Aurora A selective inhibitor LY3295668 leads to dominant mitotic arrest, apoptosis in cancer cells and shows potent preclinical antitumor efficacy

Supplementary Materials and Methods

Cell lines:

In Figure 4, all cancer cell lines were purchased first in 2014 and later as needed. A2780 was purchased from Sigma, EFM-19, KYSE-140, MKN45 and THP-1 from DSMZ, and the following ones from ATCC: A375, AsPC-1, BT-20, BT-549, CAMA-1, CAPAN-1, Capan-2, DU-4475, FADU, HCC1143, HCC1187, HCC1395, HCC1419, HCC1569, HCC1806, HCC1937, HCC1954, HCC202, HCC2218, HCC38, HCC70, HCC827, HCT-116, HCT-15, Hs-578-T, HT-1080, HuVEC, LS-411N, MCF7, MDA-MB-134-VI, MDA-MB-157, MDA-MB-175-VII, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MDA-MB-468, NCI-H1048, NCI-H1299, NCI-H1573, NCI-H1623, NCI-H1650, NCI-H1666, NCI-H1703, NCI-H1734, NCI-H1770, NCI-H1944, NCI-H1975, NCI-H1993, NCI-H2009, NCI-H2030, NCI-H209, NCI-H2122, NCI-H2126, NCI-H2171, NCI-H2347, NCI-H2405, NCI-H358, NCI-H446, NCI-H520, NCI-H524, NCI-H661, NCI-H69, OVCAR-3, SK-BR-3, SK-MEL-2, SK-OV-3, SW48, T47D, U-87-MG, UACC-812, UACC-893, ZR-75-1, ZR-75-30.

Aurora A, Aurora B enzymatic and kinase profiling assays

Aurora A and B kinase enzymatic activity assays *in vitro* were performed using Transcreener ADP-Fluorescent Polarization technology and Corning Costar 3694 plates. Testing compounds were serially diluted 1:3 to create a 10 point concentration-response curve (concentration range 20μM to 0.001μM in 4% final DMSO concentration). Aurora A enzyme reaction buffer contains 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH

7.4, 4 mM $MgCl_2$, 0.01% Triton X-100 and 2 mM dithiothreitol (DTT). 0.096 ng/µL of Aurora A enzyme was added to the reaction mixture with compound, DMSO (positive control) or 100 nM ethylenediaminetetraacetic acid (EDTA) (negative control) to pre-incubate for 30 minutes at 22 ºC. Then 20 µM adenosine triphosphate (ATP) and 150 µM Aurora A kinase activation loop (#326861, EnoGene) were added to start the reaction for 30 minutes at 22 ºC. Aurora B enzyme reaction buffer contains 37.5 mM HEPES pH 7.4, 6.25 mM $MgCl_2$, 0.0075% triton X-100 and 2.5 mM DTT with 0.1 µM INCEP peptide (#92480, GenScript). 0.39 ng/µL of Aurora B enzyme was added to pre-incubate with the compounds for 30 minutes at 22 ºC, then 10 µM ATP and 5 µM Histone H3 (KLH08-4, Anaspec) were added to start the reaction for 45 minutes at 22 ºC.

The reactions were then stopped by adding 25 µL (1:1 v:v) of ADP detection mixture containing ADP far red tracer, ADP antibody and the Stop and Detection buffer (3003-10K, Bellbrook Labs). The plates were kept in the dark for at least 2 hours to allow the displacement of ADP Alexa633 Tracer (bound to ADP Antibody) by ADP produced in the kinase reaction, and the decrease in fluorescence polarization is measured (Ultra384, Tecan). A standard curve of ADP/ATP in the above kinase buffer was used to determine ADP conversion for the compounds tested.

The difference between the median value of positive and negative controls is taken as 100% activity. A four-parameter logistic curve fit was used to generate the IC50 values using ActivityBase™ software (IDBS, Alameda CA). The assay displays a Minimum Significant Ratio (MSR) of ≤ 3.

Kinase profiling (DiscoveRx, KINOMEscan, Fremont, CA) was carried out in 96-well polystyrene plates, according to manufacturer's specifications (1). Briefly, streptavidin-coated

magnetic beads were treated with biotinylated small molecule ligands for 30 min at 25°C to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer [SeaBlock (Pierce), 1% BSA, 0.05% Tween 20, 1 mM DTT] to remove unbound ligand and to reduce nonspecific binding. The kinase assay plates were incubated at 25°C with shaking for 1 h in binding buffer (20% SeaBlock, 0.17x PBS, 0.05% Tween 20, 6 mM DTT) with kinase preparation, liganded affinity beads and LY3295668. After reaction, the affinity beads were washed extensively to remove unbound protein. Bound kinase was eluted in the presence of nonbiotinylated affinity ligands for 30 min at 25°C with shaking. The kinase concentration in elutes was measured by quantitative PCR.

