Title Page

WJD008, a Dual PI3K/mTOR inhibitor, Prevents PI3K Signaling and Inhibits the Proliferation of Transformed Cells with Oncogenic PI3K mutant

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Running title page
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A Dual PI3K/mTOR Inhibitor Prevents Oncogenic PI3K Signaling

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ABBREVIATIONS:

PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; SRB, sulforhodamin B; Grp1, general receptor for phosphoinositides, isoform 1; PH domain, pleckstrin homology domain; p70S6K, the 70-kDa ribosomal S6 kinase; (eIF)4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; PTEN, phosphatase and tensin homologue deleted from chromosome 10; DNA-PK, DNA-dependent protein kinase; ATM, Ataxia Telangiectasia Mutated; ATR, ATM- and Rad3-related; PIKK, PI3K-related protein kinase; H2AX, a member of the histone H2A

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family; PDK1, phosphatidylinositol 3-phosphate-dependent kinase 1; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; IC_{50}, the half maximal inhibitory concentration; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; DTT, 1,4-dithiothreitol; ELISA, enzyme-linked immunosorbent assay; CPT, camptothecin; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; PAGE, polyacrylamide gel eletrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

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Abstract

The phosphatidylinositol-3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) signaling pathway is often constitutively activated in various human cancers, providing validated targets for cancer therapy. Among a series of 5-cyano-6-morpholino-4-substituted-pyrimidine analogues designed and synthesized based on PI3K target, WJD008 was selected for further pharmacological characterization due to its potent activity against PI3K signaling. WJD008 inhibited kinase activity of PI3Kα and mTOR with less activity against PIKK family members. In cellular settings, WJD008 abrogated IGF-I-activated PI3K-Akt-mTOR signaling cascade and blocked the membrane translocation of a pleckstrin homology domain containing EGFP-Grp1-PH fusion protein, suggesting down-regulation of PtdIns(3,4,5)P_3 output induced by WJD008 resulted in inactivation of PI3K pathway. Consequently, WJD008 arrested cells in G1 phase without induction of apoptosis. Furthermore, WJD008 reversed the hyper-activation of the PI3K pathway caused by the oncogenic mutation of p110α H1047R and suppressed the proliferation and clonogenesis of transformed RK3E cells harboring this mutant. WJD008 was superior to the pan-PI3K inhibitor wortmannin against proliferation of a panel of cancer cells independent of their status of PI3K pathway or tissue originations. In summary, WJD008 is a potent dual PI3K/mTOR modulator with anti-proliferative and anti-clonogenic activity in tumor cells as well as transformed cells with *PIK3CA* mutant, which provides new clues for design and development of this chemical scaffold as anticancer drug.

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Introduction

Phosphatidylinositol 3-kinase (PI3K) are a family of lipid kinases, which are responsible for the generation of 3-phosphorylated inositides and play central roles in diverse cellular responses such as proliferation, survival, mobility and metabolism (Vivanco and Sawyers, 2002). The PI3K pathway is frequently deregulated in a wide range of tumor types as a result of genetic and epigenetic aberrations. Except for hyper-activation of the upstream growth factor signaling (Douglas et al., 2006; Hollestelle et al., 2007) and mutation in or loss of PTEN (Harima et al., 2001; Byun et al., 2003), oncogenic mutations in *PIK3CA* itself provide further evidence of the role of PI3K in tumorigenesis and validation of PI3K as an attractive target for cancer therapy (Samuels et al., 2004). Mutations in *PIK3CA*, which encodes the p110α catalytic subunit, have been reported to occur at high frequency in a number of human cancers (27% glioblastomas, 25% gastric cancers, 8% breast cancers and 4% lung cancers). Interestingly, more than 80% of the mutations in *PIK3CA* cluster in two small conserved regions, i.e. E545K in helical domain and H1047R in kinase domain (Samuels et al., 2004). Despite the suggested different functions (Zhao and Vogt, 2008; Chaussade et al., 2009), these two *PIK3CA* mutation hotspots, leads to full activation of the catalytic subunit (Samuels et al., 2004) and transformation (Zhao et al., 2005; Bader et al., 2006). Genetic ablation of the PI3K mutant allele in the colorectal cancer cell lines HCT116 and DLD-1 resulted in the reduction of their oncogenic properties (Samuels et al., 2005). Therefore, discovery and development of the anti-cancer drugs targeting PI3K especially mutated ones has been an attractive strategy in tumor chemotherapy.

With multiple efforts under way in academia and industry to develop clinically relevant

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inhibitors of this signaling pathway, a number of pan-specific or isoform-specific PI3K inhibitors have been identified and developed as potential molecularly targeted anticancer therapy. The first set of PI3K inhibitors has entered clinical trials. Of them, NVP-BEZ235 (Maira et al., 2008), NVP-BGT226, XL765, SF1126 [(Garlich et al., 2008) and reviewed in (Brachmann et al., 2009)] are all reported active against phosphatidylinositol-3 kinase related kinases (PIKKs), including mTOR, which is also the downstream effectors of PI3K and participates in the negative feedback loop targeting an upstream component of the signaling chain (Wullschleger et al., 2006).

In spite of the effort made in the past decade, the identified PI3K inhibitors is far from enough to meet clinical need as well as probing the complicated PI3K/mTOR pathway. To this end, we designed and synthesized a series of 5-cyano-6-morpholino-4-substituted-pyrimidine analogues in an aim of discovering new modulators of PI3K-Akt-mTOR pathway using a structure-based design approach. Of them, WJD008 distinguished itself as a potent dual PI3K/mTOR inhibitor with potential against *PIK3CA* mutant transformed cells. Highlighted by the methodology of target-based synthesis, the discovery of WJD008 provides a promising novel chemical template for discovery and development of PI3K inhibitors, especially those targeting mutated PI3K.

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Material and Methods

Chemicals

A series of 5-cyano-6-morpholino-4-substituted-pyrimidine analogues was synthesized as potential PI3K inhibitors based on the structures reported previously (Hayakawa et al., 2006). Wortmannin and camptothecin (Furuta et al., 2003)was purchased from Sigma (St. Louis, Mo). All the compounds were prepared at 10 mmol/L or 20 mmol/L in 100% DMSO and aliquots were stored at -20^{o}C. All compounds were diluted to desired concentrations immediately before each experiment. Final DMSO concentration was kept below 0.2% in control and compound-treated cells.

