1521-0103/348/3/432–441$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.113.210724
J Pharmacol Exp Ther 348:432–441, March 2014

DW09849, a Selective Phosphatidylinositol 3-Kinase (PI3K) Inhibitor, Prevents PI3K Signaling and Preferentially Inhibits Proliferation of Cells Containing the Oncogenic Mutation p110α (H1047R)

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Received October 22, 2013; accepted December 19, 2013

ABSTRACT

Phosphatidylinositol 3-kinase, α isoform (PI3Kα) plays essential roles in cell metabolism, growth, and proliferation and has been validated as a promising anticancer target. In an effort to search for new PI3Kα-selective inhibitors, DW series compounds were designed and synthesized aiming to reduce the off-target effects of their parent compound PIK-75 [2-methyl-5-nitro-1-benzenesulfonic acid 2-[(6-bromoimidazo[1,2-a]pyridin-3-yl)methylene]-1-methylhydrazide], which was reported to selectively target PI3Kα. A series of compounds named DW series potently inhibited the kinase activity of PI3Kα with little activity against PI3K-related protein kinases and a panel of 15 tyrosine kinases. Similar to PIK-75, DW series compounds were more potent to inhibit PI3Kα among four class I PI3K isoforms, whereas a representative compound DW09849 [(*E*)-*N*′-((6-bromoimidazo[1,2-a] pyridin-3-yl)methylene)-*N*-ethyl-2-methyl-5-nitrobenzohydrazide] displayed distinct binding mode compared with PIK-75. Although DW series compounds inhibited proliferation of rhabdomyosarcoma RH30 cells at elevated 50% inhibitory concentrations (IC_{50}) in comparison with PIK-75, they were more selective than PIK-75 to inhibit PI3K signaling in the cellular context. In particular, DW09849 significantly and persistently blocked PI3K/protein kinase B signaling in RH30 cells, which consequently arrested RH30 cells in the G_1 phase. Moreover, DW09849 selectively suppressed the proliferation and clonogenesis of transformed RK3E/HR cells harboring oncogenic mutation of p110α H1047R, as well as a panel of human breast cancer cells containing mutated PI3Kα, which is consistent with the finding that DW09849 demonstrated preference against H1047R mutated PI3Kα in molecular docking stimulation. These results suggest that DW series compounds, especially DW09849, selectively targeting PI3Kα with less off-target effects than PIK-75, provide new clues for the design and discovery of new specific PI3Kα inhibitors for cancer therapy.

Introduction

The phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases defined by their ability to phosphorylate the 3′-OH of phosphoinositides, which were grouped into three different classes (I, II, and III) based on their lipid substrate specificity and sequence homology (Vanhaesebroeck et al., 1997; Parekh et al., 2000). Class I PI3Ks have been most studied, which were divided into four isoforms according to their catalytic subunits: p110α, p110β, p110δ, and p110γ. PI3K signaling leads to activation of multiple pathways, including protein kinase B (Akt)/mammalian target of rapamycin (mTOR), which plays a central role in regulating cell growth, survival, proliferation, motility, and morphology (Cantley, 2002; Vivanco and Sawyers, 2002; Bader et al., 2005). Hyperactivation of PI3K signaling resulting from abnormal activation of upstream molecules (e.g., tyrosine kinase receptors), gain-of-function mutations of PI3K

This work was supported by National Science & Technology Major Project "Key New Drug Creation and Manufacturing Program" [Grant 2012ZX09301-001], National Natural Science Foundation of China [Grants 81321092 and 81373445], and Knowledge Innovation Program of Chinese Academy of Sciences [Grant KSCX2-EW-Q-3]. L.-h.M. gratefully acknowledges the support of the Sanofi-Aventis Shanghai Institutes for Biological Sciences Scholarship Program.
J.-l.L. and G.-r.G. contributed equally to this work, W.-h.D. and L.-h.M. contributed equally to this work.
dx.doi.org/10.1124/jpet.113.210724.

ABBREVIATIONS: Akt/AKT, protein kinase B; ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; BIBW2992, *N*-[4-[(3-chloro-4-fluorophenyl)amino]-7-[[(3S)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4(dimethylamino)-2-butenamide, afatinib; CAL-101, 5-fluoro-3-phenyl-2-([*S*])-1-[9*H*-purin-6-ylamino]-propyl)-3*H*-quinazolin-4-one, idelalisib; DNA-PK, DNA-dependent protein kinase; DW09849, (*E*)-*N*′-((6-bromoimidazo[1,2-*a*]pyridin-3-yl) methylene)-*N*-ethyl-2-methyl-5-nitrobenzohydrazide; DW09855, (*E*)-*N*-allyl-*N*′-((6-bromoimidazo[1,2-a]pyridin-3-yl)methylene)-2-methyl-5-nitrobenzohydrazide; DW09861, (*E*)-*N*′-((6-bromoimidazo[1,2-a]pyridin-3-yl)methylene)-*N*,2-dimethyl-5-nitrobenzothiohydrazide; 4E-BP1, phospho-eukaryotic initiation factor 4E-binding protein 1; ELISA, enzyme-linked immunosorbent assay; H2A histone family, member X; IGF-1, insulin-like growth factor-1; mTOR, mammalian target of rapamycin; OD, optical density; PDB, Protein Data Bank; PI3K, phosphatidylinositol 3-kinase; PIK-75, 2-methyl-5-nitro-1-benzenesulfonic acid 2-[(6-bromoimidazo[1,2-a]pyridin-3-yl)methylene]-1-methylhydrazide; PIK3CA, phosphatidylinositol 3-kinase, catalytic subunit α; PIKK, PI3K-related protein kinase; p70S6K, the 70-kDa ribosomal S6 kinase; SRB, sulforhodamine B; SN-38, 7-ethyl-10-hydroxy-camptothecin.