LY3295668 in vivo kinase profile was also performed using KiNativ™ (ActivX, La Jolla, CA) kinase target profiling, an ATP acyl phosphate desthiobiotin probe based chemoproteomics platform to profile the selectivity of kinase inhibitors in lysates according to manufacturer's recommendations. The KiNativ assay measures the ability of small molecules to protect kinases present in cell extract from binding to and forming an adduct with a lysine-reactive ATP-biotin (2, 3).

Cell and tumor lysate preparations

Small cell lung cancer (SCLC) NCI-H446 cell lysate was prepared according to Meso Scale Diagnostics (MSD) manufacturer specifications using lysis buffer [Tris 25 mM, pH 7.5; Leupeptin 10 µg/mL; Trypsin-Chymotrypsin 10 µg/mL; tosyl phenylalanyl chloromethyl ketone (TPCK) 10 µg/mL; Aprotinin 10 µg/mL; β-glycerophosphate 60 mM; Triton X-100 1%; Sodium pyrophosphate ($Na_2H_2P_2O_7$) 2.5 mM; NaCl 150 mM; EDTA 15 mM; ethylene glycol tetraacetic acid (EGTA) 5 mM; Nalpha-4-Tosyl-L-arginine methyl ester hydrochloride (TAME) 2 mM; 4-nitrophenyl

phosphate disodium salt hexahydrate (PNPP) 15 mM; benzamidine 5 mM; sodium vanadate 1 mM; sodium fluoride 10 mM; phenylmethanesulfonylfluoride (PMSF) 50 µg/mL; DTT 1 mM; okadaic acid 1 µM; Microcystine 1 µM] plus complete EDTA free tablet (#1873580; Roche). The cells were incubated with lysis buffer for 15 minutes on ice with agitation.

Tumor tissue samples were first chilled in liquid nitrogen, placed on foil sheets with the mortar and pestle chilled on a dry ice container. The tumor tissue samples were ground according to MSD manufacturer specifications with the pestle and the pulverized tumor tissues were transferred to a tube containing Lysing Maxtrix D beads (#6913-500; MP Biomedicals) and 0.6mL of lysis buffer as described above. The tubes were shaken vigorously for 30 seconds at speed 6.0 in Bio101 fastPrep (#Bio 101; Thermo Seweant). Supernatants from cell and tumor lysates were collected after spinning down the buffer at 10,000g for 10 minutes at 4°C in a table top centrifuge.

Total Histone extraction kit (#OP-0006; EpiQuik) was used to extract total histone from the tumor tissue pellets after centrifugation according to manufactures specifications.

The supernatant protein concentration was measured by Biorad DC-Protein assay (#500-0111, BioRad) and normalized to 1 mg/mL. 25 µL of the lysates were used for Aurora A phosphor-Thr288 or histone H3 phospho-Ser10 MSD assays, respectively.

Aurora A inhibition by Aurora A P-Thr288 MSD cell-based assay

The cell-based and tumor lysate Aurora A P-Thr288 phosphorylation inhibition assay was performed using 96-well ELISA Meso Scale Discovery (MSD) plates (K150 JCD Whole Cell Lysate Kit, Meso Scale Discovery, MD) according to manufacturer's instruction. The plate was first blocked with 150 µL/well of Blocking Solution-A (final concentration 3% BSA) by shaking at room temp for 1 hour and then washed with MSD TrisWash Buffer three times. 25µL

of 1 mg/mL cell lysates were dispensed onto the plate and incubated for additional 3 hours at room temperature and then washed with MSD TrisWash buffer three times. Detection antibody (SULFO-TAGTM anti-AuroraA Phospho-T288) was diluted 50-fold in 1% BSA antibody dilution buffer and 25 µl/well was added into the plate to shake for 1 hour at room temperature. The plate was washed 3 times with MSD TrisWash Buffer before 150 µL/well 2X MSD Read Buffer T was added into the plate. The plate was read on a MSD SECTORTM Imager 6000 instrument. Percentage of inhibition was defined as [(100-(MSD value-MSD maximum inhibition value)/(MSD minimum inhibition value- MSD maximum inhibition value)]*100. The "maximum" and "minimum" were defined by 2 µM positive control Alisertib vs. DMSO negative control value alone.

