Supporting Information

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SI Materials and Methods

Chemistry. IBPR001 and IBPR002 were synthesized from 4-chloro-6-(4-methoxyphenyl)furo[2,3-*d*]pyrimidine (**A**) which was obtained as reported in WO 2005092896 A1 (Fig. S1). First, the reaction of starting material **A** and 4-(2-aminoethyl)aniline under basic conditions provided the S_NAr reaction product **B**. The $-NH_2$ group of **B** then was converted to phenyl urea by reacting with phenyl-isocyanate. Demethylation of **C** by treating with BBr_3 in CH_2Cl_2 gave the key –OH–bearing intermediate **D**. As a final step, the hydroxyl group acted as an anchor, and the appropriate solubilizing groups were attached to the core structure by using an S_N2 reaction to provide the products IBPR001 and IBPR002 (compounds **1** and **2**, Fig. 1*A*). Spectral profiles of IBPR001 and IBPR002 are provided in Fig. S1.

Aurora A in Vitro Kinase Assay. The in vitro Aurora A luminescent kinase assay was performed as described (1). Briefly, test compounds were diluted from 10-mM stocks (in DMSO) with the reaction buffer [50 mM Tris·HCl (pH 7.4), 10 mM NaCl, 10 mM $MgCl_2$, and 100 μg/mL BSA]. Then 25 μL of the diluted solution was incubated with 105 ng (in 10 μL) of the purified Aurora A protein at 25 °C for 15 min in 96-well U-bottom plates (Nunc) followed by the addition of 0.1 mM tetra(LRRWSLG) peptide substrate (5 μL), 5 μM ATP (5 μL), and 1 mM DTT (5 μL). After 90 min of incubation at 37 °C, 50 μL of Kinase-Glo Plus Reagent (Promega) was added to the reactions, followed by 20-min incubation at 25 °C. Then 70 μL of the reaction mixture was transferred to 96-well black plates (Nunc) for quantification of the ATP remaining in the solution. The luminescence was recorded by a luminometer (Victor2 V, 1420 multilabel plate counter; Perkin-Elmer).

Expression and Purification of Aurora A for Crystallization. The Aurora A catalytic domain, amino acids 123–401 with one mutation at residue 288 (T288D), was fused with six His tags in a pET-28a (Novagen) vector and expressed in BL21(DE3) *Escherichia coli*. Cells were grown at 37 °C to reach OD_{600} = 0.4, followed by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubation at 27 °C for another 6 h. Cells then were harvested and resuspended in a lysis buffer [40 mM Hepes (pH 7.5) and 50 mM NaCl]. After sonication to disrupt the cell membrane, the lysate was centrifuged, and the supernatant was collected. The target protein was purified with nickel affinity chromatography following the procedures suggested by the supplier (GE Biosciences). Fractions containing the recombinant Aurora A protein were digested with tobacco etch virus (TEV) protease (Invitrogen) for 44 h at 4 °C to cleave the His tags. The mixture then was loaded onto a desalting column (GE Biosciences) to remove the His tags and exchange buffer for the following crystallization trials. The purified Aurora A protein finally was concentrated to 7.5 mg/mL in a protein buffer [40 mM Hepes (pH 7.5), 50 mM NaCl, and 1 mM DTT].

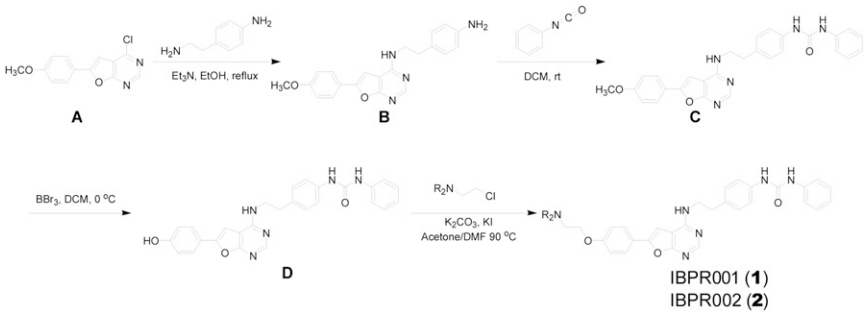
Crystallization and Structure Determination. Crystals of the Aurora A/IBPR001 and Aurora A/IBPR002 complex were grown by the hanging-drop method. Nine microliters of the purified Aurora A protein [7.5 mg/mL in a buffer containing 40 mM Hepes (pH 7.5), 50 mM NaCl, and 1 mM DTT] was mixed with 1.0 μL of 10 mM IBPR001 or IBPR002 (in DMSO) and was incubated for 6 h on ice. A drop of the 1.5-μL complex solution was added to 1.5 μL of reservoir solution (22% PEG400 and 0.1 M ammonium sulfate). The crystals were grown after 3- to 7-d incubation at 18 °C. The Aurora A/VX-680 complex crystals were prepared by soaking native Aurora A crystals for 1 h in a solution [40 mM Hepes (pH 7.5), 50 mM NaCl, and 1 mM DTT] containing 1.0 mM of VX-680. The complex crystals then were flash-frozen in liquid nitrogen, and the diffraction data were collected on beamlines BL13B1 and BL13C1 at the National Synchrotron Radiation Research Centre (Taiwan) and beamline SP12B2 at the SPring-8 (Japan). Data were processed by Denzo (HKL Research) and reduced with Scalepack (HKL Research). The structures were solved by molecular replacement in MOLREP (CCP4 Software Suite) using the published Aurora A structure [Protein Data Bank (PDB) ID: 1MQ4] as the search model. Refinement was performed by REFMAC5 (2), and model-building was carried out with the program O11 (3). Statistics of the complex structures are summarized in Table S2.