Cell Lines and Cell Culture

The transformed rat kidney epithelial cell lines RK3E/NT and RK3E/p110α(H1047R) were kindly provided by Professor Peter K. Vogt (The Scripps Research Institute). Chinese hamster ovary cell line CHO-K1, Human breast cancer cell line MDA-MB-468 and BT-474, breast cell line MCF-10A, squamous carcinoma cell line HeLa, colon cancer cell line SW1116, prostate cancer cell line PC-3 and DU-145, and glioblastoma cell line M059J and M059K were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Human breast cancer cell line MCF-7, ovarian cancer cell line SKOV-3, colon cancer cell line HCT-116, and stomach cancer cell line MKN28 and MKN45 were obtained from the Japanese Foundation of Cancer Research (Tokyo, Japan). Rhabdomyosarcoma cell line Rh-30 was a gift from the St. Jude Children's Research Hospital (Memphis, TN). Human lung adenocarcinoma cell line A549 was from the

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National Cancer Institute (NCI, Bethesda, MD). Human hepatocellular carcinoma cell line SMMC-7721 and Zip-177 was a gift from the Second Military Medical School (Shanghai, China).

MKN28, MKN45, HCT-116, SKOV-3 and MCF-7 cells were maintained in DMEM (Gibico, Grand Island, NY) with 10% FBS (Gibico), penicillin (100 IU/mL), 100 mg/L streptomycin (100 μg/mL and additional 3 μg/mL puromycin were included in the medium in the case of RK3E). A549 and PC-3 cells were maintained in Ham's F12 (Gibico) with 10% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL). CHO-K1 transfected with pEGFP-C1-Grp1-PH were selected in the presence of 700 ng/mL of G418 (Calbiochem, San Diego, CA) and maintained in the same medium containing 350 ng/mL of G418. SMMC-7721, Zip-177, M059J, M059K, DU-145, Rh30, SW1116, HeLa and MDA-MB-468 were maintained in RPMI 1640 (Gibico) supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL), and HEPES (10 mmol/L, pH 7.4). BT-474 cells were incubated in the aforementioned media supplemented with 1 mmol/L sodium pyruvate. All cells were cultured in humidified atmosphere of 95% air and 5% CO_2 at 37 ^oC.

In vitro PI3K Kinase Assay

The catalytic activity of PI3K was measured by Kinase-Glo Assay as previous described (Serra et al., 2008). Briefly, the kinase reactions were preformed in 96-well white plates (Greiner). Each well was loaded with 1 μL of tested compounds (in 10% DMSO) and 25 μL reaction buffer [10mmol/L Tris-HCl pH 7.5, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L DTT, and 0.05% CHAPS] containing 10 μg/mL substrate

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D-*myo*-Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2; Echelon Biosciences Inc., UT) and 100 μM PI3 Kinase (p110α/p85α) (Millipore, Bedford, Mass). The reaction was initiated by addition of 25 μL of 1 μmol/L ATP prepared in the reaction buffer and terminated by the addition of 50 μL Kinase-Glo buffer (Promega, Madison, WI) after incubation for 60 min. The plates were read in Novostar Microplate Reader (BMG LABTECH, Offenburg, Germany) for luminescence detection.

ELISA-based mTOR kinase assay

A GST-tagged truncated human TOR (animo acid 1360-2549) was expressed using the Bac-to-BacTM baculovirus expression system (Invitrogen, Carlsbad, CA, USA) and purified in Glutathione SepharoseTM 4B(GE Healthcare, Buckinghamshire, UK). The kinase activity of the purified truncated mTOR kinase was determined by a ELISA-based assay. The kinase reaction was performed in 96-well plates pre-coated with 10 μg /ml of p70S6K1(amino acid 332-415) as a substrate. Ninety microliter of kinase assay buffer (10 mM Hepes (pH 7.4), 50 mM NaCl, 50 mM b-glycerophosphate, 10 mM $MnCl_2$, 0.5 mM DTT, 0.25 μM microcystin LR, and 100 μg/mL BSA) containing 5 μM ATP was added to each well. The reaction was initiated by the addition of 1.8 μg/mL Gst-tagged truncated human TOR. After incubation for 2 h at 37°C, the plate was washed three times with PBS containing 0.1% Tween 20 (T-PBS). Next, 100-μl antibody against phosphorylated p70S6K1 at Thr389 (1A5,Cell Signaling) in 1:4000 dilution was added into each well. After 1-h incubation at room temperature, the plate was washed three times. Mouse anti-mouse IgG horseradish peroxidase (100 μl, 1:2000 dilution) diluted in T-PBS containing 5 mg/ml BSA was added. The plate was incubated at room temperature for 1 h

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and then with 100-µl solution (0.1% H2O2, 2 mg/ml o-phenylenediamine in citrate buffer 0.1 M, pH 5.5) after secondary antibody was washed out. The reaction was terminated by adding 100 µl of 2 M of H_2SO_4 and OD value at 490 nm was measured using a multi-well spectrophotometer (VERSAmax).

Western Blot Analysis

Rh30 and PC-3 cells grown to 50% confluence in 12-well plates continued to be incubated in serum-free medium for 24 h. After exposure to tested compounds for 1 h, cells were stimulated with 50 ng/mL IGF-I for 10 min. Cells were then collected and subjected to Western blot analysis as previously described (Zhang et al., 2005) with the antibodies against phospho-PDK1 (S241) (#3061), phospho-Akt (T308) (#9275), phospho-Akt (S473) (#9271), phospho-mTOR (S2448) (#2971), phospho-p70-S6K (T389) (#9205; Cell Signaling Technology, Beverly, MA) and phospho-4E-BP1 (T70) (sc-18092-R) (Santa Cruz Biotechnology, Santa Cruz, CA). Membrane detected phosphorylated proteins were stripped with Re-blot plus mild solution (Chemicon International, Temecula, CA) and re-blotted with antibodies against corresponding total PDK1 (#3062), Akt (#9272), mTOR (#2972; Cell Signaling Technology), S6K (#ab9366), 4E-BP1 (#ab2606) (Abcam, Cambridge, UK). To detect γ-H2AX, Rh30 cells grown in 12-well plates were pre-treated with WJD008 or wortmannin for 0.5 h, and were then co-treated with 1 µM camptothecin for 2 h. Cells were collected and subjected to Western blot analysis using antibodies against phospho-H2AX (S139) (#2577; Cell Signaling Technology). GAPDH (KangChen Bio-tech Inc., Shanghai, China) were used

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as loading control.

