estimate of the true binding affinity of TPX2 for AurA.

We also expressed an N-terminally GST tagged version of TPX2(1−43) in *Escherichia coli* and analyzed its binding to AurA. The results were virtually identical to those using synthetic TPX2(1−43) (data not shown). Synthetic TPX2 was used for all subsequent experiments.

Substrate Double Titration of AurA and the AurA−TPX2 Complex. The experiment described above confirms the stimulating effect of TPX2 on AurA activity and also provides an estimate of the binding affinity between these two proteins. Since the experiment was carried out at concentrations of both substrates close to their K_m values, this stimulating effect could be a result of an increased turnover number, an increased substrate binding affinity, or both, in the presence of TPX2. To further investigate the effect of TPX2 on AurA catalysis, we carried out substrate double-titration experiments with either free AurA or the AurA−TPX2 complex. TPX2, when present, was at 40 nM ($20K_d$) to ensure almost complete formation (>95%) of the AurA−TPX2 complex. Intersecting double-reciprocal plots are obtained for reactions catalyzed by AurA and the AurA−TPX2 complex, indicating that both enzyme forms follow a sequential, rather than a ping-pong, kinetic mechanism (Figure 2) (*24*, *25*). The kinetic constants generated in these experiments are summarized in Table 2. The V_{max} value is not affected significantly by TPX2. However, when TPX2 binds, the K_m values for ATP and the peptide substrate are reduced by 2.5- and 2.6-fold, respectively, and their K_d values are lowered by 14- and 14.7-fold, respectively. These results indicate that TPX2 binding induces an AurA conformation with enhanced affinity for both substrates, a conclusion that agrees with the crystal structure (*9*). A negative binding synergism ($K_m/K_d = 4$ for ATP and the peptide substrates) between the two substrates is observed with the AurA−TPX2 complex that is absent with free AurA. This suggests that TPX2 binding induces communication between the two substrate binding sites. We believe that the increase in AurA activity by TPX2 is not likely due to additional T288 phosphorylation for two reasons. First, the reaction time course is linear under all experimental conditions and does not show any increase in reaction rate over time. Second, if more AurA is activated, we would expect to see an increase in k_{cat}, which was not observed.

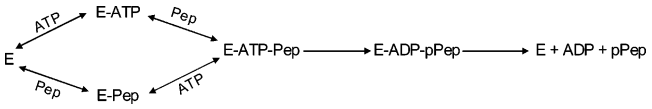
Table 3: Inhibition Constants for the Inhibition of AurA in the Presence or Absence of TPX2[a]

variable substrate	TPX2	inhibitor	K_{is} (μM)	K_{ii} (μM)	α	inhibition pattern[d]
ATP	no	ADP	13 ± 1	270 ± 80	21	C
peptide	no	ADP	25 ± 5	32 ± 3	1.3	NC
ATP	no	staurosporine	2.3 ± 0.3	57 ± 20	25	C
peptide	no	staurosporine	2.9 ± 0.5	9.3 ± 1.1	3.2	NC
ATP	no	A-peptide	180 ± 18	160 ± 6	0.9	NC
peptide	no	A-peptide[b]	65 ± 20	740 ± 350	11	C
ATP	no	P-peptide	320 ± 40	450 ± 30	1.4	NC
peptide	no	P-peptide	270 ± 60	1300 ± 200	4.8	NC
ATP	yes	ADP	6.9 ± 0.7	>1000		C
peptide	yes	ADP	20 ± 4	32 ± 2	1.6	NC
ATP	yes	staurosporine	6.1 ± 1.0	>1000		C
peptide	yes	staurosporine	14 ± 3	24 ± 2	1.7	NC
ATP	yes	A-peptide	150 ± 30	190 ± 10	1.3	NC
peptide	yes	A-peptide	50 ± 5	700 ± 100	14	C
ATP	yes	P-peptide	500 ± 200	300 ± 20	0.6	NC
peptide	yes	P-peptide	90 ± 9	540 ± 50	6	NC
ATP[c]	yes	P-peptide	800 ± 200	520 ± 30	0.7	NC

[a] Inhibition experiments were carried out as described in Materials and Methods and in the text. Two product inhibitors, ADP (0, 12, 30, and 75 μM) and P-peptide (0, 120, 300, and 750 μM against AurA and 0, 80, 200, and 500 μM against the AurA−TPX2 complex), and two dead-end inhibitors, staurosporine (0, 4.8, 12, and 30 μM) and A-peptide (0, 48, 120, and 300 μM), were used in the inhibition studies. Data were fitted to eq 3 to generate inhibition constants and the α values, on the basis of which the inhibition pattern was determined. [b] A-Peptide inhibition against peptide substrate with AurA was carried out at 0, 30, 60, and 120 μM. [c] Reaction carried out at a saturating peptide substrate concentration (100 μM). [d] C, competitive; NC, noncompetitive.