p110α, aberrations of downstream effectors (e.g., AKT), as well as loss of the lipid phosphatase PTEN (phosphatase and tensin homolog) have been frequently found in a wide range of tumor types (Kok et al., 2009). In particular, the gene encoding p110α (PIK3CA) has been uncovered as one of the most frequently mutated oncogenes in human tumors (Kan et al., 2010). Interestingly, mutations at three hot spots (E542K, E545K, and H1047R) represent 80% of all PIK3CA mutations (Samuels et al., 2004), which were proved to be oncogenic (Isakoff et al., 2005; Zhao et al., 2005). Therefore, discovery and development of anticancer drugs targeting PI3Kα, especially mutated ones, has been an attractive strategy for tumor therapy.

With multiple efforts under way in academia and industry to develop clinically relevant inhibitors against PI3K, a number of inhibitors have entered clinical trials, including PI3K selective inhibitors (either isoform-specific or pan-class I PI3K inhibitors) and dual PI3K/mTOR inhibitors (Pal and Mandal, 2012). Among them, dual PI3K/mTOR inhibitors target both PI3K and mTOR and avoid reactivation of PI3K induced by mTOR inhibition (Li et al., 2010), pan-PI3K inhibitors target all isoforms of class I PI3Kα (Kong and Yamori, 2010), and isoform-specific PI3K inhibitors preferentially target one isoform of class I (Engelman, 2009). Although most drug candidates now in clinical trials are pan-PI3K inhibitors or dual PI3K/mTOR inhibitors, generation of more selective agents is supposed to be advantageous to reduce side effects. A convincing example is the p110δ-selective inhibitor CAL-101 [5-fluoro-3-phenyl-2-([*S*)]-1-[9*H*-purin-6-ylamino]-propyl)-3*H*-quinazolin-4-one], which demonstrated remarkable clinical efficacy in certain hematologic diseases, including B-cell lymphoma (Ciraolo et al., 2011). Given the important role of p110α in cell transformation and tumorigenesis, targeting p110α represents an attractive strategy for cancer therapy. Moreover, selective inhibition of p110α and its mutants may inhibit the growth of cancer while avoiding the toxicity induced by inhibition of other PI3K isoforms (Sabbah et al., 2010).

Thus, to search for new selective PI3Kα inhibitors, we focused our attention on compound PIK-75 [2-methyl-5-nitro-1-benzenesulfonic acid 2-[(6-bromoimidazo[1,2-a]pyridin-3-yl)methylene]-1-methylhydrazide], an imidazo[1,2-α] pyridine derivative, which displays more than 100-fold p110α selectivity over p110β and p110γ, with an IC_{50} value of 0.3 nM (Hayakawa et al., 2007). Previous studies indicated its wide off-target effects (Knight et al., 2006; Jamieson et al., 2011), which may impede its further development. We sought to optimize the compound to reduce its off-target effects, while maintaining its PI3K inhibitory activity. We synthesized a series of PIK-75 analogs, namely, DW compounds. We found that DW compounds, especially DW09849 [(*E*)-*N*′-((6-bromoimidazo[1,2-a]pyridin-3-yl)methylene)-*N*-ethyl-2-methyl-5-nitrobenzohydrazide], displayed more specific activity against PI3Kα than PIK-75, which is further supported by the results obtained from molecular docking stimulation. DW09849 significantly inhibited PI3K signaling and consequently arrested tumor cells in the G_1 phase. Moreover, DW09849 demonstrated higher potency to inhibit proliferation and colony formation of p110α H1047R mutated RK3E/HR cells than those parent cells, making it a promising candidate for further structure optimization to selective PI3Kα targeting.

Materials and Methods

A series of imidazo[1,2-α]pyridine analogs were synthesized as potential PI3K inhibitors based on the structures reported previously (Hayakawa et al., 2007), with purity ≥95% (G. R. Gao et al., manuscript in preparation). PIK-75 and 7-ethyl-10-hydroxy-camptothecin (SN-38) were purchased from Selleck Chemicals (Selleck, Houston, TX). All compounds were prepared at 10 mM in 100% dimethylsulfoxide, and aliquots were stored at −20°C. All compounds were diluted to the desired concentrations immediately before each experiment. Final dimethylsulfoxide concentration was kept below 0.2% in control and compound-treated cells.