Aurora B inhibition by Histone H3 P-Ser10 inhibition (1 hour) high throughput cell based assay

Histone H3 P-Ser10 inhibition assay was performed in NCI-H446 cells on Acumen platform. Compounds in DMSO were serially diluted 3-fold from the highest concentration of 40 µM and incubated with cells for 1 hour before fixed with 4% formaldehyde (Sigma) for 45 minutes at room temperature. The short term 1 hour compound incubation measures the Aurora B kinase activity on its kinase substrate histone H3 P-Ser10 inhibition, which distinguishes the Aurora A inhibition induced mitotic arrests by histone H3 P-Ser10 increase with long term (24 hours) compound incubation demonstrated. The cells were then permeated with cold methanol (Sigma) for 15 minutes. Primary antibody (Histone H3 P-Ser10) in 3 % Skim Milk /PBS buffer was incubated at 4C overnight, then with secondary antibody (goat anti-rabbit Alexa Flour 488) for 1 hour at room temperature and finally with propidium iodide (Sigma) containing 50 ug/ml of RNase

(Sigma) before being analyzed on Acumen Explorer Laser-scanning fluorescence microplate cytometer (TTP LabTech Ltd.) for fluorescent intensity read.

Mitotic arrest phenotypic and cell proliferation inhibition by histone H3 P-Ser10 and DNA content multiplexing (24 hours) cell-based assay

HeLa cells were seeded in 96-well plates and incubated overnight. Compounds in DMSO were serially diluted 3-fold from the highest concentration of 20 µM, incubated with cells for 24 hours and fixed with Prefer (Anatech Ltd.) for 30 min at room temperature. The cells were then permeated with 0.1% Triton-X100 in PBS at 4C for 15 minutes and processed as in above Acumen protocol although in this case the primary antibody was diluted in 1% BSA/PBS. Plate was analyzed on Acumen Explorer Laser-scanning fluorescence microplate cytometer (TTP LabTch Ltd.) for H3 P-Ser10 signal, DNA histogram profiles to demonstrate mitotic arrest phenotypes, as well as cell number for proliferation inhibition. See (4) for further details.

Aurora A P-Thr288 and histone H3 P-Ser10 *in vivo* target inhibition assays

NCI-H446 and NCI-H69 xenograft tumor lysates were used for in vivo target inhibition (IVTI). The cells were maintained as recommended by vendor, harvested, washed, and resuspended in a 1:1 mixture of serum free media and matrigel (354234, BD Biosciences). Athymic nude female mice (Harlan, 7-8 weeks) were injected subcutaneously at 6 x 10^6 /mouse in the rear flank. Tumor volume was estimated by using the formula: $V = L*W*0.536$ where L = larger of measured diameter and W = smaller of perpendicular diameter, All in vivo studies were performed according to the Institutional Animal Care and Use Protocols.

Four animals were used for each treatment group and six animals in the vehicle group. Formulated LY3295668 was administrated orally (P.O.) in mice carrying tumors in a final

volume of 0.2 ml when the mean tumor volume was approximately 150 to 200 mm^3. At the indicated time point post dosing, animals were asphyxiated with CO2 and the xenograft tumors harvested by surgical removal, flash frozen in liquid nitrogen, and stored at -80C until analyzed. Tumors were homogenized at 4C to make tumor lysates and subsequently analyzed for Aurora A P-Thr288 inhibition MSD assay as described above.

The tumor pellets after homogenization were process with histone extraction kid for H3 P-Ser10 inhibition MSD assay in a 96-well ELISA plate format (K150 EWD-3 JCD Whole Cell Lysate Kit, Meso Scale Discovery, MD). Blocking Solution-A (final concentration 3% BSA) was added to a MULTI-SPOT Histone H3 4-Spot plate at 150 µL/well followed by shaking at room temperature for 1 hour. After washing 3 times with MSD TrisWash Buffer, 25µL/well histone extract was dispensed onto the plate. The plate was incubated for additional 3 hours at room temperature, and washed 3 times with MSD TrisWash buffer. Detection SULFO-TAG anti-histone H3 P-Ser10 antibody was diluted 50-fold into 3 mL 1% BSA antibody dilution buffer (1 ml 3% BSA with 2 mL MSD washing buffer; 0.01% of blocker D-M), and 25 µL/well was added into the plate followed with shaking at room temperature for 1 hour. Plate was then washed 3 times with MSD TrisWash Buffer, and added at 150 µL/well of 2-fold MSD Read Buffer T to the plate. The plate was read immediately with a MSD SECTORTM Imager 6000 instrument. The percentage of inhibition was defined as [100-(compound treatment group mean MSD reading/vehicle group mean MSD reading)]*100, and analyzed with SAS software (SAS Institute Inc.).

