Specific Small-Molecule Activator of Aurora Kinase A Induces Autophosphorylation in a Cell-Free System[⊥]

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Aurora kinases are essential for chromosomal segregation and cell division and thereby important for maintaining the proper genomic integrity. There are three classes of aurora kinases in humans: A, B, and C. Aurora kinase A is frequently overexpressed in various cancers. The link of the overexpression and tumorigenesis is yet to be understood. By employing virtual screening, we have found that anacardic acid, a pentadecane aliphatic chain containing hydroxylcarboxylic acid, from cashew nut shell liquid could be docked in Aurora kinases A and B. Remarkably, we found that anacardic acid could potently activate the Aurora kinase A mediated phosphorylation of histone H3, but at a similar concentration the activity of aurora kinase B remained unaffected in vitro. Mechanistically, anacardic acid induces the structural changes and also the autophosphorylation of the aurora kinase A to enhance the enzyme activity. This data thus indicate anacardic acid as the first small-molecule activator of Aurora kinase, which could be highly useful for probing the function of hyperactive (overexpressed) Aurora kinase A.

Introduction

The Aurora family of serine/threonine protein kinase in mammals includes three paralogues, Aurora-A, -B, and -C. These kinases play important role in chromosome segregation and cell division.[1] The expression and activity of Aurora kinases are tightly associated with cell cycle. Aurora kinases are overexpressed in mitotically active cells, such as thymus, testis, spleen, intestine, and bone marrow.[2] Aurora kinase A (AURA[a])plays critical role in mitotic spindle formation, centrosome maturation, and segregation. Dysfunction of Aurora A disrupts cell cycle progression. Overexpression of Aurora A and B is often observed in the human tumors. Aurora kinase B (AURB) is involved in chromosomal condensation, alignment and separation[3,4] and kinetochore−microtubule attachment and cytokinesis.[5] In vivo AURB is present in a complex containing INCENP, survivin, and Borealin.[6,7] AURA and AURB have several interacting partners and substrates that have direct implications in cell cycle. Proteins like TPX2, a microtubule binding protein,[8] and Ajuba, a LIM domain protein,[9] can bind to and activate AURA. This binding of substrates to AURA and consequential autophosphorylation of AURA prevents the dephosphorylation of the enzyme by type I phosphatases and thus keeps it in active form in the cytoplasm.[10] Recently, it was shown that Bora interacts with AURA and activates the AURA kinase activity in vitro.[11] It has been reported earlier that overexpression of Bora (activator of AURA) can rescue defects caused by mutations in AURA.

Aurora kinases, particularly AURA, are known to frequently overexpress in various malignancies such as colorectal, breast, gastric, prostate, ovarian, colon, and cervical tumors.[2,12,13] Recently, by use of microarray analysis, it was shown that AURA overexpresses in human pancreatic tumors.[14,15] The overexpression of AURA in the cancerous tissue is shown to be due to the amplification of the AURA gene.[2,12,13] Overexpression of AURA in normal cells results in centrosome abnormalities and induces aneuploidy.[16] It was previously shown that ectopic overexpression of AURA can transform the cells, suggesting its oncogenic role. AURA overexpression thus could result in the abnormal chromosome segregation and aberrant entrance into mitosis.[17] Since AURA is up-regulated and/or amplified largely in cancerous cells compared to normal cells, the enzyme is gaining immense importance as a potential therapeutic target.

In this report we show that anacardic acid, isolated from cashew (*Anacardium occidentale*) nut shell liquid, could bind to Aurora kinases in silico. Remarkably, anacardic acid could strongly activate the kinase activity of AURA but not the activity of AURB. Using spectrofluorimetry, we have shown that the association constant (K_a) of ATP (substrate) to AURA increases in presence of anacardic acid and vice versa. Furthermore, anacardic acid induces autophosphorylation of AURA. Since autophosphorylation is known to trigger the kinase activity, these data suggest that anacardic acid mediated alteration of enzyme structure and activation of autophosphorylation are the underlying mechanisms for inducing Aurora kinase A activity.

Results

Anacardic Acid Enhances AURA Kinase Activity. All the Aurora kinases have been closely linked to cancer progression. This observation made these enzymes an attractive target for the treatment of cancer. We were interested to find out the small-molecule natural compounds that would alter the activity of

[⊥] X-ray crystallographic data of anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, $C_{22}H_{36}O_3$) were submitted to the Cambridge Crystallographic Data Centre (deposition number CCDC662069).