Cell Lines and Cell Culture. The transformed rat kidney epithelial cells RK3E/NT and RK3E/HR were provided by Dr. Peter K. Vogt (The Scripps Research Institute, La Jolla, CA) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 3 μg/ml puromycin. Rhabdomyosarcoma RH30 cells were a gift from the St. Jude Children's Research Hospital (Memphis, TN) and were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). HCT116, T47D, MCF-7, MDA-MB-361, BT474, BT20, MDA-MB-231, MDA-MB-468, SK-BR-3, BT549, MDA-MB-435, ZR-75-30, and HCC1937 cells were obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained in recommended culture media (all culture media were from Invitrogen). MDA-MB-231 and MDA-MB-361 were cultured in a humidified atmosphere of 95% air at 37°C; the remaining cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Phosphorylated Akt (Ser473) Enzyme-Linked Immunosorbent Assay. Cellular phosphorylated Akt at Ser473 was detected by enzyme-linked immunosorbent assay (ELISA) as described previously (Li et al., 2010). In brief, RH30 cells were grown to 60%–70% confluence and continued to be incubated in serum-free medium for 24 hours. After exposed to tested compounds at 10 μM for 1 hour, cells were stimulated with 100 ng/ml insulin-like growth factor-1 (IGF-1) for 10 minutes at 37°C. In parallel, ELISA test plates (Corning Life Sciences, Lowell, MA) were prepared with the goat anti-Akt1 C-20 coating antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by incubation with 5%–10% bovine serum albumin solution for 2 hours at room temperature. At the end of the treatment, cells were lysed and the cell lysate (50 μl) was transferred into the ELISA plates coated with Akt1 antibody and incubated for 3 hours on ice. After being washed with phosphate-buffered saline-Tween 20 three times, 50 μl of diluted antibody against phosphorylated Akt at Ser473 (Cell Signaling Technology, Cambridge, MA) was added, and plates were incubated overnight at 4°C. After the plate was washed with phosphate-buffered saline-Tween 20 three times, horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) was added and incubated for 2 hours, and the immune complexes were assayed with 100 μl of substrate (3,3′,5,5′-tetramethylbenzidine dihydrochloride). The OD value at 450 nm was read on a Multiwell spectrophotometer (Versa-Max; Molecular Devices, Sunnyvale, CA), and the inhibitory rate on phosphorylation of Akt at Ser473 was calculated by using the formula [OD_{450} (nm) control cells − OD_{450} (nm) treated cells]/OD_{450} (nm) control cells ×100%.

PI3K Kinase Assay. A glutathione S-transferase–tagged human p110α and regulatory full-length p85 were coexpressed in a Bac-to-Bac Baculovirus expression system (Invitrogen) and then purified using Glutathione-Sepharose 4B resin (GE Healthcare, Pittsburgh, PA). The kinase activity of the purified p110α/p85α was determined by the PI3K HTRF Assay (Millipore, Billerica, MA) according to the manufacturer's protocol. In brief, PI3Kα/p85 complex was incubated in the assay buffer containing 10 μM phosphatidylinositol 4,5-bisphosphate (PIP2) in a white 384-well plate (PerkinElmer Life and Analytical Sciences, Waltham, MA). The reaction was initiated by adding ATP to a final concentration of 5 μM and allowed to proceed at room temperature for

30 minutes. After adding the stop solution and the detection mixture, the plate was sealed and incubated at room temperature overnight. The intensity of the light emission was measured on an EnVision Multilable Reader (PerkinElmer Life and Analytical Sciences) in time-resolved fluorescence resonance energy transfer (TR-FRET) mode (excitation at 320 nm and emission at 665 nm).

mTOR Kinase Assay. A glutathione S-transferase–tagged truncated human mTOR (amino acids 1360–2549) was expressed and purified as described previously (Li et al., 2010). The kinase activity of the purified truncated mTOR was determined using LANCE Ultra time-resolved fluorescence resonance energy transfer assay (PerkinElmer Life and Analytical Sciences) following the manufacturer's instructions (Chen et al., 2012).

Biochemical Tyrosine Kinase Assays. Effects of tested compounds on the kinase activity of a panel of tyrosine kinases were measured using kinase assays according to the procedure described previously (Zhong et al., 2005).

Western Blot Analysis. RH30 cells grown to 80%–90% confluence were exposed to tested compounds for 1 hour, and cells were then collected and subjected to standard Western blot analysis with antibodies against Akt, phospho-Akt (Thr308), phospho-Akt (Ser473), phospho-eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; Thr37/46), 4E-BP1 (Cell Signaling Technology), phospho-p70S6K (Thr389) (Abcam plc, Cambridge, UK), and p70S6K (Epitomics Inc., Burlingame, CA). To detect γ-H2AX (H2A histone family, member X), HCT116 cells were pretreated with tested compounds for 1 hour and then cotreated with 1 μM SN-38 for 2 hours. Cells were collected and subjected to Western blot analysis using antibodies against γ-H2AX (Cell Signaling Technology). Actin (Sigma-Aldrich) was used as a loading control.