Phosphorylated Akt (S473) ELISA Assay

Rh30 cells were grown to 50% confluence in 12-well plates and continued to be incubated in serum-free medium for 24 h. After exposed to tested compounds for 1 h, cells were stimulated with 50 ng/mL IGF-I for 10 min at 37 ^{o}C. In parallel, ELISA test plates (Corning) were prepared with the anti-Akt coating antibody (Santa Cruz). At the end of treatment, cells were lysed and the cell lysate (50 μL) were transferred into ELISA plates and incubated for 3 h at 4 ^{o}C. After three washes, 50 μL diluted antibody against phosphorylated Akt at Ser 473 (Cell Signaling Technology) was added and plates were incubated overnight at 4 ^{o}C. After the plate being washed with PBST for three times, the horseradish peroxidase-conjugated secondary antibody (Sigma) was added and incubated for 2 h, and the immune complexes assayed with 100 μL substrate (TMB). The OD value at 450 nm was read on a multi-well spectrophotometer VERSAmax (Molecular Devices, Sunnyvale, CA) and IC_{50} value was determined with the sigmoid dose-response curves by Graphpad Prism 4.

Grp1-PH Translocation Assay

The CHO-K1 cells were transfected with pEGFP-C1-Grp1-PH (gift from Professor Tamas Balla, National Institute of Health) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. CHO-K1 cells stably expressing pEGFP-C1-Grp1-PH were selected in the presence of 700 ng/mL G418 and maintained in the same medium containing 350 ng/mL G418. 5×10^3 cells were seeded in 96-well

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black plate (Greiner). On the next day, cells were incubated in serum-free media for 24 h followed by treatment of WJD008 or wortmannin for 1 h. After addition of IGF-I (250 ng/mL) for 10 min to trigger PI3K activation, the cells were washed with ice-cold PBS and fixed with 4% paraformaldehye for 10 min at room temperature. The fluorescent images were captured with a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Cell Cycle Analysis

Exponentially growing Rh30 were seeded in 6-well plate at a density of 2×10^5 (Costar). On the next day, cells were treated with WJD008 at indicated concentrations for 72 h. Cells were collected for cell number counting (Z1 coulter particle counter, Beckman coulter) and the rest were fixed in ice-cold 70% ethanol. DNA content was measured with a FACS Calibur™ (Becton-Dickinson, Mountain View, CA) and cell cycle distribution was analyzed using ModFIT LT software ((Verity Software House, Inc., Topsham, ME). A total of 10,000 cells were analyzed from each sample.

SRB Assay

Cell proliferation was evaluated by sulforhodamine B staining assay (Skehan et al., 1990). Briefly, cells seeded in 96-well plates were treated with tested compounds for 72 h. The medium was removed and the cells adhered to the plate were then fixed with 10% trichloroacetic acid and stained with sulforhodamine B (Sigma). After 3 washings using 1% acetum, sulforhodamine B was dissolved in 100 μL buffer containing 10 mmol/L Tris-base and the OD value was measured at 520 nm using a multi-well

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spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA). The inhibitory rate on cell proliferation was calculated using the formula: ($OD520nm_{control\ cells}$ − $OD520nm_{treated\ cells}$)/$OD520nm_{control\ cells}$ × 100%. The average IC_{50} values were from at least three independent tests.

Colony Formation Assay

RK3E/NT and RK3E/p110α(H1047R) (single-cell suspension) were seeded in 6-well plates at a density of 200 cells per well. On the next day, cells were treated with tested compounds at indicated concentrations for one week. Adherent cells were stained with 0.1% violet after fixation of methanol, and visible colonies (≥50 cells) were counted.

Data Analysis Statistics

Data were presented as mean ± SD from at least three independent experiments and differences were considered significant when $P<0.05$ as determined by Student's *t* test.

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Results

Structures of WJD series compounds and their effects on PI3K-mediated signaling

Among PI3K inhibitors recently reported in the literatures (Marone et al., 2008; Yap et al., 2008), tricyclic pyridofuropyrimidine PI-103 strongly inhibits all four class I isoforms and mTOR at nanomolar range (Figure 1A) (Hayakawa et al., 2007; Raynaud et al., 2007). Its thienopyrimidine analogue **2** is a selective p110α inhibitor that inhibits tumor cell proliferation (Hayakawa et al., 2006). In this study, we chose compound **2** as our lead compound for further optimization because of its good selectivity for p110α. It has been reported that cyano group and thio atom have similar lipophilicity property, and they were used as bioisosteres in estrogen receptor ligand design successfully (Meyers et al., 2001; Schopfer et al., 2002). Herein, we simplified the thienopyrimidine scaffold into 5-cyanopyrimidine ring, kept the morpholine and 3-substituted-phenyl ring which bind to the ATP-binding pocket (Knight et al., 2006) of PI3K. We then synthesized a series of 5-cyano-6-morpholino-4-substituted-pyrimidine analogues **3**, named WJD as potential PI3K inhibitors (Figure 1A).

To determine whether WJD series compounds have any effect on PI3K-mediated signaling in Rh30 cells, the effect of those compounds on IGF-I induced Akt phosphorylation was evaluated. As shown in Figure 1B, WJD008 distinguished itself among the WJD series compounds with potent activity in inhibiting phosphorylation of Akt at S473 upon the stimulation of IGF-I. In order to further confirm this effect, ELISA assay was employed to measure phosphorylation Akt triggered by IGF-I in the presence of WJD008 and found that WJD008 significantly suppressed this process with an IC_{50}

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value of 0.32±0.02 μM (Figure 1C).

WJD008 potently inhibits the catalytic activity of PI3Kα and mTOR.