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[a] Abbreviations: AURA, Aurora kinase A; AURB, Aurora kinase B.

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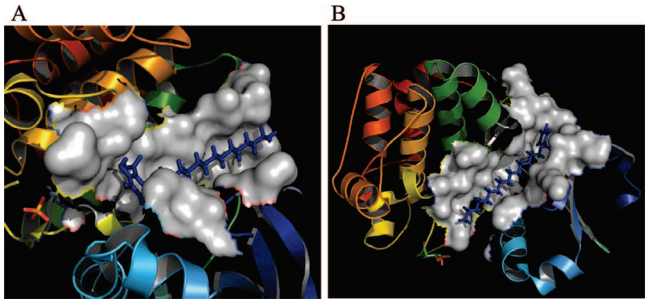

Figure 1. (A) Aurora kinase A−anacardic acid complex. Crystal structure of Aurora kinase (1MQ4) is 388 amino acid residues long, and it interacts with the anacardic acid between amino acid residues 126 and 129. The ligand is in blue, whereas the interacting part of the protein has been projected as a metallic white surface. (B) Aurora kinase B−anacardic acid complex. Crystal structure of Aurora kinase (2BFY) is 356 amino acid residues long, and it interacts with the anacardic acid between amino acid residues 142 and 154. The ligand is in blue, and the interacting part of the protein has been projected as a metallic white surface.

Aurora kinases. We adopted an in silico approach to screen the natural molecules. For this purpose several compounds from natural sources were docked on AURA and AURB using Hex 4.5 software. Out of many small molecules, we found that anacardic acid could interact with both AURA (amino acid residues 126−129) and AURB (amino acid residues 142−154) (parts A and B of Figure 1). In order to validate the docking studies, we investigated the effect of anacardic acid on the histone H3 phosphorylation ability of AURA and AURB. Interestingly, we found that anacardic acid could enhance the kinase activity of AURA by about 4- to 5-fold as estimated by phosphorimager analysis (Figure 2A, compare lane 2 vs lanes 3 and 4). Surprisingly, anacardic acid did not affect the kinase activity of AURB at similar concentration (Figure 2A, compare lane 6 vs lanes 7 and 8 and Figure 2B). In order to confirm the specificity of activation of AURA kinase by anacardic acid, we have done dose-dependent activation assays. For this purpose the enzyme was preincubated with increasing concentrations of anacardic acid (5−50 μM) and then subjected to histone H3 phosphorylation assay using [γ-^{32}P]-ATP. The results show that there is a dose dependent activation of AURA by anacardic acid (parts C and D of Figure 2, compare lane 2 vs lanes 3−6). These results suggest that anacardic acid is a small-molecule activator of Aurora kinase, which is specific to AURA.

Anacardic Acid Increases the Association Constant (K_a) of AURA to Its Substrate ATP. In order to find out the mechanism of activation of AURA activity by anacardic acid, we have done spectrofluorometric assays. Equilibrium binding of ATP and anacardic Acid to AURA was measured by fluorescence titration at 20 °C. AURA fluorescence spectrum was studied by exciting the sample at 280 nm and recording the emission spectrum in the range of 290–400 nm. In the absence of substrate, fluorescence was observed in the range of 290–400 nm, with a maximum at 301 nm. However, in the presence of substrate (ATP), the fluorescence in the region 290–400 nm declined as a result of the quenching of its intrinsic fluorescence (λ_{max} = 301 nm). The effect of ATP and anacardic acid binding to AURA was studied by titration of the ligand from a stock solution of 0.5 and 1000 μM, respectively, and the corresponding decrease in the fluorescence intensity was monitored at 301 nm. A double-reciprocal plot of the fluorescence intensity and ligand concentration, at a fixed concentration of AURA, resulted in the fluorescence intensity at infinite ligand concentration (F_α). A representative titration curve is shown in Figure 3. The results show that K_a of ATP increased by ∼5-fold in the presence of anacardic acid (50 μM). Similarly K_a of anacardic acid increased by ∼2-fold in the presence of saturating concentration of ATP (Table 1). Taken together, these data suggest that anacardic acid induces a structural change in the Aurora kinase A, which in turn enhances the ATP recruitment by the enzyme and thereby activates the process of phosphorylation.