Tumor Xenograft. Male athymic nu/nu nude mice (BioLASCO) at age 6 wk were housed in sterile cages maintained under 12-h light/dark cycles with controlled temperature and humidity. Mice were inoculated s.c. with 1×10^6 HCT116 cells resuspended in saline. The sizes of the xenografted tumors were measured by a digital caliper (GMC-190; Goldsun Electronics Co.) and were calculated using the algorithm: tumor volume (mm^3) = length × $(\text{width})^2/2$. Body weight and tumor size were measured twice a week.

Drug Treatment. IBPR002 and VX-680 were dissolved completely in a vehicle mixture of DMSO/Cremophor EL/saline (10/20/70%). When the growing tumor xenograft reached a size ≥100 mm^3, the xenograft tumor-bearing nude mice were treated i.v. with IBPR002 or VX-680 via the tail vein in a dose regimen of 50 $mg \cdot kg^{-1} \cdot d^{-1}$, five daily doses per week, for two consecutive weeks. The control group was treated with the vehicle mixture only. Tumor size and animal body weight were measured twice a week after the s.c. inoculation of the tumor cells. The procedure and use of the animals were approved by the Institutional Animal Care and Use Committee of the National Health Research Institutes (Zhunan, Miaoli, Taiwan). Tumor growth was analyzed for a statistically significant difference using ANOVA followed by the Student–Newman–Keuls test. $P < 0.05$ was considered a significant difference between groups.

In Vitro Kinase Assay for Hepatoma Up-Regulated Protein Phosphorylation. Full-length cDNA of hepatoma up-regulated protein (HURP) was amplified by PCR and cloned in a pcDNA3 vector (Invitrogen) that contains two FLAG tags at the 5′ end of HURP. The FLAG-HURP expression plasmid was transfected (by Poly-Jet; SignaGen Laboratories) to 293T cells. Cells were treated with 5 μg/mL MG132 for 2 h after 24 h of transfection. Total cell lysates were harvested, and cellular supernatants were immunoprecipitated with mouse anti-FLAG agarose (Sigma-Aldrich) in RIPA buffer at 4 °C for 16 h. The immunoprecipitated products were subjected to the in vitro Aurora A kinase assay in a kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 0.5 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 50 μM ATP] that contained 0.37 μM active Aurora A (Millipore) and/or 10 μM Aurora A inhibitors (MLN8237, AZD1152, or IBPR001) and were incubated at 37 °C for 45 min. The products were analyzed by SDS/PAGE and blotted with rabbit anti-FLAG antibody (Sigma-Aldrich) to detect the molecular size of HURP by Western analysis.

1. Coumar MS, et al. (2008) Aurora kinase A inhibitors: Identification, SAR exploration and molecular modeling of 6,7-dihydro-4H-pyrazolo-[1,5-a]pyrrolo[3,4-d]pyrimidine-5,8-dione scaffold. *Bioorg Med Chem Lett* 18(5):1623–1627.
2. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53(Pt 3): 240–255.
3. Jones TA, Zou JY, Cowan SW, Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* 47(Pt 2):110–119.



Scheme A 4-Chlorofuro[2,3-*d*]pyrimidine (**A**) was obtained as reported in WO 2005092896 A1.