Class I PI3K Isoform Selectivity Assay. To detect the PI3K isoform selectivity of the tested compounds, RH30 cells stably expressing myristoylated p110α, p110β, p110δ, or p110γ, respectively, were established and named as RH30-p110α, RH30-p110β, RH30-p110δ, or RH30-p110γ cells. Cells were grown to 60%–70% confluence and continued to be incubated in serum-free medium for 15 hours. After being exposed to tested compounds for 2 hours, cells were harvested and subjected to Western blot analysis.

Cell Cycle Analysis. RH30 cells grown to 50%–60% confluence were treated with tested compounds at indicated concentrations for 24 hours. Cells were harvested and fixed with ice-cold 70% ethanol. After being stained with propidium iodide, DNA content was measured with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and cell cycle distribution was analyzed using ModFit LT software (Verity Software House, Topsham, ME). A total of 10,000 cells were analyzed from each sample.

Antiproliferative Assay. The sulforhodamine B (SRB) assay was used to assess cell proliferation, and IC_{50} values were determined by the four-parameter logit method as described previously (Chen et al., 2012). The average IC_{50} values were obtained from at least three independent tests.

Colony Formation Assay. RK3E/NT and RK3E/HR cells were seeded in six-well plates at a density of 200 cells per well. The next day, cells were exposed to tested compounds at indicated concentrations for 1 week. Cells were then stained with 0.1% violet after fixation of 90% ethanol, and visible colonies (≥50 cells) were counted.

Docking Simulation. The crystal structures of PI3Kα [Protein Data Bank (PDB) code 2RD0] and the PI3Kα H1047R mutant (PDB code 3HIZ) were retrieved from the Protein Data Bank. All missing residues within the kinase domain (Gly725 to Ile1094) were added and refined by Discovery Studio (NeoTrident Technology LTD., Shanghai, China). Five models were generated for each protein, and the model with minimum energy was selected to conduct docking simulation. DW09849 and PIK-75 were docked into the binding site of PI3Kα with Autodock 4.2 (Sanner, 1999). The number of trial runs was set to 100, whereas other parameters were left as default in the Lamarckian genetic algorithm. Docked conformations were clustered using a tolerance of 2Å and ranked by binding energy. The most populated cluster was picked and presented. Ligand-protein complexes were written by AutodockTool 1.5.4 (The Scripps Research Institute, Jupiter, FL) and presented by PyMOL 1.3 (Schrödinger, LLC, Portland, OR).

Data Analysis. Data are presented as the mean ± S.D. from at least three independent experiments, and differences were considered significant at $P < 0.05$ as determined by Student's t test.

Results

Structure of DW Compounds and Their Selective Activity to Inhibit PI3Kα Kinase Activity. PIK-75 has been reported to selectively inhibit PI3Kα with high potency, but its wide off-target effects impeded its further development (Knight et al., 2006; Jamieson et al., 2011). In an effort to discover new PI3Kα inhibitors, PIK-75 was used as a lead compound for structure optimization, and DW compounds were synthesized and evaluated for their activity against PI3Kα (G. R. Gao et al., manuscript in preparation). Three new compounds (DW09849, DW09855, DW09861) stood out by their potent PI3Kα inhibitory activity with IC_{50} values comparable to PIK-75 (Table 1); therefore, these compounds were chosen for further investigation. Among them, DW09849 possesses improved water solubility (3.27 ± 0.59 μg/ml) at 25°C compared with PIK-75 (less than 0.224 μg/ml). All of the compounds are quite stable up to 100-hour incubation at 37°C. To confirm their capability to inhibit PI3K at the cellular level, we detected phosphorylated Akt at Ser473, which has been recognized as a faithful readout of PI3K activity in cells (Leevers et al., 1999). As RH30 cells express high levels of IGF-II (Minniti et al., 1995), which results in constitutive activation of the PI3K signaling pathway, these cells were exposed to 10 μM DW compounds, and phosphorylated Akt was measured by ELISA. As shown in Table 1, DW compounds, which are more potent than PIK-75, completely inhibited IGF-1–induced phosphorylation of Akt. DW compounds significantly inhibited the proliferation of Rh30 cells with IC_{50} values in the micromolar range. However, we found DW compounds were less potent than PIK-75 in this assay, suggesting DW compounds might be more selective than PIK-75 against PI3K.

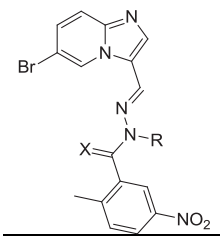
PI3K-related protein kinases (PIKKs), consisting of high molecular weight enzymes with a catalytic core similar to PI3Ks, include mTOR (the target of rapamycin), which is downstream in the PI3K pathway, and DNA-PK (DNA-dependent protein kinase), ATM (the ataxia telangiectasia mutated gene product), and ATR (ataxia telangiectasia related), which are pivotal in DNA repair (Workman et al., 2010). Most of the PI3K inhibitors so far displayed off-target effects against other PIKK members because of the sequence similarity of the kinase domain. We detected the effect of DW compounds on the kinase activity of mTOR. As shown in Fig. 1A, PIK-75, DW09849, and DW09855 displayed much less activity against mTOR than PI3Kα, with IC_{50} values of 2.67, 3.48, and 4.83 μM, respectively, and DW09861 failed to inhibit the activity of mTOR by 50% at 10 μM.