Inhibition of AurA and the AurA−TPX2 Complex by ADP and Staurosporine. Our substrate double-titration study suggested that AurA follows a sequential mechanism. However, these data alone cannot differentiate a random from compulsory binding order of the substrates. Product and dead-end inhibition studies therefore were carried out to further define the kinetic mechanism and to investigate whether the formation of an AurA−TPX2 complex changes the catalytic mechanism. The initial velocity of AurA reactions was measured at several, fixed inhibitor concentrations with one substrate at its K_m and the other titrated over a range of concentrations. The data were fitted to a mixed-type inhibition model to generate the α values (K_{ii}/K_{is}) which are summarized in Table 3. Inhibition is considered essentially competitive when the α value is >10, indicating a >10-fold higher affinity of the inhibitor toward free E than for the E−S complex. Product inhibition studies with AurA

Scheme 1: Rapid Equilibrium Random Kinetic Mechanism of AurA in the Presence or Absence of TPX2



indicate that ADP is competitive against ATP and noncompetitive against the peptide substrate ($\alpha = 1.3$). When tested with the AurA−TPX2 complex, ADP remains competitive against ATP and noncompetitive against the peptide substrate ($\alpha = 1.6$) (Table 3). Dead-end inhibition studies with staurosporine indicate that with AurA, staurosporine is competitive against ATP and noncompetitive against the peptide substrate ($\alpha = 3.2$). Likewise, when tested with the AurA−TPX2 complex, staurosporine remains competitive against ATP and noncompetitive against the peptide substrate ($\alpha = 1.7$) (Table 3). The noncompetitive pattern against the peptide indicates that staurosporine is able to bind to the enzyme before the peptide substrate. Taken together, these results limit the mechanistic possibilities to a rapid-equilibrium random mechanism or a compulsory ordered mechanism with ATP binding first and ADP being released last (*25*).

Inhibition of AurA and the AurA−TPX2 Complex by an Alanine-Peptide and a Phosphopeptide. To further distinguish between the rapid equilibrium random and compulsory ordered mechanism, peptide product inhibitor (phosphopeptide, P-peptide) and dead-end inhibitor (alanine-peptide, A-peptide) were used against AurA and the AurA−TPX2 complex to examine their inhibition patterns (Table 3). Both peptide inhibitors are noncompetitive against ATP ($\alpha = 1.4$ and 0.6 for P-peptide against AurA and the AurA−TPX2 complex, respectively; $\alpha = 0.9$ and 1.3 for A-peptide against AurA and the AurA−TPX2 complex, respectively) which in conjunction with the inhibition results for ADP and staurosporine suggest a rapid equilibrium random mechanism.

A rapid equilibrium random mechanism would predict that both the A-peptide and the P-peptide would be competitive against the peptide substrate. Indeed, the A-peptide is competitive against this substrate. However, experiments with the P-peptide show some degree of mixed-type inhibition versus the peptide substrate ($\alpha = 4.8$ and 6 for AurA and the AurA−TPX2 complex, respectively). This type of inhibition pattern would be consistent with a compulsory ordered mechanism but is inconsistent with the rest of our data.

Since the P-peptide is a fairly weak inhibitor, with a much weaker affinity for the enzyme than the substrate peptide, the inhibition experiments were carried out at high concentrations of the P-peptide. It is possible that the P-peptide may have nonspecific interactions with the enzyme or may form aggregates at high micromolar concentrations, resulting in the observed mixed inhibition. To further distinguish the rapid equilibrium random from the compulsorily ordered kinetic mechanisms, inhibition by P-peptide was carried out at a saturating peptide substrate concentration ($20K_m$). Under this condition, P-peptide should behave as an uncompetitive inhibitor against ATP if the reaction mechanism is compulsory ordered with ATP binding first (*25*). Our results indicate that P-peptide remains a mixed-type inhibitor against ATP with a similar α value (0.7) as obtained at a nonsaturating peptide substrate concentration (Table 3). Therefore, a rapid equilibrium random kinetic mechanism is most consistent with our data (Scheme 1). However, our results do not rule out a random mechanism with a preferred order of ATP binding first. Additional analysis needs to be carried out to test this mechanistic possibility.