Anacardic Acid Enhances AURA Activity by Increasing the V_{max} of the Reaction. In order to further investigate the mechanism of activation of kinase activity of AURA by anacardic acid, we performed kinetic analysis of the anacardic acid mediated activation of AURA. The kinase assays were done with increasing concentrations of ATP (2.5−15 μM) in the presence of DMSO (Figure 4A, lanes 1−6) or 50 μM anacardic acid (Figure 4A, lanes 7−12). The reaction mixture was subjected to TCA (25%) precipitation, and histones were resolved using 15% PAGE. The radioactive signal was quantitated using phosphorimager analyzer (Figure 4B). The enzyme activity was analyzed using SigmaPlot. Results show that the V_{max} of the reaction is increased by ∼6-fold in the presence of anacardic acid while K_m is increased by ∼2-fold (Table 2).

Furthermore, we have also performed the time course assay for the activation of the kinase. The kinase assay reaction mixtures were incubated in the presence of DMSO (Figure 4C, lanes 6−10) or anacardic acid (Figure 4C, lanes 1−5) for 5, 10, 15, 20, and 25 min. The TCA precipitated reaction mixture was resolved using 15% PAGE, and the radioactive signal was quantitated and plotted. The graph shows that anacardic acid can enhance the rate of reaction (Figure 4D). These data suggest that because of faster recruitment of ATP upon anacardic acid binding to the AURA, the rate of phosphorylation is enhanced dramatically.

AURA Kinase Activity Enhancement Is through Enhancement of Autophosphorylation. Previous studies have shown that autophosphorylation of AURA at Thr-288 residue can enhance the kinase activity of AURA. The threonine residue that gets autophosphorylated is highly conserved among the aurora kinase family members (Supporting Information Figure 1). To investigate the effect of anacardic acid on autophosphorylation of AURA, we have incubated the enzyme with [γ-^{32}P]ATP, histone H3, and anacardic acid. The results show that phosphorylation of histone H3 was enhanced by about 4- to 5-fold in the presence of anacardic acid (Figure 5A, autoradiogram; compare lane 2 vs lanes 3 and 4). On longer exposure we see that phosphorylation of AURA also has been enhanced by anacardic acid by ∼2-fold (Figure 5A, autoradiogram; compare lane 2 vs lanes 3 and 4). Significantly, under similar conditions anacardic acid could not enhance the autophosphorylation activity of AURB (Figure 5B, autoradiogram, lane 2 vs lanes 3 and 4). These results convincingly establish that apart from increasing the K_a of AURA to that of ATP, anacardic acid could also specifically induce autophosphorylation of AURA, which presumably is partially responsible for the activation of AURA kinase activity.

Discussion

We have shown that the natural compound anacardic acid dramatically enhances the kinase activity of AURA but not the AURB. To best of our knowledge, this would be the first report of any small-molecule activator of any of the Aurora kinases. Humans have three different Aurora kinases: A, B, and C. Structurally, although different Aurora kinases have size differences ranging from 403 amino acids to 309 amino acids, they possess a highly conserved catalytic domain. It is important to