We next determined the effect of DW compounds on the activity of DNA-PK, ATM, and ATR by measuring the level of phosphorylated H2AX (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 Ser139 upon DNA strand breaks induced by SN-38 (Satoh et al., 1994). Therefore, γ-H2AX was examined

TABLE 1

Structure of DW compounds and their activity to inhibit PI3K and cell proliferation

Concentrations that cause 50% inhibition (IC_{50}) of the tested compounds against PI3Kα were determined by HTRF assay. Data shown are the mean ± S.D. of three separate experiments performed in triplicate. Inhibitory rate (IR) of the compounds to inhibit AKT phosphorylation at Ser473 was measured by ELISA after RH30 cells were exposed to the compounds at 10 μM for 1 hour. IC_{50} values of the compounds to inhibit cell proliferation in RH30 cells were determined by SRB assay.

Compound		X	R	HTRF IC_{50}	ELISA IR	SRB IC_{50}
				nM	%	*μM*
PIK-75		—	—	13.26 ± 3.92	99.5	0.14 ± 0.03
	DW09849	O	CH_3CH_2	47.81 ± 1 5.91	103.32	2.05 ± 0.11
	DW09855	O	CH_2=$CHCH_2$	69.47 ± 13.25	108	4.39 ± 1.99
	DW09861	S	CH_3	20.28 ± 5.78	109.6	1.74 ± 0.23

O, oxygen atom; S, sulfur atom.

to evaluate the activity of DW compounds against these kinases. As shown in Fig. 1B, HCT116 cells displayed a robust increase of γ-H2AX after treatment with SN-38 (1 μM). Cotreatment with PIK-75 partially abrogated the upregulation of γ-H2AX. By contrast, DW compounds had a negligible effect on the phosphorylation of γ-H2AX, indicating DW compounds are more selective than PIK-75 against PI3K with little activity against ATM, ATR, and DNA-PK in HCT116 cells.

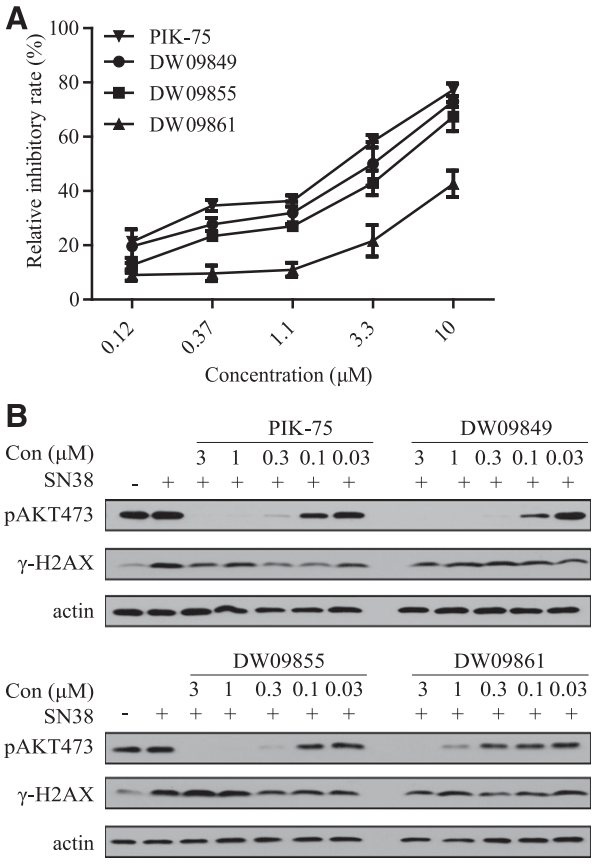


Fig. 1. DW compounds possess little activity against PIKKs. (A) Inhibitory rate of the tested compounds against mTOR were determined with LANCE assay. Data shown are the mean ± S.D. of three separate experiments performed in triplicate. (B) HCT116 cells were pretreated with tested compounds for 1 hour and then cotreated with 1 μM SN-38 for 2 hours. Cell lysates were then subjected to Western blot analysis. Data shown are representative of three independent experiments. Con, concentration.

We also tested the effects of DW compounds on the kinase activity of a panel of tyrosine kinases that play important roles in human tumor. As presented in Table 2, DW compounds exhibited little activity against tested tyrosine kinases at 10 μM. It should be noted that PIK-75 displayed significant activity against epidermal growth factor receptor/T790M/L858R–mutated epidermal growth factor receptor, which is comparable to the positive compound BIBW2992 [*N*-[4-[(3-chloro-4-fluorophenyl)amino]-7-[[(3*S*)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4(dimethylamino)-2-butenamide].