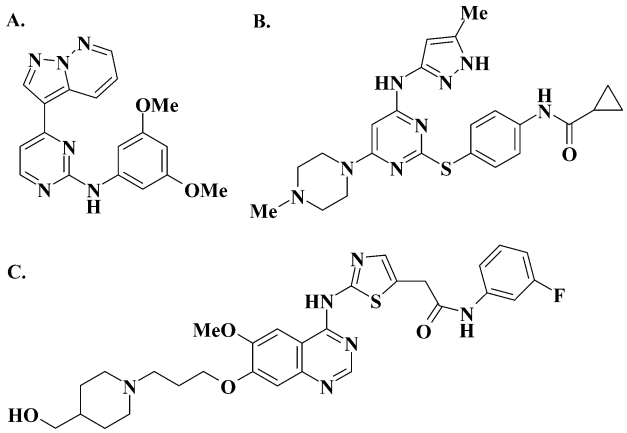


FIGURE 3: Chemical structures of AurA inhibitors GW801372X (A), VX-680 (B), and GSK623906A (C).

For both AurA and the AurA−TPX2 complex, identical inhibition patterns were observed, indicating that the presence of TPX2 does not change the kinetic mechanism of AurA-catalyzed reactions.

Effect of TPX2 on AurA Inhibition. Since TPX2 is a physiologically important binding partner of AurA and regulates AurA activity by altering the K_m for ATP and peptide substrates, it is of interest to investigate whether TPX2 binding alters the SAR of AurA inhibitors. We have determined the inhibitory potency of three AurA inhibitors, GW801372X, VX-680, and GSK623906A, against AurA and the AurA−TPX2 complex. The structures of these three compounds are shown in Figure 3. VX-680, an aminopyrazole and also known as MK0457, is a potent inhibitor of AurA, -B, and -C currently in clinical development by Merck & Co., Inc., and Vertex Pharmaceuticals Inc. (*16*). GSK632906A, a quinazoline, is a close analogue of an Aurora inhibitor described as compound 13 by AstraZeneca, a crystal structure of which has recently been reported (*17*). GW801372X is a pyrazolopyridazine compound from the GSK collection. It was reported to be a GSK3 inhibitor (*27*) that also inhibits AurA. Concentration−response plots for AurA inhibition by these three compounds are shown in Figure 4, and their K_i values calculated from eq 5 are listed in Table 4. Hill coefficients for all the inhibition curves are close to 1. While no shift in potency is observed for GW801372X, 4.2- and 18-fold IC_{50} (or K_i) shifts are observed for VX-680 and GSK623906A, respectively, both with a decreased potency against the AurA−TPX2 complex (Table 4).

Computer Modeling. The three compounds characterized in our biochemical assays described above were docked using the Flo+ program to the crystal structure of AurA with the T288D activating mutation and AZ compound 13 [PDB entry 2c6e (*17*)]. As GSK632906A is a close analogue of AZ compound 13, its docking was used as a test to see whether we could reproduce the crystallographic pose. The lowest-energy pose minimized in MOE has a rmsd of 0.8 Å with respect to AZ compound 13. For VX-680, since the crystal coordinates of the AurA−VX-680 complex have not yet been