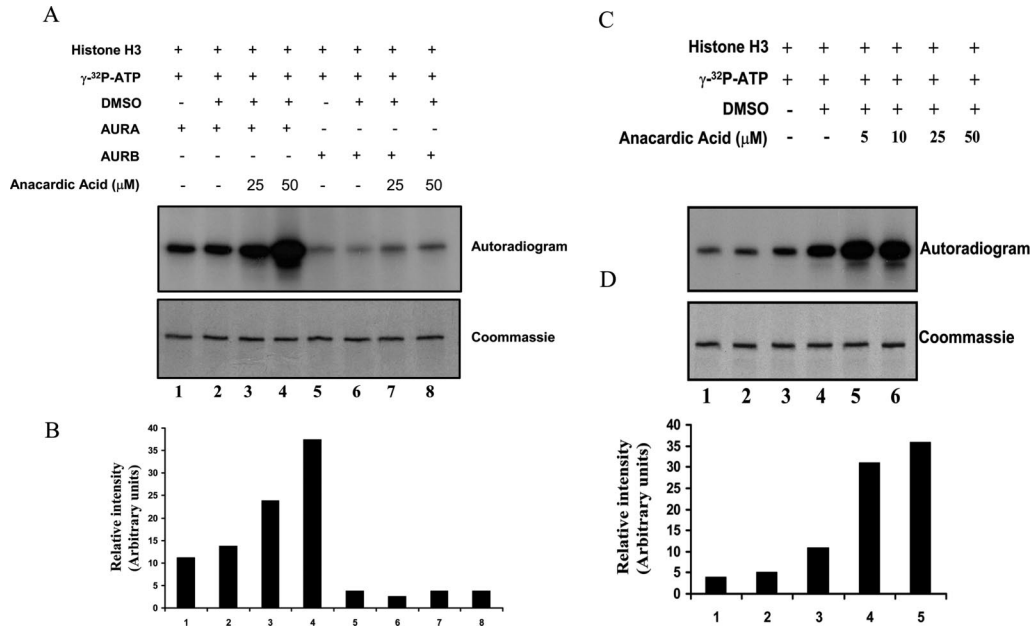

Figure 2. (A) Anacardic acid enhances AURA kinase activity. Kinase assays were performed by incubating 1.5 μg of histone H3 with either AURA or AURB (40 ng) and [γ-^{32}P]ATP in the presence or absence of anacardic acid (25 and 50 μM). The reaction mixtures were precipitated using 25% TCA, resolved on a 15% SDS−PAGE and subjected to autoradiography: lane 1, histone H3 with AURA and [γ-^{32}P]ATP alone; lane 2, histone H3 incubated with AURA, [γ-^{32}P]ATP, and DMSO as control; lanes 3 and 4, histone H3 with AURA, [γ-^{32}P]ATP, and anacardic acid (25 and 50 μM) dissolved in DMSO; lane 5, histone H3 with AURB and [γ-^{32}P]ATP alone; lane 6, histone H3 incubated with AURB, [γ-^{32}P]ATP, and DMSO as control; lanes 7 and 8, histone H3 with AURB, [γ-^{32}P]ATP and anacardic acid (25 and 50 μM) dissolved in DMSO. (B) Bar graph shows the relative intensities of the phosphorylated products that were quantitated using a phosphoimager analyzer (Fuji). (C, D) Dose dependent activation of AURA kinase activity by anacardic acid. Kinase assays were performed in the absence or presence of increasing concentrations of anacardic acid (5–50 μM), and the relative intensities of the phosphorylated products were estimated using a phosphoimager analyzer.

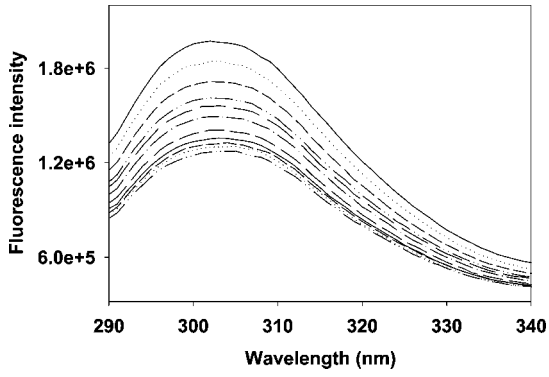

Figure 3. Emission spectra for the intrinsic protein fluorescence (λ_{max} = 301 nm) of Aurora kinase A (AURA) at 20 °C. Aliquots of 2 μL of ATP from stock solutions of 0.5 μM were added to 200 μL of Aurora kinase A (2 μM in 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EGTA, 10 mM MgCl2, 0.1% β-mercaptoethanol), and the changes in fluorescence intensities were monitored between 290 and 400 nm. Samples were excited at 280 nm. Quenching of intrinsic protein fluorescence of AURA (290–400 nm range; λ_{max} = 301 nm) occurred as a function of increasing concentrations of the substrate ATP.

Table 1. Binding Constants (K_a) of ATP and Anacardic Acid to AURA at 20 °C, Using the Changes in Protein Fluorescence Intensity at 301 nm

serial number	titrated with ligand	saturated with	association constant (K_a)
1	anacardic acid	none	5.2μM^{-1}
2	anacardic acid	ATP (200 nM)	10.1μM^{-1}
3	ATP	none	5.7nM^{-1}
4	ATP	anacardic acid (50 μM)	27.5nM^{-1}

point out that the diversity of kinases is observed in the N-terminal domain. Though anacardic acid shows strong binding to both AURA and AURB kinases in our in silico analysis, it could only activate AURA close to 5-fold. In several cancers AURA gene gets amplified and thereby its expression is amplified. The link of overexpression of AURA and tumorigenesis is not well established. The specific activator anacardic acid may be useful to establish this link further.

