Targeting activated Akt with GDC-0068, a novel selective Akt inhibitor that is efficacious in multiple tumor models

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Supplementary Materials:

Supplemental Tables:

Supplemental Figures:

Supplementary Materials and Methods

Enzymatic assays. The assay for the determination of Akt1/2/3, and PKA kinase activity employs the IMAP™ fluorescence polarization (FP) phosphorylation detection reagent (IMAP™ Screening Express Kit, Molecular Devices Catalog R8073) to detect fluorescently labeled peptide substrates that have been phosphorylated by the respective kinases. The Akt1 enzyme employed in these studies consisted of a recombinant baculovirus-expressed, amino-terminal, poly-histidine-tagged, full-length, wild-type human form (Genbank accession number: M63167) and was obtained from Millipore (Catalog 14-276; Lot D8MN034U; Dundee, Scotland). The PKA enzyme employed in these studies consisted of the recombinant untagged human isolated catalytic subunit of PKA (Genbank accession number: X07767) expressed in *E. coli* obtained from Invitrogen (Catalog 14-440, Lot 26698U; Madison, WI). Inhibitor, enzyme (9 nM Akt1 or 100 pM PKA), and substrate (100 nM Crosstide, Catalog R7110, Molecular Devices, Sunnyvale, CA) were incubated with 5 μM ATP in assay buffer (10 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 0.1% BSA [w/v], final DMSO 2% [v/v]) for 60 minutes at ambient temperature in a 5 μL reaction volume. Reactions were initiated by addition of enzyme+peptide substrate to ATP solutions. IMAP™ Binding Reagent (15 μL) was added to terminate the reaction and the stopped reactions were incubated for a minimum of 30 minutes at room temperature (rt).

pPRAS40 In Cell Western Assay. Cells were plated in 96-well plates (Grenier Cat 655946) at a density of 20,000 cells/well and incubated for 16–24 hours at 37 °C, 5% CO_2. Cells were treated with 0–25 μM inhibitor for 1.5 hours at 37 °C. Medium above the cells was removed and each well was supplemented with fixation solution (3.7% [v/v]

formaldehyde in phosphate-buffered saline [PBS]) for 20 minutes at rt. Cells were permeabilized with a 10-minute exposure to 100% methanol (-20 °C) and subsequently rehydrated in PBS and blocked in blocking buffer (Catalog 927-40000; LI-COR Inc.; Lincoln, NE) for 60 minutes at rt. A primary antibody solution consisting of an antibody specific for Thr246-phosphorylated PRAS40 (rabbit polyclonal antibody, 1:500 dilution, Cat AS1011; Calbiochem; San Diego, CA) and a signal-normalizing antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, mouse monoclonal, 1 µg/mL final concentration, Cat RDI-TRK5-6C5, Fitzgerald Industries Inc.; Concord, MA) in blocking buffer was applied to each of the wells and incubated overnight at 4 °C. Wells were then washed with PBS containing 0.05% (v/v) Tween-20 and treated with a secondary antibody solution containing fluorophore-conjugated antibodies specific for rabbit (Alexa680 fluorophore-conjugated goat anti-rabbit IgG, Cat AS21109; Invitrogen; Madison, WI) and mouse IgG (IRDye800 fluorophore-conjugated goat anti-mouse IgG, Cat 610-132-121; Rockland Inc.; Gilbertsville, PA) and incubated for 1 hour at rt. Wells were washed in PBS with 0.05% (v/v) Tween-20 and then imaged and quantified on a LI-COR Aerius™ imager (LI-COR Inc.; Lincoln, NE). Phospho-PRAS40 signal was normalized to the GAPDH signal to control for well-to-well variation in cell number.