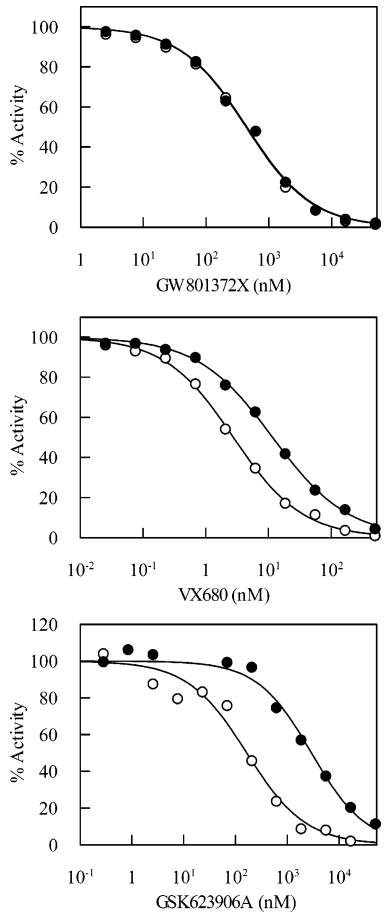


FIGURE 4: Effect of TPX2 on the potency of AurA inhibitors. IC_{50} values of three AurA inhibitors (GW801372X, VX-680, and GSK623906A) were determined for AurA (2 nM) in the absence or presence of TPX2 (40 nM). Concentrations of ATP and the peptide substrate were fixed close to their K_m values ([peptide] = 20 μM; [ATP] = 4 and 20 μM in the presence and absence of TPX2). Data were fitted to eq 4 to generate IC_{50} values [(○) data obtained in the absence of TPX2 and (●) data obtained in the presence of TPX2]. TPX2-induced IC_{50} shifts of 0-, 4.2-, and 18-fold were determined with GW801372X, VX-680, and GSK623906A, respectively.

Table 4: Inhibition Constant (K_i)[a] of Three AurA Inhibitors against AurA and the AurA−TPX2 Complex

compound	AurA K_i (μM)	AurA−TPX2 K_i (μM)	K_i (fold shift)	$\Delta\Delta G_{binding}$ (kcal/mol)[b]
GW801372X	0.22 ± 0.02	0.22 ± 0.01	1.0	0
VX-680	0.0014 ± 0.0001	0.0059 ± 0.0003	4.2	0.8
GSK623906A	0.082 ± 0.004	1.5 ± 0.2	18	1.7

[a] K_i values were calculated from eq 5. [b] Calculated at 25 °C from the K_i values (*34*).

released to the public, the rmsd between the predicted binding mode and the crystal structure cannot be determined. However, the lowest-energy pose does have an orientation similar to that in the crystal structure of the AurA−VX-680 complex reported by Cheetham et al. (*23*). Therefore, our model appears to predict the overall modes of binding of compounds to AurA. Figure 5 shows a comparison of the predicted binding modes of the three compounds. All three inhibitors characterized in our biochemical studies are ATP competitive inhibitors of AurA. Crystal structures of AZ compound 13 and VX-680 complexed with AurA as well

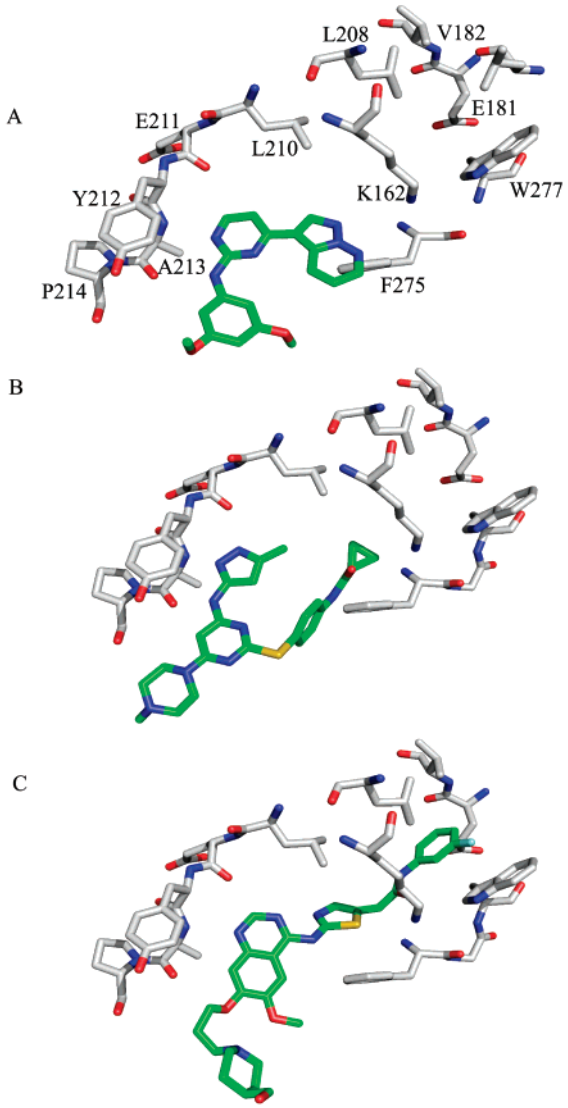


FIGURE 5: Comparison of binding modes of GW801372 (A), VX-680 (B), and GSK623906A (C) to AurA modeled on the basis of the AurA−AZ compound 13 structure (PDB entry 2c6e).