There are several mechanisms by which the Aurora kinases′ activity is regulated. One of the most important factors is the autophosphorylation. Aurora kinase needs to be associated with the substrate to be activated by autophosphorylation. Interestingly, we have found that anacardic acid induces the autophosphorylation of Aurora kinase A. Probably binding of anacardic acid to AURA induces a certain structural change in the enzyme that acts as a signal for the autophosphorylation. It would be interesting to find out whether the same principle is involved during the autophosphorylation by substrate binding. However, we have found that anacardic acid could not activate the autophosphorylation of AURB, suggesting the importance of autophosphorylation in the small-molecule mediated enhancement of AURA activity.

Small-molecule mediated activation of AURA in vivo would mimic the overexpression of AURA as it happens in tumorigenesis. This phenomenon could be useful for understanding the role of AURA in cell cycle and metastasis. Furthermore, the function of AURA in cell division, maintenance of genomic integrity, and chromosome segregation could be studied using small-molecule modulators. Anacardic acid was discovered as the first natural, nonspecific inhibitor of histone acetyltransferases.[22] Significantly, the amide derivate of anacardic acid, CTPB, was found to be the only known p300 specific HAT activator. It would be interesting to find out the chemical moieties of anacardic acid responsible for the activation of Aurora kinase A and inhibition of histone acetyltransferases. This chemical analysis could be highly useful for understanding

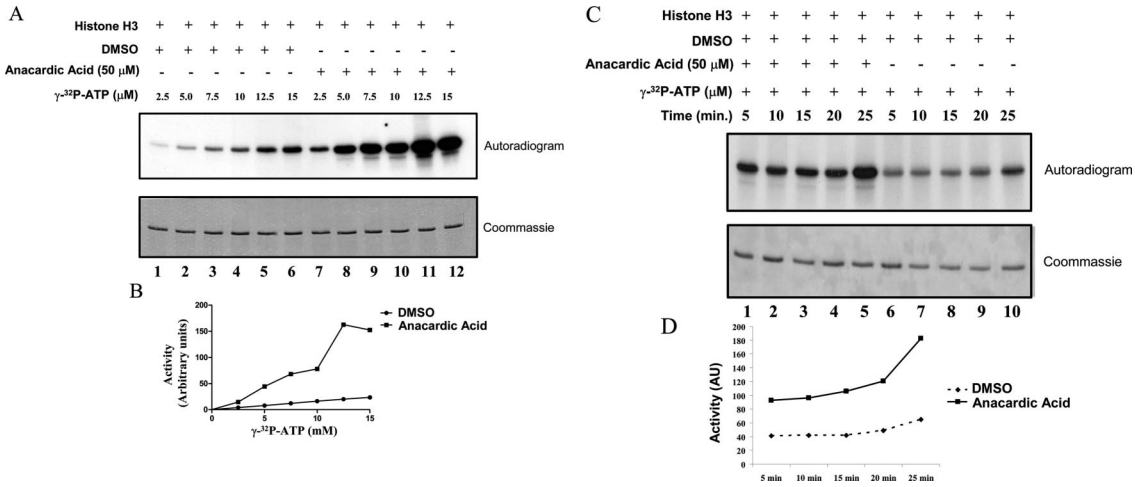

Figure 4. (A, B) Kinetic analysis of AURA activation by anacardic acid. Kinase assays were done using fixed concentrations of AURA and substrate (H3) and increasing concentrations of ATP in the presence of DMSO (control) or anacardic acid. The relative percent of activity (arbitrary units) is plotted against substrate concentration (ATP). (C, D) Time dependent activation of AURA by anacardic acid. AURA, H3, and ATP were incubated in the presence of DMSO or anacardic acid for 5, 10, 15, 20, and 25 min. The relative activity (AU, arbitrary units) is plotted against time.