Since WJD008 displayed most potent activity in blocking IGF-I-triggered Akt activation among this series of compounds, we examined whether this effect is due to its inhibition on the catalytic activity of PI3K using an ATP depletion (Kinase-Glo) assay (Koresawa and Okabe, 2004). As shown in Figure 2A, WJD008 dose-dependently inhibited the kinase activity of PI3Kα with an IC_{50} value of 1.72 ± 0.66 nM. Considering the important role of mTOR in PI3K-Akt-mTOR pathway and its highly homologues catalytic domain with PI3K, we detected whether WJD008 inhibits the activity of mTOR by an ELISA-based assay. WJD008 inhibited the kinase activity of mTOR with an IC_{50} of 3.42 μM (Fig. 2B). Further study indicated that that increasing concentrations of ATP reduced the inhibitory activity of WJ008 against mTOR (data not shown), suggesting that WJD008 is an ATP-competitive inhibitor. It should be noted that concentration of ATP used in mTOR assay is much higher than that used in PI3K assay, which might result in less potency of WJD008 against mTOR. Therefore, WJD008 is likely a dual PI3K/mTOR inhibitor.

Besides mTOR, DNA-dependent protein kinase (DNA-PK), Ataxia Telangiectasia Mutated (ATM) and ATM- and Rad3-related (ATR) are three serine/threonine protein kinases sharing highly homologues catalytic domain with PI3K (Jackson, 1997) and are therefore assigned as members of PI3K-related protein kinase (PIKK) family. A number of PI3K inhibitors, such as LY294002 and wortmannin, have been reported to inhibit these PIKK kinases (Izzard et al., 1999). We next determined the effect of WJD008 on the activity of DNA-PK, ATM and ATR by measuring the level of phosphorylated H2AX

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(γ-H2AX) in DNA-damaged cells. DNA-PK, ATM and ATR are involved in the DNA repair machinery and their activation is required for the phosphorylation of H2AX at Ser 139 upon DNA strand breaks induced by camptothecin (CPT) (Furuta et al., 2003). Therefore, the cellular readouts were used to evaluate the potential activity of WJD008 against these kinases. As shown in Figure 2C, Rh30 cells displayed a robust increase of γ-H2AX after treatment with CPT (10 μM). Similar to the effect induced by wortmannin, co-treatment with WJD008 completely abrogated the up-regulation of γ-H2AX at the concentration up to 40 μM, whereas the effect was mild at the concentrations below 8 μM. The result indicates that high concentration of WJD008 also inhibits the kinase activity of ATM, ATR and/or DNA-PK in Rh30 cells.

WJD008 blocks the PI3K-Akt-mTOR signaling in tumor cells

Since WJD008 had been demonstrated to potently inhibit the kinase activity of PI3K and mTOR as well as to abrogate activation of Akt, we next determined in detail how WJD008 could abrogate PI3K/mTOR-mediated signaling pathway stimulated by growth factors in tumor cells. Serum-deprived Rh30 cells with intrinsically hyper-activated PI3K-Akt-mTOR pathway was treated with various concentrations of WJD008 followed by the stimulation of IGF-I. As illustrated in Figure 3A (left), the level of phosphorylated phosphatidylinositol 3-phosphate-dependent kinase 1 (PDK1) was not affected by the stimulation of IGF-I or pre-incubation of WJD008, which is similar with the previous report that phosphorylation of PDK1 at Ser 241 was insensitive to serum deprivation or treatment with wortmannin (Pullen et al., 1998; Casamayor et al., 1999). Contrarily, WJD008 inhibited phosphorylation of Akt at Thr 308 and Ser 473 in a dose-dependent

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manner. Moreover, the phosphorylation levels of mTOR at Ser 2448 and its two best characterized downstream effective molecules, p70 S6 kinase 1 (p70S6K) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) all reduced in the presence of increasing concentrations of WJD008. Meanwhile, the total protein level of aforementioned downstream components in PI3K pathway remained unchanged, suggesting that down-regulation of phosphorylated proteins by WJD008 is not due to loss of total proteins. The inhibitory activity of WJD008 on the PI3K-Akt-mTOR signaling was also confirmed using prostate cancer PC-3 cells (Figure 3A, right).

In order to further verify that down-regulated PI3K signaling is due to inhibition of PI3K activity in tumor cells, the effect of WJD008 on the redistribution of Grp1-PH domain following stimulation of IGF-I were determined in CHO-K1 cells expressing Grp1-PH fused to GFP. Grp1-PH domain is shown a specific binding affinity to PtdIns(3,4,5)P_3 rather than PtdIns(4,5)P_2 in cells and aggregates around the cell membrane responding to the recruitment of PtdIns(3,4,5)P_3 (Gray et al., 1999). As shown in Figure 3B, activation of PI3K and production of PtdIns(3,4,5)P_3 resulted in translocation of GFP-Grp1-PH to cellular membrane, which was indicated as the fluorescent foci near the membrane. However, pre-incubation of 10 μM WJD008 for 1 h before IGF-I stimulation completely prevented the translocation of GFP-Grp1-PH fusion protein to plasma membrane, which was consistent with its inhibition of PI3K signaling in Rh30 cells. A similar effect was observed when cells were treated with wortmannin, but not with rapamycin (data not shown). Thus, it appears that the inhibition of the activity of PI3K by WJD008 results in down-regulation of PtdIns(3,4,5)P_3 and further blocks PI3K-Akt-mTOR signaling.

WJD008 induces G1-phase arrest without apoptosis in tumor cells

It has been reported that some PI3K inhibitors induces apoptosis though most of them, such as PI-103 and ZSTK474, induce G1-phase arrest instead of apoptosis (Yaguchi et al., 2006; Raynaud et al., 2007; Maira et al., 2008). Hence, we examined the effect of WJD008 on the cell cycle distribution of Rh30 cells. As presented in Figure 4A, WJD008 arrested cells in G1 phase in a dose-dependent manner. Cell population in G1 phase increased from 43.60% (DMSO control) to 55.09% and 63.74% upon treatment of the cells with 5 or 10 μM WJD008, respectively. Similarly, cell population in G1 phase increased in the presence of Wortmannin, but at a less extent. To exclude WJD008-induced cytotoxicity, we counted the cell number in parallel while performing the FACS. As shown in Figure 4B, fold change in cell number relative to that seeded was plotted. This is no loss in cell number after treatment with WJD008 up to 10 μM and over 95% of cells were viable observed by trypan-blue staining (data not show). However, obvious sub-G1 peak failed to appear following WJD008 treatment even at 10 μM for 72 h, indicating that WJD008 is unable to induce apoptosis in Rh30 cells and its ability to induce G1 phase arrest may at least partially result in inhibition of proliferation of tumor cells.