DW Compounds Selectively Inhibit p110α. PIK-75 was reported to be highly selective against p110α among class I PI3K isoforms, but its activity in isoform-mediated cellular signaling has not been detected. To this end, we generated a set of RH30 cell lines that stably express a myristoylated-tagged p110 catalytic subunit (p110α, p110β, p110γ, or p110δ, respectively) of human class I PI3K at the N terminus, namely, RH30-p110α, RH30-p110β, RH30-p110δ, or RH30-p110γ (Wang et al., 2013). In these cells, endogenous PI3K is inactivated under serum-free culture, whereas the ectopically expressed p110 isoforms are membrane-anchoraged and constitutively active because of myristoylation at the N terminus. Therefore, isoform-specific PI3K activity in these cells can be blocked by the corresponding inhibitors (Wang et al., 2013). This panel of cells was treated with serial diluted concentrations of DW compounds and phosphorylated Akt as the readout of PI3K activity was detected. In line with previously reported results, PIK-75 significantly inhibited p110α-mediated AKT phosphorylation at Ser473 at 0.1 μM, whereas it had little inhibitory activity against the other three isoforms at the same concentration (Fig. 2A). DW09849 and DW09855 significantly inhibited phosphorylation of Akt at the concentration of 0.03 μM in RH30-p110α cells, whereas they exhibited a negligible effect at the same concentration in RH30-p110β, RH30-p110δ, and RH30-p110γ cells. Unexpectedly, DW09861 displayed selectivity against p110γ, which deserves further investigation. Thus, DW09849 and DW09855 exhibited a similar selectivity profile with PIK-75 against class I PI3K but with higher potency.

TABLE 2
Effects of DW compounds on the activity of a panel of tyrosine kinases
The inhibitory activity of PIK-75 and DW compounds against 15 tyrosine kinases were measured with ELISA at a concentration of 10 μM.

Compound	Inhibitory Rate at the Concentration of 10 μM								
	PIK-75	DW09849	DW09855	DW09861	Su11248	BIBW2992	Dasatinib	PF2341066	PD173074
					%				
Flt-1	55.9	66.1	64.2	34.9	86.7				
KDR	46.0	49.5	50.1	37.2	90.4				
c-Kit	43.7	41.7	43.3	32.8	87.3				
PDGFR -α	45.3	31.6	37.3	20.3	80.9				
PDGFR -β	56.4	54.2	54.7	41.0	87.4				
RET	58.4	53.9	56.8	49.5	87.6				
EGFR	54.7	24.6	27.6	11.7		86.9			
ErbB2	66.7	43.6	49.8	50.4		86.1			
EGFR/ T790M/ L858R	85.4	65.1	60.7	49.3		83.4			
ErbB4	15.1	9.9	3.1	24.2		83.1			
Src	0	9.8	25.4	5.1			94.1		
Abl	62.2	46.1	62.7	36.6			91.0		
EPH-A2	65.5	56.2	73.0	54.4			84.4		
RON	0	0	0	22.3				92.7	
FGFR1	62.0	58.8	57.9	53.1					87.5

Abl, Abelson murine leukemia viral oncogene homolog 1; EGFR, epidermal growth factor receptor; EPH-A2, ephrin type-A receptor 2; Erb2, human epidermal growth factor receptor 2; FGFR, fibroblast growth factor receptor; Flt-1, fms-related tyrosine kinase; KDR, kinase-insert domain-containing receptor; KIT, stem cell factor receptor; PDGFR, platelet-derived growth factor receptor; PD173074, *N*-[2-[[4-(diethylamino)butyl]amino-6-(3,5-dimethoxyphenyl)pyrido[2,3-d]pyrimidin-7-yl]-*N*′-(1,1-dimethylethyl)urea; PF2341066, 3-[(1*R*)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl)pyridin-2-amine; RET, rearranged during transfection; RON, macrophage-stimulating protein receptor; Src, sarcoma; Su11248, *N*-(2-diethylaminoethyl)-5-[(*Z*)-(5-fluoro-2-oxo-1*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide.

As DW09849 inhibited the kinase activity of PI3Kα and prevented PI3Kα-mediated cell signaling, a molecular docking approach was performed to predict their potential binding sites and possible binding mode between DW09849 and p110α. As shown in Fig. 2B, a horizontal flip was observed between DW09849 and PIK-75 (the most populated cluster) and formed a "head to tail" structure, which may explain the specificity of DW09849 against PI3K. The potential binding modes of DW09849 and PIK-75 were investigated in a close-up view of the binding interface. DW09849 formed hydrogen bonds with Val851, Ser854, and Lys802, whereas PIK-75 interacted with Val851 and Tyr836. As Ser854 was reported to play an important role in the ligand-complex interaction of the PI3Kα H1047R mutant (Sabbah et al., 2010), this observation suggested that DW09849 may possess a preference for the PI3Kα H1047R mutant.