Table 2. Kinetic Parameters of Activation of AURA by Anacardic Acid[a]

kinetic parameters	DMSO	anacardic acid
V_{max} (mmol min^{-1} mg^{-1})	10.5	62.5
K_m (mM), ATP	17.34	40.25
K_{cat} (s^{-1})	47.2	281.5
K_{cat}/K_m (s^{-1} mM^{-1})	2.72	6.99

[a] AURA was incubated with DMSO (control) or anacardic acid in the presence of increasing concentrations (2.5–15 μM) of ATP.

the chemistry as well as its utility and for exploiting these unique natural products for different purposes. Since cells are less permeable to anacardic acid, synthesis and characterization of novel cell permeable small molecules similar to anacardic acid (activator) could be a potent tool to probe the function of Aurora kinase in normal cell.

Experimental Section

Virtual Screening of Druglike Compounds by Docking. The crystal structure of Aurora kinase A (1MQ4) and Aurora kinase B (2BFY) were retrieved from the PDB database. AURA and AURB structures were docked with several compounds to find out their interaction sites in these kinases. Molecular simulation and the docking of AURA and AURB structures with anacardic acid were performed using Hex 4.5 software. The docking calculations were done using 3D parametric functions of both the kinases and anacardic acid structures, which are used to encode surface shape and electrostatic charge and potential distributions. The parametric functions are based on expansions of real orthogonal spherical polar basis functions. The docking was performed in full rotation mode. Both kinases and ligand were taken at 180 ranges for 20 000 solutions.

X-ray Structure Determination of Anacardic Acid. The needle-shaped quality crystals of the anacardic acid grown from a mixture of chloroform, hexane, ethyl acetate, and methanol (1:1:2:1) by slow evaporation at room temperature were chosen after examination under an optical microscope and coated with epoxy before mounting. X-ray diffraction intensities were measured by ω scans using Bruker SMART diffractometer attached to a CCD area detector and a graphite monochromator for the Mo Kα radiation (50 kV, 40 mA). A hemisphere of reciprocal space was collected using the SMART software with 2θ setting of the detector at 28°. Data reduction was performed using the SAINT program (Bruker). The phase problem was solved by direct methods (SHELXS97), and the non-hydrogen atoms were refined anisotropically by means of the full matrix least-squares procedure using the SHELXL97 program. All the hydrogen atoms were located using geometrical considerations and refined using the "riding-model" method. The absolute structure of anacardic acid is shown in Figure 6A.

Cloning, Baculovirus Expression, and Purification of Aurora Kinases A and B. Full length AURA and AURB were cloned by PCR based cloning using cDNA prepared from HEK293 cells. AURA and AURB baculoviruses were constructed using commercially available kits (Invitrogen catalog numbers K2400-20, K2420-20, K2525-20, K2535-20, K2435-20, K2435-20, K2635-20, 12562-013, and 12562-039) according to manufacturer's protocol. AURA and AURB enzymes were expressed as C-terminal His_6-tagged proteins and purified using Ni-NTA affinity purification. The protein profiles of the enzymes were analyzed by 12% PAGE (parts B and C of Figure 6).

Purification of Recombinant Histone H3. The bacterially expressed recombinant histone (*Xenopus*) H3, which comes in inclusion bodies, was purified by denaturation in 8 M urea followed by renaturation as described elsewhere.[18] H3 was dialyzed against buffer containing 10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 20% glycerol v/v, 0.05% NP40, 0.02% 2-mercaptoethanol, and 1 mM EDTA and analyzed using 15% PAGE (Figure 6D).

Isolation of Anacardic Acid. Anacardic acid was isolated from cashew (*Anacardium occidentale* L.) nut shell liquid (CNSL) as described elsewhere with few modifications.[19] Briefly, CNSL (100 g) was dissolved in (95%) methanol (500 mL), and calcium hydroxide (50 g) was added under stirring for 6 h. The precipitated calcium anacardate was filtered and washed with methanol and dried under vacuum. Calcium anacardate was suspended in distilled water (500 mL), and 11 M HCl (50 mL) was added and stirred for 1 h. The resultant solution was extracted with ethyl acetate. The organic layer was washed with distilled water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to yield anacardic acid (monoene, diene, and triene). The anacardic acid−ene mixture was reduced using catalytic hydrogenator with 10% Pd/C. The identity of the compound was confirmed by ^{13}C NMR, ^{1}H NMR, and X-ray crystallography.