as computer modeling of the binding modes of GW801372X and GSK623906A indicate that GW801372X completely fits within the ATP site; however, VX-680 approaches the adjacent hydrophobic back pocket behind the catalytic lysine residue, K162, and GSK623906A fully occupies this back pocket (Figure 5).

The $\Delta\Delta G_{binding}$ values, calculated using K_i measurements from our biochemical studies (Table 4), correlate with the degree to which the inhibitors access this hydrophobic pocket. To accommodate compounds like GSK623906A or AZ compound 13 that reach into the back pocket, the C helix must shift outward. Since this helix is sandwiched between the two regions of TPX2, its movement is restricted when TPX2 is bound to AurA. As shown in Figure 6, the outward movement of the C helix causes steric clashes of its residues (for example, R179 and H176) with TPX2. The restricted movement of this C helix in the presence of TPX2 results in a reduction in the size and accessibility of the back pocket, as shown by the comparison of the crystal structures of the AurA−TPX2−ADP (PDB entry 1ol5) and AurA−AZ compound 13 (PDB entry 2c6e) complexes (Figure 7A,B). Our modeling based on the AurA structure suggests that the three

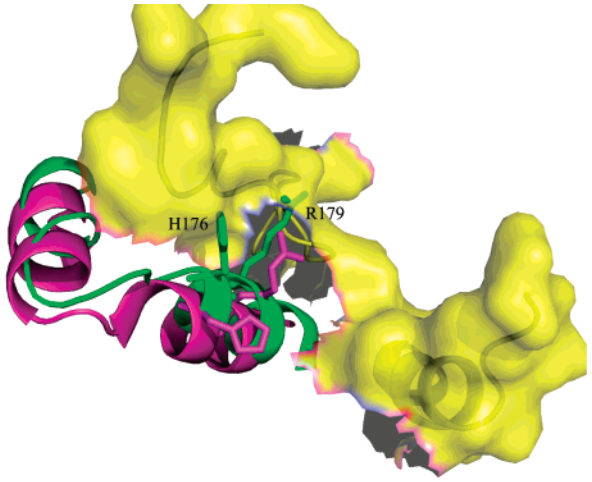


FIGURE 6: Side view of the N-terminal domain of two superimposed AurA crystal structures. The C helix is colored magenta from the crystal structure of the AurA−TPX2−ADP complex (PDB entry 1ol5) and green from the crystal structure of the AurA−AZ compound 13 complex (PDB entry 2c6e). The yellow surface represents TPX2. This view illustrates the conformational change in the C helix that is needed to accommodate compounds in the hydrophobic back pocket. Since the C helix shifts up in the presence of AZ compound 13, R179 and H176 would have steric clashes with TPX2.

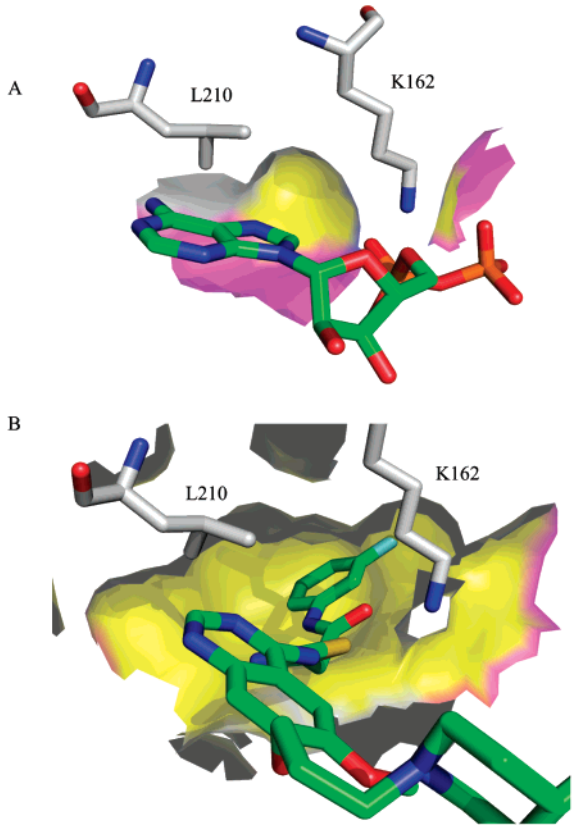


FIGURE 7: Size of the back pocket (yellow surface) which is reduced in the AurA−TPX2−ADP structure (A, PDB entry 1ol5) compared to the AurA−AZ compound 13 structure (B, PDB entry 2c6e).