WJD008 inhibits p110α exhibting oncogenic H1047R mutation in cells

H1047R is one of the mutation hot spots in the *PIK3CA*, which results in hyper-activation of PI3K and transformation. In order to evaluate the potential ability of WJD008 against cells harboring mutated p110α H1047R, we examined the antiproliferative activity of WJD008 in transformed RK3E cells (Fu et al., 2005) stably expressing p110α H1047R

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(HR) by retroviral infection. The oncogenic mutation H1047R induced constitutive phosphorylation of Akt even when cells were deprived of serum (Figure 5A), which was also observed in the breast cancer cells exogenously expressing the mutant (Serra et al., 2008). When treated with WJD008 for 1 h followed by IGF-I stimulation, the phosphorylation level of Akt was more vulnerable to WJD008 in H1047R mutant cells than that in none-transfected (NT) cells (Figure 5A), suggesting that WJD008 was potent against p110α H1047R.

To determine whether blockage of PI3K signaling result in inhibition of proliferation of RK3E cells with mutant PI3K, SRB assay was employed to measure the effect of WJD008 on the proliferation of these cells. Though WJD008 and wortmannin displayed similar potency against RK3E/NT cells with IC50s of 16.29 ± 7.72 or 25.79 ± 4.35 μM, respectively, there is a sharp divergence in their activities against H1047R mutant cells (Figure 5B). WJD008 was active to inhibit the proliferation of H1047R mutant cells with an IC_{50} of 23.06 ± 9.52 μM, while wortmannin had little effect on the proliferation of this cell line and the inhibitory rate was about 25% even as the concentration reached 40 μM. The antiproliferative activity of WJD008 was also detected in H1047R mutant cells with colony formation assays. As expected, the mutant transfected cells are more clonogenic than NT cells (Fig. 5C). The colonies of mutant H1047R cells formed decreased in a concentration-dependent manner in the presence of WJD008 with an IC_{50} of 12.91 μM, and almost no colony could be detected upon treatment with 40 μM WJD008 (with inhibition rate of 98.77 %). Wortmannin was much less potent against colony formation of mutant H1047R cells compared to WJD008 and induced an inhibition by 66.83% at 40 μM ($P<0.01$; Fig. 4C and D). Collectively, these findings substantiate that WJD008 is

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capable of repressing the proliferation and clonogenesis potential of both RK3E/NT and RK3E/p110α(H1047R) cells.

WJD008 possesses potent antiproliferative activity in cancer cells

The anti-proliferative activity of WJD008 was measured with SRB assay against a panel of human tumor cells, including liver cancer, lung cancer, stomach cancer, glioblastoma, prostate cancer, rhabdomyosarcoma, colon cancer, ovarian cancer, squamous carcinoma and breast cancer (Fig. 6). The average IC_{50} values of WJD008 and wortmannin against the 17 human tumor cell lines tested were 16.45 and 23.73 μM ($P<0.05$), respectively. Though WJD008 and wortmannin shared similar profile of antiproliferative activity in some cell lines such as Zip-177, A549 and Rh30 cells, they did display distinctive activities in certain cell types. In order to gain insight into the antiproliferative properties of WJD008 in the tumor cells with aberrant PI3K signaling, the cell lines were classified into 5 groups according to their PI3K statuses: PTEN-negative (PC-3 and MDA-MB-468), upstream deregulation of growth factor (Rh30), H1047R mutant (HCT-116 and SKOV-3), E545K mutant (MCF-7) and other cell lines without any reported deregulation of PI3K pathway so far (Ikediobi et al., 2006; Marty et al., 2008). WJD008 is active in cells exhibiting deregulation of the PI3K pathway by various means (Fig. 6). Of particular note, WJD008 is more potent than wortmannin against MCF-7 cells with oncognenic E545K mutation in PI3K (Fig. 6). Together with the fact that WJD008 is active in H1047R mutant cells (Fig. 5 & Fig. 6), oncogenic mutations in PI3K known at present unlikely confer resistance to this inhibitor.

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Discussion

WJD008, among a series of 5-cyano-6-morpholino-4-substituted-pyrimidine analogues stood out for its potent activity against PI3K/mTOR axis. In this study, we identified the cellular target of WJD008 and characterized its mechanism of action. Though WJD008 was designed based on a p110α-specific inhibitor, WJD008 potently inhibited the kinase activity of both p110α and mTOR with less activity against PIKK family members. In cellular context, WJD008 inhibited several key components in PI3K-Akt-mTOR pathway in cascade, such as PtdIns(3,4,5)P_3, Akt, mTOR as well as its downstream effectors p70S6K and 4E-BP1, which resulted in the G1 phase arrest. WJD008 was further noted for its antiproliferative activity against a panel of tumor cells and transformed cells expressing *PIK3CA* H1047R mutant.

It has been reported that mTOR allosteric inhibitor rapamycin analogs are cytostatic in tumor cells (Easton and Houghton, 2004; Takeuchi et al., 2005). However, it was somewhat disappointing when these inhibitors were tested clinically, partially due to retrograde hyperphosphorylation of Akt (Margolin et al., 2005). The recent discovery of the dual PI3K/mTOR inhibitors, such as NVP-BEZ235 and PI-103 (Raynaud et al., 2007; Maira et al., 2008; Serra et al., 2008) has shed the light on vertical blockade of multiple molecules in the PI3K pathway to overcome the feedback loops and achieve the effective cytostasis in cancers exhibiting deregulation of this pathway. In this study, as a novel dual PI3K/mTOR inhibitor, WJD008 well translated its potent activity into cells against the PI3K-Akt-mTOR axis. WJD008 prevented PtdIns(3,4,5)P_3 production, Akt and mTOR activation as well as its down-stream effectors p70S6K and 4E-BP1. It is noteworthy that WJD008 completely abrogated the phosphorylation of Akt at Ser 473 in spite of its

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activity in inhibiting the catalytic activity of TOR comlex 1, which might due to simultaneous inhibition of PI3K and possibly TOR complex 2 . Thus, WJD008 functions on several levels to block both the growth factor- and nutrient-sensing pathways and the inhibitory efficacy of PI3K and mTOR was augmented mutually. The characterization of WJD008 so far supports the emerging consensus of inhibition of PI3K and mTOR in combination as a mechanistic rationale for the cancer therapeutic options.