Fluorescence Titration of AURA−Anacardic Acid Binding. Equilibrium binding of ATP and anacardic acid to AURA was measured by fluorescence titration at 20 °C using a Jobin-Yvon Horiba spectrofluorimeter (Edison, NJ) (band-pass of 3 and 5 nm for the excitation and emission monochromator, respectively). The fluorescence spectrum of AURA was studied by exciting the samples at 280 nm and recording the emission spectrum in the range of 290–400 nm. In the absence of substrate, fluorescence was

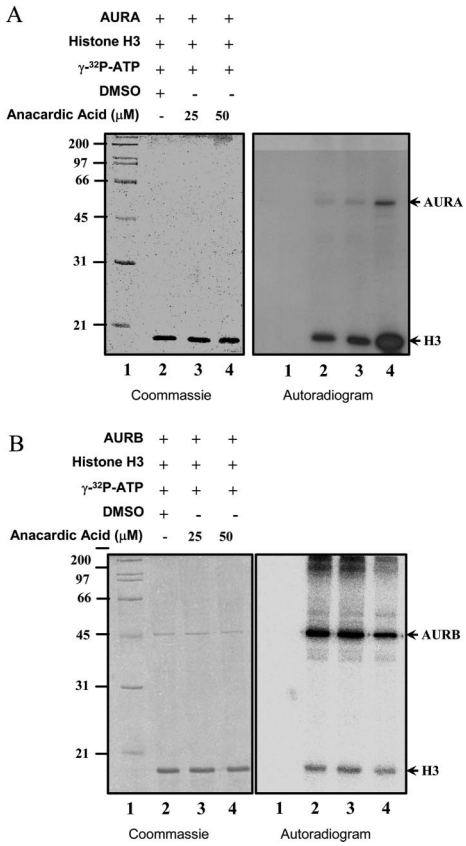

Figure 5. (A) Anacardic acid enhances autophosphorylation of AURA. Kinase assay was performed by incubating 1.5 μg of histone H3 with either AURA (40 ng) or AURB (120 ng) and [γ-^{32}P]ATP in the presence or absence of anacardic acid (25 and 50 μM). The reaction mixtures were precipitated using 25% TCA, resolved on a 15% SDS−PAGE and subjected to autoradiography: lane 1, protein molecular weight marker; lane 2, histone H3 with AURA and [γ-^{32}P]ATP alone; lanes 3 and 4, histone H3 with AURA, [γ-^{32}P]ATP, and anacardic acid (25 and 50 μM) dissolved in DMSO. (B) Anacardic acid does not affect the autophosphorylation of AURB: lane 1, protein molecular weight marker; lane 2, histone H3 with AURB, DMSO, and [γ-^{32}P]ATP; lanes 3 and 4, histone H3 with AURB, [γ-^{32}P]ATP, and anacardic acid (25 and 50 μM) dissolved in DMSO.

observed in the range of 290–400 nm, with a maximum at 301 nm. However, in the presence of substrate (ATP), the fluorescence in the region 290–400 nm declined as a result of the quenching of its intrinsic fluorescence (λ_{max} = 301 nm). Aliquots of 2 μL of ATP (from stock solutions of 0.5 and 5 μM) were added to 5 μM of AURA in 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EGTA, 10 mM MgCl2, 0.1% β-mercaptoethanol. The solution was mixed after the addition of each aliquot, and the fluorescence intensity in the 290–400 nm region was recorded as the average of three readings. Samples were excited at 280 nm. The effect of ATP and anacardic acid binding to AURA was studied by titration of the ligand from a stock solution of 0.5 and 1000 μM, respectively, and the corresponding decrease in the fluorescence intensity was monitored at 301 nm. A double-reciprocal plot of the fluorescence intensity and ligand concentration, from the data obtained by titration of a fixed concentration of AURA with ligand, gave the fluorescence intensity at infinite ligand concentration (F_α).

Correction for the inner filter effect was performed according to the following equation:

$$F_c = Fanti\log[(A_{ex} + A_{em})/2] \quad (1)$$

where F_c and F are the corrected and measured fluorescence intensities, respectively,[20] and A_{ex} and A_{em} are the solution absorbance values at the excitation and emission wavelengths, respectively.