compounds reach into different depths of the back pocket. Therefore, we hypothesize that TPX2 binding reduces the affinity of back pocket-binding inhibitors by restricting the conformational changes required to accommodate the large back pocket binding groups and altering the binding interactions into less favorable ones.

DISCUSSION

Aurora kinases have important binding partners in vivo that regulate their activation, catalytic activity, and biological functions. The inner centromere protein (INCENP), for example, is required for the full activation of AurB (*28*). It may also be involved in the regulation of AurC. Similarly, TPX2 has been found to stimulate AurA activation and its kinase activity (*8*−*11*, *29*). In the work presented here, we have carried out a detailed kinetic analysis of the effect of TPX2 binding to AurA. We find that TPX2 binds to AurA with high affinity and alters the binding interactions with both ATP and a peptide substrate. Our kinetic experiments suggest that AurA catalyzes the phosphorylation of a peptide substrate via a rapid equilibrium random mechanism. TPX2 binding does not alter the kinetic mechanism of AurA. However, the presence of TPX2 changes the SAR for inhibitors that bind at the ATP active site. Our hypothesis based on the modeling studies is that TPX2 reduces the binding affinity of inhibitors that extend into the hydrophobic back pocket beyond the ATP site.

Effect of TPX2 on AurA Kinetics. In the recently published crystal structure of AurA and TPX2 (*9*), TPX2 makes two contacts with the AurA kinase domain. The interactions between TPX2 and AurA help mold the activation loop into a conformation that is ready for substrate binding. It also provides a lever arm-like mechanism that causes the rotation of phosphorylated T288 away from the solvent-exposed position found in free AurA, thus protecting it from dephosphorylation by PP1. TPX2 therefore facilitates T288 phosphorylation by both stimulating the AurA kinase activity and antagonizing the effect of PP1.

We have confirmed the stimulating effect of TPX2 and discovered that it does so by increasing the binding affinities of both ATP and the peptide substrate rather than by increasing the intrinsic catalytic activity (k_{cat}) of AurA. Our kinetic data agree with the mechanism of activation predicted from the crystal structure (*9*). It has been shown that without TPX2, the activation loop is in a conformation not suitable for substrate binding. However, this loop is quite flexible in the absence of TPX2, and substrate binding could induce an active or active-like conformation that confers the basal kinase activity. It was proposed that the substrate-induced conformation is less active than the TPX2-induced one (*9*). In light of the kinetic data presented here, however, it is reasonable to conclude that substrate binding can induce a fully active AurA conformation, since the catalytic rate at saturating amounts of substrates (k_{cat}) does not change significantly whether TPX2 is present. The primary effect of TPX2 is to increase the binding affinity of the substrates so that the maximum velocity can be reached at lower substrate concentrations. TPX2 achieves this effect by establishing the active conformation of the activation loop for substrate binding. The kinetic constants determined from the double-titration experiments indicate a negative binding effect between the two substrates only when TPX2 is present. One possibility is that a conformational communication between the two substrate binding sites is established through TPX2 so that a change in one site affects the conformation of the other. It is also possible that the binding of TPX2 limits the conformational freedom of the substrate binding pockets. Therefore, the induced conformational changes in

one site upon binding of substrate to the other site may not be fully relieved due to the structural constraints exerted by TPX2.

Although TPX2 binding significantly affects the affinities of ATP and the peptide substrate, it does not alter the overall kinetic mechanism of AurA. This result probably reflects the accessory role of TPX2 in AurA catalysis. As discussed previously, substrates are able to bind AurA in the absence of TPX2, and this binding induces a fully active AurA conformation. TPX2 helps stabilize such an active conformation. Hence, it facilitates, but is not essential for, substrate binding.