Spectral data

IBPR001: 1-(4-{2-[(6-{4-[2-(dimethylamino)ethoxy]phenyl}furo[2,3-*d*]pyrimidin-4-yl)amino]ethyl}phenyl)-3-phenylurea ^{1}H NMR (300 MHz, CD_3OD) δ 2.35 (s, 6H), 2.78 (t, *J* = 5.4 Hz, 2H), 2.91 (t, *J* = 7.2 Hz, 2H), 3.78 (t, *J* = 7.2 Hz, 2H), 4.15(t, *J* = 5.4 Hz, 2H), 6.96-6.98 (m, 1H), 6.99 (s, 1H), 7.04 (d, *J* = 9.0 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 7.34 (d, *J* = 8.7 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.74 (d, *J* = 8.7 Hz, 2H), 8.01 (brs, 1H), 8.20 (s, 1H); LC-MS (ESI) *m/z* 537 ($[M+H]^+$); HPLC Purity 97.8%

IBPR002: 1-(4-{2-[(6-{4-[2-(4-hydroxypiperidin-1-yl)ethoxy]phenyl}furo[2,3-*d*]pyrimidin-4-yl)amino]ethyl}phenyl)-3-phenylurea ^{1}H NMR (300 MHz, $CDCl_3$) δ1.37-1.44 (m, 2H), 1.90-1.99 (m, 2H), 2.85 (t, *J* = 5.7 Hz, 2H), 2.95-2.92 (m, 2H), 3.23-3.17 (m, 2H), 3.41-3.39 (m, 1H), 3.78-3.81 (m, 4H), 4.14 (t, *J* = 5.7 Hz, 2H), 6.77 (s, 1H), 6.94 (d, *J* = 8.7 Hz, 2H), 7.00-7.05 (m, 1H), 7.13 (d, *J* = 8.4 Hz, 2H), 7.26-7.44 (m, 7H), 7.70 (d, *J* = 9.3 Hz, 2H), 8.29 (s, 1H); LC-MS (ESI) *m/z* 593 ($[M+H]^+$); HPLC Purity 95.7%.

Fig. S1. Schematics for chemical synthesis of IBPR001 and IBPR002. The chemical synthesis approach is described in *SI Materials and Methods*. The NMR, liquid chromatography-MS (electrospray ionization), and HPLC profiles of IBPR001 and IBPR002 are presented below the scheme.