We next compared the potential binding mode of DW09849 with wild-type PI3Kα (PDB code 2RD0) and H1047R mutant (PDB code 3HIZ). The same hydrogen bonds were found in both the DW09849-3HIZ complex and the DW09849-2RD0 complex. The *N*′-ethyl group of compounds pointed into a shallow lipophilic cavity surrounded by Tyr856, Asn918, Met922, and Ile932. Compared with wild-type PI3Kα, the H1047R mutant formed a generally tighter binding pocket that was caused by the leaning inward of residues Ser774, Asp933, and Lys802 (Fig. 2C). By calculating the potential binding energy, DW09849 possessed better affinity with the H1047R mutant (–8.6 kcal/mol) than with wild-type PI3Kα (−7.52 kcal/mol).

DW Compounds Block PI3K/mTOR Signaling Pathway in RH30 Cells. As we have demonstrated the ability of DW compounds to inhibit PI3K activity, we next determined in detail how DW compounds could abrogate the PI3K-mediated signaling pathway in RH30 cells. RH30 cells were incubated with tested compounds at various concentrations for 1 hour. As expected, phosphorylation of Akt both at Thr308 and Ser473 was inhibited by all four compounds in a dose-dependent manner (Fig. 3A). However, DW compounds were more potent than PIK-75 against PI3K-mediated signaling. In particular, DW09849 completely inhibited phosphorylation of Akt at Ser473 at 0.3 μM. Phosphorylation of p70S6K1, one of the key mTOR downstream effectors, was also inhibited. It is interesting to note that all compounds failed to inhibit phosphorylation of 4E-BP1, another important mTOR downstream substrate (Fig. 3A). It has been reported elsewhere that compounds such as rapalogs effectively inhibited p70S6K1 phosphorylation but only partially inhibited phosphorylation of 4E-BP1 (Ma and Blenis, 2009). This phenomenon might be dependent on cell types. To determine the time course of DW09849 on the PI3K signaling pathway, we treated RH30 cells with 0.1 μM DW09849 or PIK-75 for different times. As shown in Fig. 3B, both DW09849 and PIK-75 robustly inhibited PI3K signaling, indicated by decreased phosphorylation of AKT at Thr308 and Ser473. However, inhibition of AKT phosphorylation by PIK-75 was ready to recover, and the level of phosphorylated Akt restored within 1.5 hour. Meanwhile, inhibition of PI3K/AKT signaling by DW09849 persisted up to 24 hours. However, inhibition of phosphorylated p70S6K1 by PIK-75 or DW09849 recovered quickly, which might be attributed to the following reasons: first, inhibition of PI3Kα might be compensated by other isoforms of PI3K, which results in phosphorylation of Akt and S6K1; second, mTOR may also be activated by other upstream regulators upon PI3K inhibition, which lead to S6K1 phosphorylation.

DW09849 Induces G_1-Phase Arrest without Apoptosis in RH30 Cells. Cancer is characterized by uncontrolled cell proliferation, and the PI3K signaling pathway plays an important role in cell cycle regulation (Marone et al., 2008). We tested the effect of DW09849 on cell cycle distribution of RH30 cells. As presented in Fig. 4, after exposure to increasing concentrations of DW09849 for 24 hours, cell population in the G_1/G_0 phase accumulated in a dose-dependent manner. Cell population in the G_1 phase increased from 46.6% (control) to