WJD008 impeded the PI3K signaling in cells with hyper-activated pathway either due to enhanced growth factor signaling (demonstrated as Rh30 cells) (Minniti et al., 1995) or due to loss of PTEN (demonstrated as PC-3 cells). In addition, WJD008 not only displayed potent antiproliferative activity in cells that harbor *PIK3CA* mutants (HCT-116, SKOV-3 and MCF-7) or PTEN-negative (PC-3 and MDA-MB-468), but also is active in cells with no reported deregulated PI3K pathway so far (Ikediobi et al., 2006; Marty et al., 2008). Thus, WJD008 blocked proliferation in all the cancer cell lines tested irrespective of their PI3K status. This notion was further supported by the experiments using isogenic *PIK3CA* wild type or H1047R mutant RK3E cells. WJD008 blocked PI3K signaling pathway as well as proliferation in both wild type or H1047R mutant RK3E cells. Differentiated from WJD008, wortmannin displayed less potency in RK3E/p110α(H1047R) cells compared to WJD008 even though it possessed similar activity with WJD008 in RK3E/NT cell. Since increased PI3K signaling in tumor cells may results from vertical (such as hyper-activated receptor signaling), horizontal (such as PTEN loss) or presence of activating PI3K mutation, WJD008 was inferred to lead to antiproliferative outcome regardless of activation modes of PI3K and target both p110α wild type and H1047R mutant. The fact is of great importance as it indicates WJD008

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may have the potential for wider applicability in cancer therapy. On the other hand, as WJD008 possessed wide spectra cytostatic activity, its potential side effect should be carefully monitored.

In summary, WJD008 functions as a dual PI3K/mTOR inhibitor and inhibits activated signaling in both wild-type and mutated p110α in cellular models. With research continuing to dig the insights into the structure-based machinery of this kind of compounds, we look forward to discovering PI3K inhibitors with more potent efficacy in cancer therapy.

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We appreciate Professor Peter K. Vogt (The Scripps Research Institute) for the RK3E/NT and RK3E/p110α(H1047R) cells and Professor Tamas Balla (National Institute of Health) for the pEGFP-C1-Grp1-PH plasmid.

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Footnotes

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legends for Figures

Fig. 1. The structure and effect of WJD series compounds on PI3K-mediated signaling in tumor cells.. A, chemical structure of WJD series compounds. B, Rh30 Cells were treated with the indicated compounds at the concentration of 20 μM for 1 h and then stimulated with IGF-I (50 ng/mL) for 10 min. The cells were lysed and the proteins were separated on SDS-PAGE. Typical Western blots for phosphorylated Akt (Ser473) and GAPDH. C, WJD008dose-dependently down-regulated phosphorylated Akt (S473). Starved Rh30 cells were incubated with increasing concentrations of WJD008 for 1 h followed by stimulation with 50 ng/mL IGF-I for 10 min. Cells were then lysed and cell extracts were analyzed by ELISA to measure the level of phosphorylated Akt at S473.

Fig. 2. WJD008 potently inhibits the catalytic activity of PI3Kα and mTOR. A, PI3Kα activity was assessed by Kinase-Glo assay as described in Material and Methods. Data were fitted and the IC_{50} was determined by GraphPad Prism 4 Software. B, WJD008 inhibits the activity of mTOR. mTOR kinase activity was measured by ELISA assay as described in Material and Methods. Data were fitted and the IC_{50} was determined by GraphPad Prism 4 Software. C, effect of WJD008 on the ATM- and DNA-PK- mediated DNA damage response. Rh30 cells were exposed to 10 μM camptothecin (CPT) for 2 h after preincubation with indicated concentrations of WJD008 or wortmannin (WT) or vehicle control (-) for 0.5 h. Cells were then lysed and cell extracts were analyzed by Western blot for γ-H2AX. Representative images are presented.

Fig. 3. WJD008 blocks the PI3K-Akt-mTOR signaling in tumor cells. A, WJD008 depresses IGF-triggered activation of PI3K-Akt-mTOR signaling pathway.

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Serum-deprived Rh30 (left panel) and PC-3 (right panel) cells were pre-incubated with the indicated concentrations of WJD008 or wortmannin (WT) for 60 min followed by stimulation of IGF-I (50 ng/mL) for 10 min. Cells were collected for Western blot to analyze the levels of indicated proteins. Arrows indicate the protein detected. B, WJD0008 blocks translocation of GFP-Grp1-PH to cellular membrane. CHO-GFP-Grp1-PH cells were starved, pre-incubated with the compounds at the indicated concentrations for 1 h and stimulated with IGF-I (250 ng/mL) for 10 min. Fluorescent pictures were captured with the inverted fluorescence microscope. Arrows indicate fluorescent foci. Scale bar, 50 μm. Representative images are presented.

Fig. 4. WJD008 induces G1-phase arrest without apoptosis in tumor cells. WJD008 dose-dependently induced G1 phase arrest in Rh30 cells. Rh30 cells seeded in 6-well plate were treated with indicated concentrations of WJD008 for 72 h and then cells were harvested for cell number counting or for DNA content analysis by FACS. A, Cell cycle distribution. B. Fold change in cell number, which was calculated using formula: cell number after 72h treatment / seeded cell number.