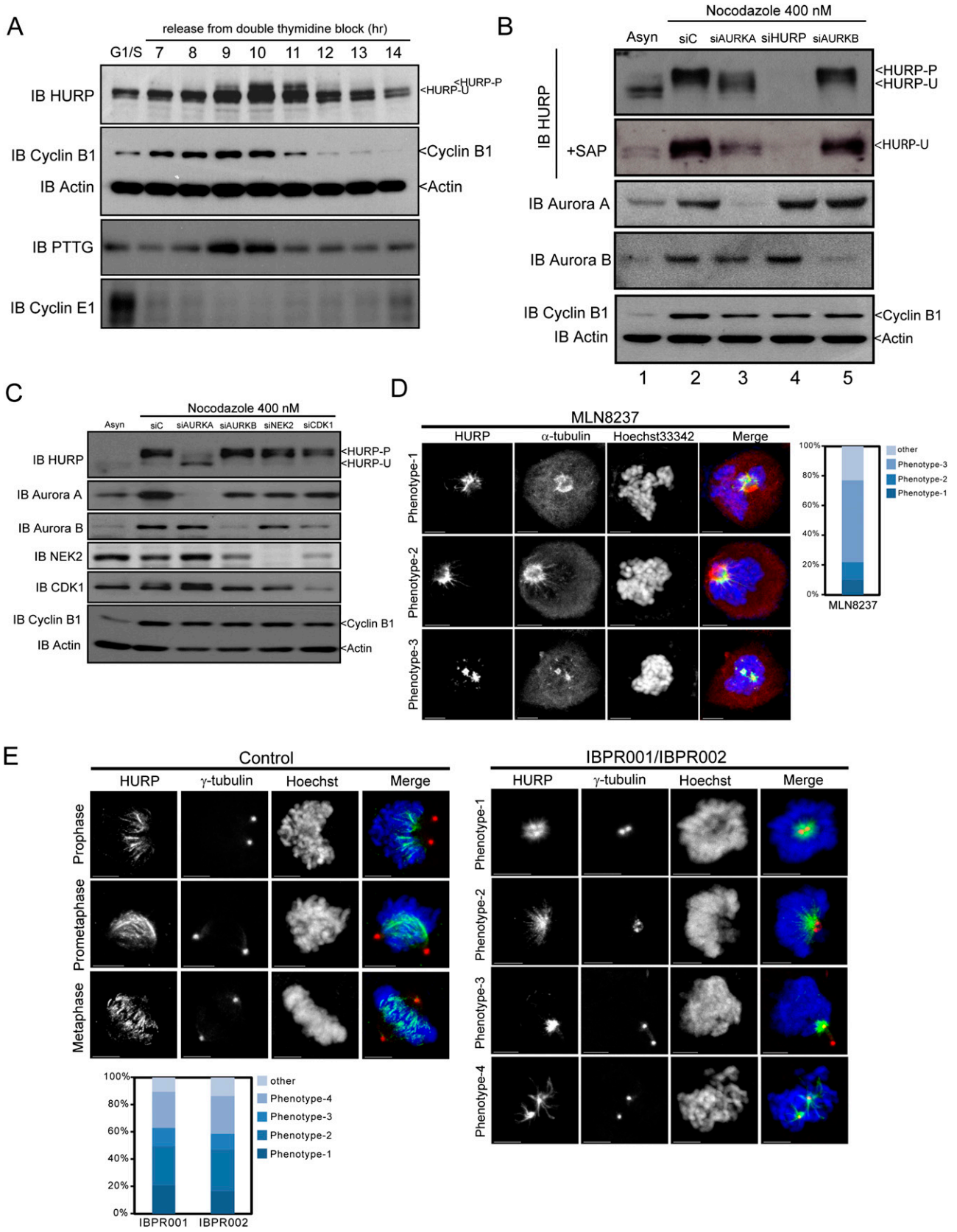


Fig. S2. Two forms of HURP are enriched in G2/M. (*A*) Immunoblot of HURP from HeLa cells released from double thymidine block. Phosphorylated HURP (HURP-P) migrates slightly more slowly than unphosphorylated HURP (HURP-U) in SDS/PAGE. Actin immunoblot was included as a loading control. Cyclin B1, pituitary tumor transforming gene (PTTG), cyclin E1, and immunoblots are shown to indicate cell-cycle stage (1). (*B*) Immunoblot of HURP from HeLa cells treated with Aurora A (*AURKA*), *HURP*, or Aurora B (*AURKB*) siRNAs. Cells were treated with 400 nM nocodazole for 24 h after 18 h of siRNA transfection to

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synchronize cells in the M phase. Lysates treated with or without shrimp alkaline phosphatase (SAP) were compared to verify phosphorylation of HURP. HURP-P was confirmed to be a phosphorylated form of HURP whose molecular size was reduced by SAP treatment. The specificity of HURP Western signals was verified by HURP knockdown using RNAi (lane 4). Asyn, asynchronized. (*C*) Immunoblot of HURP from HeLa cells treated with siRNAs targeting Aurora A (*AURKA*), Aurora B (*AURKB*), cyclin-dependent kinase 1 (*CDK1*), or NIMA-related kinase 2 (*NEK2*). Cells were treated as described in *B*. (*D*) Immunofluorescence staining for HURP and α-tubulin in cells treated with 1.0 μM MLN8237. DNA stained with Hoechst33342 is in blue. Statistics of the representative phenotypes are shown in the graph at right. (*E*) Profiles of HURP in cells treated with 1.0 μM IBPR001/IBPR002. Cells were coimmunostained with rabbit anti-HURP and mouse anti–γ-tubulin antibodies. Statistics of the representative HURP morphological phenotypes are presented in the graph at lower left. Images are maximum projection of *z*-stacks. (Scale bars: 5 μm.)

1. Zou H, McGarry TJ, Bernal T, Kirschner MW (1999) Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* 285(5426):418–422.

Table S1. In vitro kinase inhibition by IBPR002 at 1.0 μM

Protein	Inhibition at 1.0 μM, %
Aurora A	101
TRKA	91
MARK1	91
CHK2	89
RSK3	79
KCC2D	74
PKG1β	67
NEK2	61
STK22B	60
PDK1	57
CK1	56
PHKG2	52
LIMK1	49
DRAK1	43
SGK1	41
IRAK4	37
MAPKAPK2	36
RIP2	34
MLCK	33
FLT3	29
RSE	29
Insulin Receptor	28
LYN A	27
CK2α1	25
MSK1	21
CDC42BPB	19
HGFR	19
PKD2	17
ALK	14
ZAP-70	13
TYK3	11
BTK	9
JNK1	8
PIM1	7
PRKBA	6
VEGFR-1	6
IKK-1	5
MST2	3
PKCη	1
ABL	1
TEK	0
CLK2	−1
GSK3B	−1
FGFR1	−1
RET	−1
Dyrk1a	−2
SGK2	−2
Ca^{2+}/Calmodulin-Dep. II	−3
MEK1	−6
ROS1	−6
PKCγ	−7
EphA4	−11
PLK1	−14
PTK2B	−15
SRPK1	−17
ASK1	−19
CSK	−19