Cell proliferation and apoptosis assays

Cells were seeded at appropriate density (2,000-5,000 cells/well) and cultured overnight in 96-well plate (Costar 3596, Corning Inc.) before treated with LY3295668 and other reference compounds serially diluted 3-fold from the highest concentration of 20 μM. The plates were mounted into Incucyte ZOOM (Essen BioScience) for cell growth inhibition (phase contrast channel) and CASP3/7 induction (Green channel. Cell event Caspase-3/7 Green Detection Reagent C10423, Thermo Fisher Scientific) were recorded for 72 to 96 hours (5). The growth and apoptosis event curves were plotted using Basic Analyzer on Incucyte.

For the 80 cell lines viability inhibition panel, cells were seeded in 384-well plates (# 356663, Bectom Dickinson) and treated for two-doubling times according to individual cell line growth rate. Cell viability inhibition was detected by using 25 μl of Cell Titer-Glo reagent (Promega Corporation) added to each well. Plates were incubated for 45 minutes at room temperature before the signal was recorded on a luminescence plate reader (EnVision, Perkin Elmer). Percent inhibition versus 2uM Staurosporine was calculated and then Rel IC50s were obtained using the four parameter logistic model as described in Assay guidance manual.

EMT and cancer cell stemness assays

The assay details are in (6). Briefly, recombinant TGFβ was added to the NCI-H358 cell culture media to a final concentration of 10 ng/ml. Tested compounds were added at indicated concentrations and the cell culture was incubated for 6 days. Cell extracts were isolated and analyzed by western for EMT and stemness markers. Inhibition of cell proliferation was measured at day-6 by Acumen Explorer Laser-scanning fluorescence microplate cytometer (TTP LabTch Ltd.).

Antitumor growth efficacy with small cell lung cancer (SCLC) xenograft, PDX and other tumor models

All in vivo studies were performed according to the Institutional Animal Care and Use Protocols. For xenograft models, human NCI-H446, NCI-H69 cells was implanted as above. 10 animals were used per treatment group and 8 animals in the vehicle group.

The small cell lung cancer (SCLC) patient-derived tumor models (xenografts; LXFS 538, LXFS 573, LXFS 615, LXFS 650, LXFS 1129, LXFS 2156) were derived from surgical specimens from patients at Oncotest (Freiburg, Germany). Establishment and characterization of these models were performed according to the relevant Oncotest SOP (7). Briefly, following their primary implantation into nude mice (passage 1, P1), the tumor xenografts were passaged until stable growth patterns established. Stocks of early passage xenografts were frozen in liquid nitrogen according to the relevant SOP, and used for compound testing. The tumors were implanted subcutaneously in the left flank, randomized when the volume reached 80 – 200 mm^3 to start compound treatment.

LY3295668 was formulated in 20% HPBCD in 25mM pH 2 phosphate and administered orally (final volume: 0.2 mL/mouse). Tumor size and body weight were recorded and analyzed using a proprietary data capture and analysis tool (ZEUS, Eli Lilly and Company). The ZEUS system used SAS software (SAS Institutes Inc, Cary, NC) to analyze the log tumor volume data using repeated measures ANOVA model with an AR(1) variance structure. For each time point taken, treatment groups were compared to the control vehicle group. Tumor volumes were given as means +/- standard errors for each treatment group. Grand mean of all groups from baseline at randomization day was used to compute % change of T/C. with PD (>=20%); SD (0% to 20%);

PR (-80% to 0%); and CR (<-80%).

Western blot and Immunofluorescence analysis

Cell and tumor lysates were loaded on NuPage 4-12% Bis-Tris or BioRad XT BisTris SDS PAGE gels. Proteins were transferred from the gels to nitrocellulose membranes and immunoblotted with antibodies for proteins indicated, then imaged with Odyssey (LI-COR Biosciences) or FUJI (LAS-4000) system using manufacturers' protocol. Immunofluroacence staining was as described in (8).

Flow Cytometry

Treated HeLa cells were trypsinized and fixed for flow cytometry on Becton Dickinson LSRII as in (9). The data were processed for cell cycle distribution by ModFit LT (Verity, Topsham, ME).

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