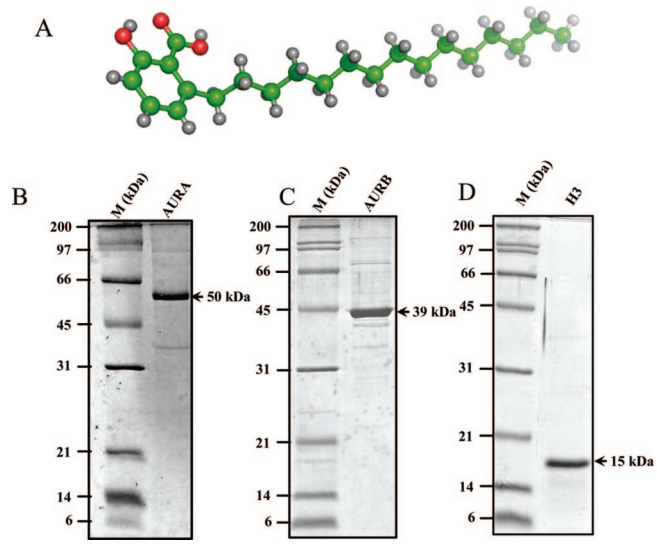

Figure 6. (A) Ball-and-stick X-ray crystal structure of anacardic acid generated by Pymol. Profiles of the proteins used in different experiments are as follows: (B) 1 μg of baculovirus expressed His$_6$-tagged AURA is resolved using 12% SDS−PAGE; (C) 1.5 μg of baculovirus expressed His$_6$-tagged AURB is resolved using 12% SDS−PAGE; (D) 800 ng of bacterially expressed recombinant *Xenopus* histone H3 was resolved using 15% SDS−PAGE.

Association Constant Determination. From the protein concentration-dependent quenching of AURA fluorescence, the K_a values for their binding were calculated by the method of Chipman et al.[21] Double-reciprocal plot of the fluorescence intensity and compounds' concentration from the data obtained by titration of a fixed concentration of AURA with anacardic acid gave the fluorescence intensity at infinite compound concentration (F_α). When log[L]$_f$ (where [L]$_f$ is the free compound concentration equal to the log term in parentheses in eq 2) was plotted against log[$(F - F_o)/(F_\alpha - F)$], the intercept on the x axis yielded the pK_b value for the AURA−anacardic acid interaction according to the following relationship for fluorescence quenching:

$$\log\left(\frac{F_0 - F}{F - F_\alpha}\right) = \log K + \log\left([P]t - [L]t\frac{\Delta F}{\Delta F_\alpha}\right) \quad (2)$$

where $\Delta F = (F - F_o)$ and $\Delta F_\alpha = (F_\alpha - F_o)$ are the fluorescence intensity changes at particular and infinite protein concentrations, respectively. F_o, F, and F_α are the fluorescence intensities of AURA in the absence, in the presence, and at infinite concentration of compound, respectively. A representative plot for the binding of anacardic acid to AURA at 20 °C is shown (Supporting Information Figure 2).

Kinase Assays. An amount of 2 μg of bacterially expressed recombinant histone H3 was incubated along with 40 ng of either baculovirus-expressed His$_6$-tagged Aurora kinase A or Aurora kinase B in a 30 μL of reaction mixture containing 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EGTA, 10 mM $MgCl_2$, 0.2% 2-mercaptoethanol, and [γ-^{32}P]ATP (specific activity of 200 Ci/mmol). The reaction mixture along with varying concentrations of anacardic acid was incubated at 30 °C for 5 min followed by addition of [γ-^{32}P]ATP and further incubation for 10 min. The reaction mixture was precipitated using 25% TCA and resolved on 15% PAGE, and autoradiography was done. The intensity of the signal was quantitated using PhosphorImager analyzer (Fuji). For kinetic analysis increasing concentrations of [γ-^{32}P]ATP (from 0 to 15 μM) were used in the reaction mixture, and the kinetic parameters were calculated using SigmaPlot.

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Supporting Information Available: Characterization of anacardic acid by ^{1}H NMR, ^{13}C NMR, and mass spectrometry; multiple alignment of aurora kinases A, B, and C; representative plot for the binding of anacardic acid to AURA. This material is available free of charge via the Internet at http://pubs.acs.org.

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