Fig. 5. WJD008 inhibits p110α exhibiting oncogenic H1047R mutation in cells. A, WJD008 inhibited PI3K (H1047R) mutant-mediated signaling. RK3E/NT and RK3E/p110α (H1047R) cells were starved and then incubated with the indicated inhibitors for 60 min followed by stimulation of IGF-I (50 ng/mL) for 10 min. Cells were then harvested and phosphorylated Akt was measured by Western blot. Total Akt and GAPDH levels are shown for loading control. B, WJD008 inhibited the proliferation

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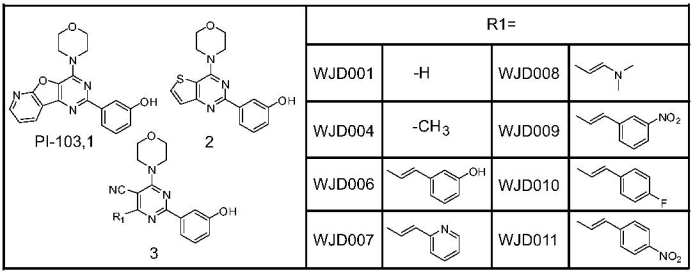
of RK3E cells expressing PI3K (H1047R) mutant. Proliferation of RK3E/NT (NT) and RK3E/p110α(H1047R) (HR) was determined using SRB assay after treatment with WJD008 (WJD) or wortmannin (WT) for 72 h at the indicated concentrations. C & D, WJD008 inhibited the colony formation of RK3E cells expressing PI3K (H1047R) mutant. Colony formation assay were carried out in RK3E/NT and RK3E/p110α (H1047R) cells treated with WJD008 or wortmannin (WT) at indicated concentrations. After incubation for 7 days, cells were stained with crystal violet and photographed. Representative images were demonstrated (C) and the number of colonies were counted by image analysis (D). ** denotes statistical significance compared to appropriate control cultures, $p<0.01$ versus WJD008 treated RK3E/p110α(H1047R). Error bars indicate the standard deviation between each experiment.

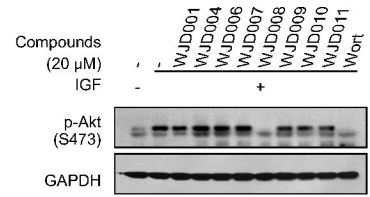
Fig. 6. WJD008 possesses potent antiproliferative activity in a panel of cancer cells. Antiproliferative activity of WJD008 and wortmannin against a wide panel of tumor cell lines was determined with SRB assay. Cells were exposed in compounds or DMSO vehicle for 72h, then fixed and stained with SRB. IC_{50}s plotted as mean ± SD (μM) were from three separate experiments.

Figure 1

A


PI-103,1
2
3
R1=
WJD001
-H
WJD008
WJD004
-CH3
WJD009
WJD006
WJD010
WJD007
WJD011

B


Compounds
(20 μM)
IGF
WJD001
WJD004
WJD006
WJD007
WJD008
WJD009
WJD010
WJD011
Wort
p-Akt
(S473)
GAPDH

C

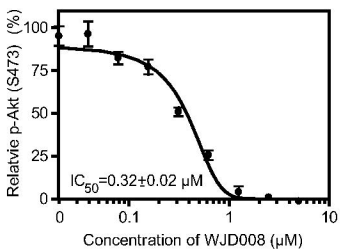

Relative p-Akt (S473) (%)
100
75
50
25
0
IC50=0.32±0.02 μM
0
0.1
1
10
Concentration of WJD008 (μM)

Figure 2

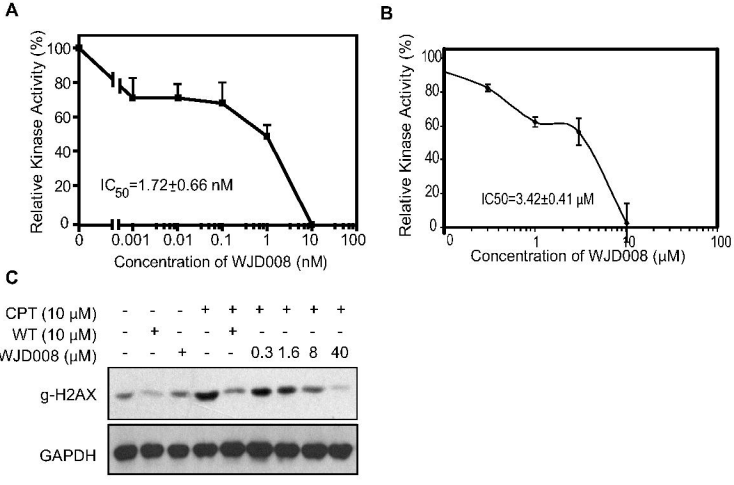

A
Relative Kinase Activity (%)
100
80
60
40
20
0
IC50=1.72±0.66 nM
0
0.001
0.01
0.1
1
10
100
Concentration of WJD008 (nM)
B
Relative Kinase Activity (%)
100
80
60
40
20
0
IC50=3.42±0.41 μM
0
1
10
100
Concentration of WJD008 (μM)
C
CPT (10 μM) - - - + + + + + +
WT (10 μM) - + - - + - - - -
WJD008 (μM) - - + - - 0.3 1.6 8 40
g-H2AX
GAPDH

Figure 3

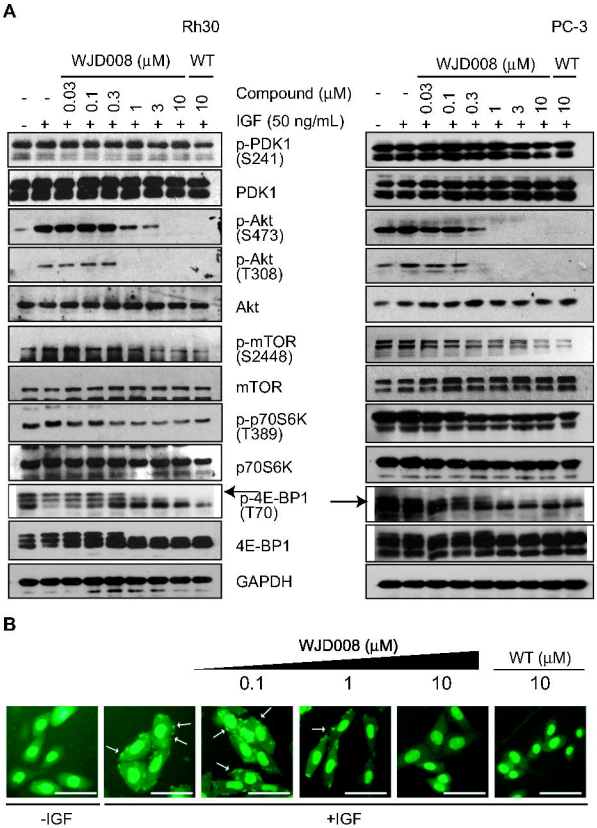

A
Rh30
PC-3
WJD008 (μM)
WT
Compound (μM)
IGF (50 ng/mL)
- - 0.03 0.1 0.3 1 3 10 10
- + + + + + + + + +
p-PDK1 (S241)
PDK1
p-Akt (S473)
p-Akt (T308)
Akt
p-mTOR (S2448)
mTOR
p-p70S6K (T389)
p70S6K
p-4E-BP1 (T70)
4E-BP1
GAPDH
B
WJD008 (μM)
WT (μM)
0.1 1 10 10
-IGF
+IGF