The biological consequences of the effect of TPX2 on the AurA kinetics need further investigation. Given the millimolar cellular concentration of ATP, one can argue that the difference in the K_m values for ATP observed here is not sufficient to induce any significant change in reaction rates in vivo. The increase in the affinity for the peptide substrate, on the other hand, could play a role in modulating AurA activity in vivo. Current data indicate that the association of TPX2 indeed induces a conformation in favor of peptide or protein substrate binding. Studies with physiologically relevant AurA protein substrates are needed to further evaluate this possibility.

In addition to AurA, the activity and activation of many other kinases are also regulated not only by phosphorylation but also by interactions with cofactors and/or regulatory proteins. For example, INCENP is required for the activation of AurB (*28*). PAK1 activation requires the binding of GTP-bound cdc42 and Rac to relieve the inhibitory interactions of the N-terminal regulatory domain (*30*). PDK1 catalysis has been shown to be greatly enhanced by PIF binding to the PDK-tide model substrate (*31*). Therefore, it is critically important to understand the effect of cofactors and use the physiologically relevant enzyme form(s) for inhibitor discovery.

Effect of TPX2 on the Inhibitor SAR. Since our kinetic data demonstrated the role of TPX2 in facilitating substrate binding, we were interested in understanding whether the potency of inhibitors that bind to the ATP pocket may also be affected by TPX2. Indeed, the three inhibitors that we studied, GW801372X, VX-680, and GSK623906A, demonstrated differential IC_{50} shifts upon TPX2 binding.

On the basis of the published cocrystal structure of AurA and AZ compound 13 (similar to GSK623906A) from AstraZeneca (*17*), computer modeling studies suggest that there are important differences in the binding interactions of these three compounds with AurA even though they all bind in the ATP pocket. Whereas the binding of GW801372X is mainly limited to the ATP site, the binding of GSK623906A extends to the hydrophobic back pocket adjacent to the ATP site. Although VX-680 does not fully occupy the back pocket, its cyclopropane ring is extended and approaches the pocket.

A comparison of the AurA−TPX2−ADP (PDB entry 1ol5) and AurA−AZ compound 13 (PDB entry 2c6e) structures suggest that to allow compounds like GSK623906A to bind to this back pocket, the C helix needs to shift up and out. This conformational change is more restricted when TPX2 is bound due to the interactions between TPX2 and the C helix. Therefore, our hypothesis is that TPX2 binding reduces the affinity of back pocket-binding inhibitors by restricting the conformational changes required to accommodate the large back pocket binding groups and altering the binding interactions to less favorable ones.

It is also interesting to note that the AurA−AZ compound 13 structure was obtained using AurA with the T288D activating mutation which was shown to have kinase activity (*32*). However, in this structure, AurA adopts a DFG-out conformation in its activation loop, typically observed in inactive kinase conformations (*17*). Bayliss et al. also noted that the activation loop of phosphorylated AurA is in an inactive conformation in the absence of TPX2 (*9*). We have shown in our biochemical studies that AurA does possess kinase activity in the absence of TPX2. Therefore, it seems that the activation loop can adopt both active (for catalysis) and inactive (as observed in crystal structures) conformations in the absence of TPX2. As observed by Bayliss et al., the binding of TPX2 stabilizes the active conformation and holds the activation loop in place for catalysis. The fact that TPX2 restricts the conformational flexibility of AurA to stabilize the active conformation is consistent with our kinetic data.

Contrary to the original belief that kinase inhibitors binding to the ATP pocket would lack the required selectivity, many such inhibitors have been shown to be very specific in the target(s) they inhibit (*33*). A number of factors contribute to the selectivity of ATP-competitive kinase inhibitors. One of these factors is the ability of many kinase inhibitors to extend their binding to regions adjacent to the ATP pocket, including the hydrophobic back pocket, since more structural heterogeneity can be found in these regions than in the ATP pocket per se. Therefore, it is at times desirable to identify inhibitors that bind to these sites. However, our current data suggest that the potency of these compounds can be altered significantly depending on whether accessory proteins are present. Given the important role of TPX2 in regulating AurA activity, the potency of inhibitors against the AurA−TPX2 complex, rather than AurA alone, likely reflects more closely their in vivo activities. Our discovery that TPX2 alters the SAR for AurA inhibition may also be more generally applicable to other kinases and enzymes with cofactors.

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