ABL, c-abl oncogene 1, receptor tyrosine kinase; ALK, anaplastic lymphoma receptor tyrosine kinase; ASK1, apoptosis signal regulating kinase 1; BTK, bruton agammaglobulinemia tyrosine kinase; CDC42BPB, CDC42 binding protein kinase beta; CHK2, checkpoint kinase 2; CK1, casein kinase 1, alpha 1; CK2α1, casein kinase 2, alpha 1 polypeptide; CLK2, CDC-like kinase 2; CSK, c-src tyrosine kinase; DRAK1, death-associated protein kinase-related 1; Dyrk1a, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A; EphA4, EPH receptor A4; FGFR1, fibroblast growth factor receptor 1; FLT3, fms-related tyrosine

kinase 3; GSK3B, glycogen synthase kinase 3 beta; HGFR, met proto-oncogene (hepatocyte growth factor receptor); IKK-1, IκB kinase 1; IRAK4, interleukin-1 receptor-associated kinase 4; JNK1, JUN N-terminal kinase 1; KCC2D, calcium/calmodulin-dependent protein kinase type II delta chain; LIMK1, LIM domain kinase 1; LYN A, v-yes-1 Yamaguchi sarcoma viral related oncogene homolog; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; MARK1, MAP/microtubule affinity-regulating kinase 1; MEK1, mitogen-activated protein kinase kinase 1 (MAP2K1); MLCK, myosin light chain kinase; MSK1, synonym RPS6KA5, ribosomal protein S6 kinase, 90 kDa, polypeptide 5; MST2, synonym STK3, serine/threonine kinase 3; NEK2, NIMA (never in mitosis gene a)-related kinase 2; PDK1, pyruvate dehydrogenase kinase, isozyme 1; PHKG2, phosphorylase kinase, gamma 2; PIM1, pim-1 oncogene; PKCη, protein kinase C, eta; PKCγ, protein kinase C, gamma; PKD2, protein kinase D2; PKG1β, protein kinase, cGMP-dependent, type I; PLK1, polo-like kinase 1; PRKBA, synonym AKT1, v-akt murine thymoma viral oncogene homolog 1; PTK2B, protein tyrosine kinase 2 beta; RET, ret proto-oncogene; RIP2, receptor-interacting serine-threonine kinase 2; ROS1, c-ros oncogene 1, receptor tyrosine kinase; RSE, receptor sectatoris; RSK3, ribosomal S6 kinase 3; SGK1, serum/glucocorticoid regulated kinase 1; SGK2, serum/glucocorticoid regulated kinase 2; SRPK1, SRSF protein kinase 1; STK22B, serine/threonine kinase 22B, spermiogenesis associated; TEK, TEK tyrosine kinase, endothelial; TRKA, TRK1-transforming tyrosine kinase protein; TYK3, synonym FER, fps/fes related tyrosine kinase; VEGFR-1, vascular endothelial growth factor receptor 1; ZAP-70, zeta-chain (TCR) associated protein kinase 70 kDa.

Table S2. X-ray data collection and structure refinement statistics of Aurora A/IBPR001 (PDB ID: 4JBO), Aurora A/IBPR002 (PDB ID: 4JBP), and Aurora A/VX-680 (PDB ID: 4JBQ) complex

Parameter	VX-680	IBPR001	IBPR002
Resolution, Å	30–2.30	30–2.50	30–2.45
Unit cell *P6122* (α= β= *90°*, γ= *120°*)			
a	82.414	80.858	80.810
b	82.414	80.858	80.810
c	170.349	170.984	174.308
Total reflections observed	271,853	62,557	55,669
Unique reflections	15,911	12,098	12,877
R_{merge} (outer shell), %	5.9 (48.0)	4.5 (41.6)	5.0 (46.4)
I/σ(I) (outer shell), %	43.2 (8.03)	33.28 (4.57)	25.14 (3.33)
Completeness (outer shell), %	99.8 (100.0)	99.2 (100.0)	99.4 (100.0)
R_{work}, %	22.44	21.97	22.25
R_{free}, %	28.23	23.57	29.07
rms bonds, Å	0.007	0.005	0.008
rms angles, °	1.155	0.953	1.308