Figure 4

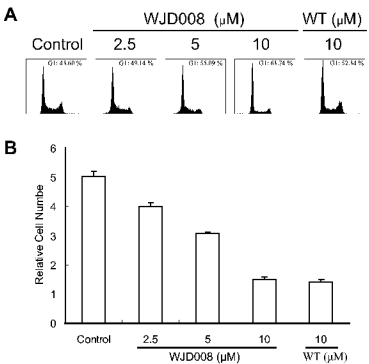

A
WJD008 (μM)
WT (μM)
Control
2.5
5
10
10
B
Relative Cell Numbe
0
1
2
3
4
5
6
Control
2.5
5
10
10
WJD008 (μM)
WT (μM)

Figure 5

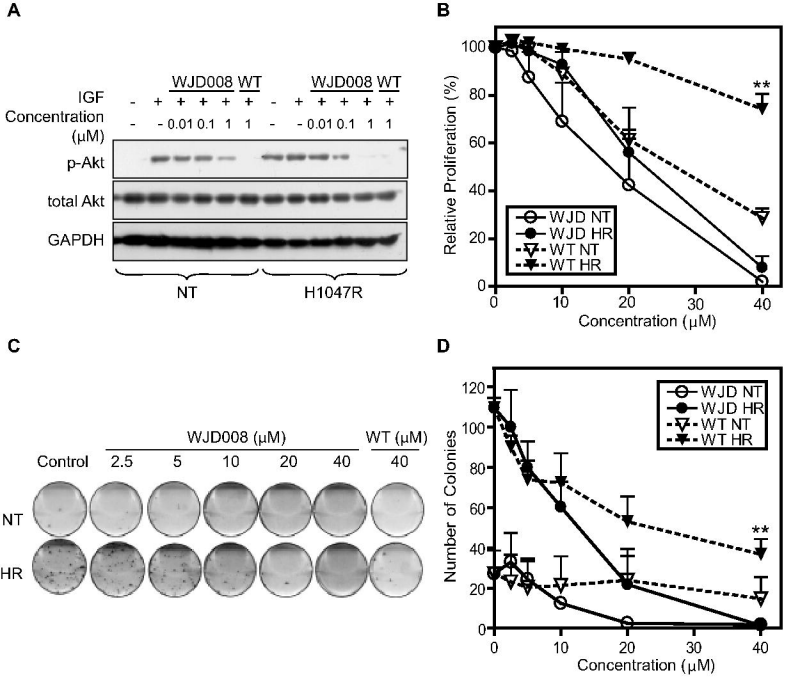
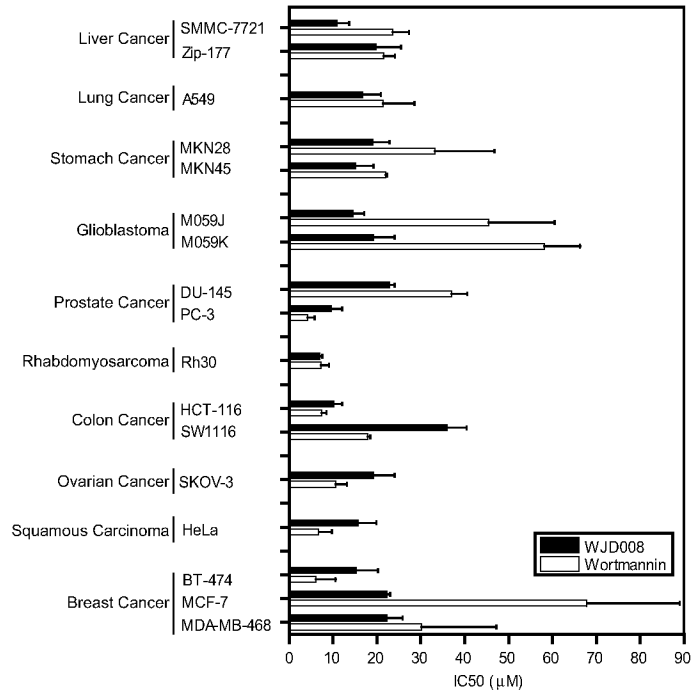

A
WJD008
WT
WJD008
WT
IGF
- + + + + + - + + + + +
Concentration (μM)
- - 0.01 0.1 1 1 - - 0.01 0.1 1 1
p-Akt
total Akt
GAPDH
NT
H1047R
B
Relative Proliferation (%)
100
80
60
40
20
0
0
10
20
30
40
Concentration (μM)
WJD NT
WJD HR
WT NT
WT HR
**
C
WJD008 (μM)
WT (μM)
Control
2.5
5
10
20
40
40
NT
HR
D
Number of Colonies
120
100
80
60
40
20
0
0
10
20
30
40
Concentration (μM)
WJD NT
WJD HR
WT NT
WT HR
**

Figure 6


Liver Cancer
SMMC-7721
Zip-177
Lung Cancer
A549
Stomach Cancer
MKN28
MKN45
Glioblastoma
M059J
M059K
Prostate Cancer
DU-145
PC-3
Rhabdomyosarcoma
Rh30
Colon Cancer
HCT-116
SW1116
Ovarian Cancer
SKOV-3
Squamous Carcinoma
HeLa
Breast Cancer
BT-474
MCF-7
MDA-MB-468
WJD008
Wortmannin
0
10
20
30
40
50
60
70
80
90
IC50 (μM)