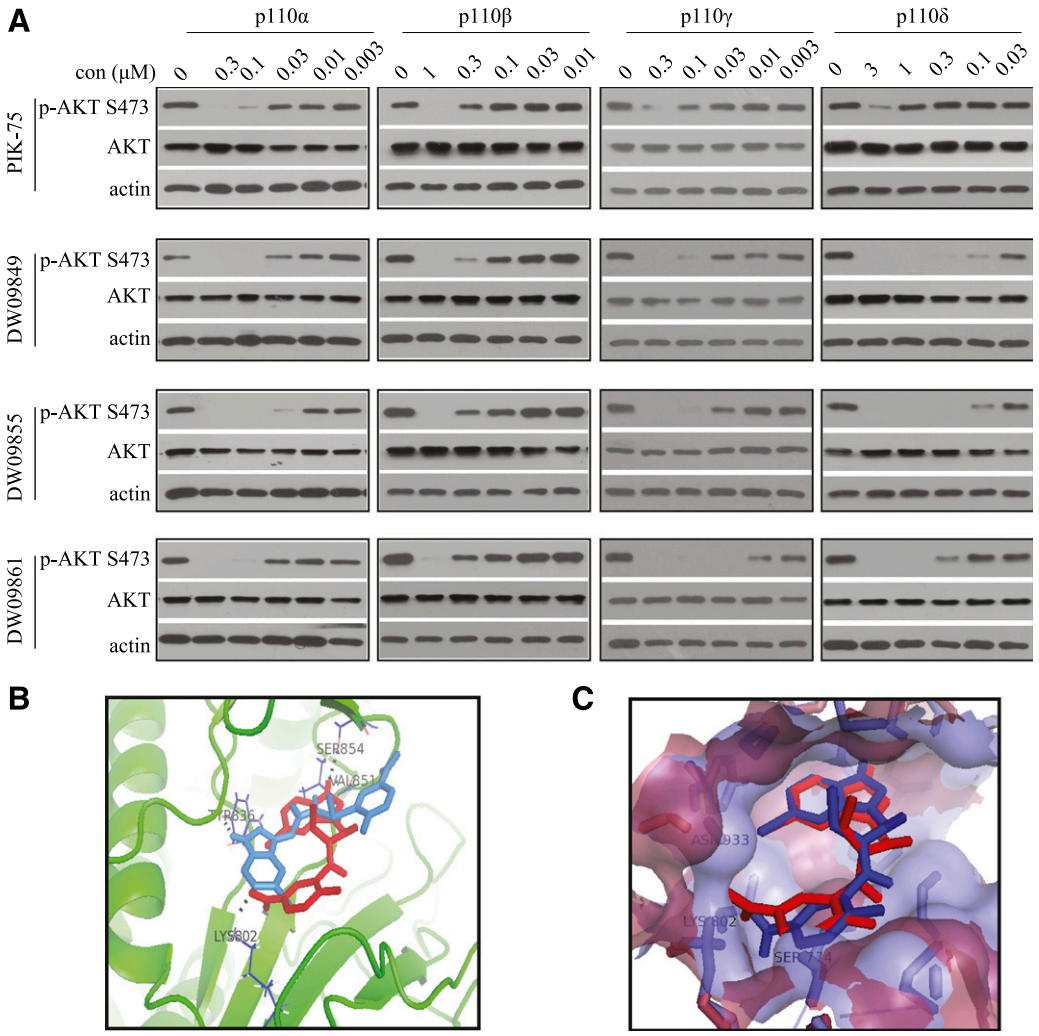


Fig. 2. The effects of DW compounds on the class I PI3K isoform-mediated signaling. (A) DW09849 preferentially inhibited activity of p110α. RH30-p110α, RH30-p110β, RH30-p110δ, and RH30-p110γ cells were cultured in serum-free media for 15 hours and then treated with various concentrations of compounds for 2 hours. Cells were collected and Western blot analyses for indicated proteins were then conducted. Data shown are representative of three independent experiments. (B) DW09849 (red) and PIK-75 (blue) displayed different binding modes with p110α. The protein is represented by illustration. The residues interacting with the compounds are shown in sticks. All of the structural diagrams were prepared using PyMOL. (C) The binding mode of DW09849 (red) with p110α implied a preference for the PI3Kα H1047R mutant. Superimposition of the DW09849-p110α complex (blue) and DW09849-p110α H1047R complex (red) indicates a tighter binding pocket of p110α H1047R, which may possibly lead to its better affinity toward DW09849. Con, concentration.

67.5% upon treatment with 3 μM DW09849. By contrast, such cell cycle arrest was not observed in cells treated with PIK-75, and cell debris was observed with relatively low concentrations of PIK-75 (data not shown), indicating the cytotoxicity of PIK-75. Thus, DW09849 displayed a typical PI3K inhibitor character by arresting cells in the G_0/G_1 phase, whereas PIK-75 displayed a complex action profile.

DW09849 Selectively Inhibits Proliferation and Colony Formation of p110α H1047R Mutated RK3E/HR Cells. The H1047R mutation is one of the most frequent mutations occurring in PIK3CA, which results in hyperactivation of PI3K and cell transformation. As normal cells do not harbor this mutation, selectively targeting oncogenic mutations would specifically target cancer cells. To evaluate whether DW09849 has any selectivity upon p110α H1047R-mutated cells, we detected the antiproliferative activity of PIK-75 and DW09849 in RK3E/NT and RK3E/HR cells (Fig. 5A). RK3E/HR is obtained from transformed RK3E cells (RK3E/NT) by stably expressing p110α H1047R by retroviral infection (Fu et al., 2005). PIK-75 displayed similar antiproliferative activity against these two cell lines, with IC_{50} values of 0.286 ± 0.144 and 0.341 ± 0.119 μM, respectively. However, DW09849 displayed marked divergence in its activity against cell proliferation. DW09849 selectively inhibited the proliferation of RK3E/HR cells which harbor the oncogenic p110α H1047R mutation, with an IC_{50} value of 0.884 ± 0.439 μM, whereas it was much less potent at inhibiting the proliferation of RK3E/NT cells (IC_{50} = 5.208 ± 1.358 μM). Additionally, colony formation assays further confirmed the preference of DW09849 against p110α H1047R mutated RK3E/HR cells. As shown in Fig. 5B, PIK-75 inhibited the clonogenesis of both cell lines with similar potency. However, DW09849 completely inhibited colony formation of RK3E/HR cells at 3 μM, whereas it displayed much less activity in the case of RK3E/NT cells at the same concentration. We further extended our experiments to a panel of breast cancer cells as PI3Kα is frequently mutated in human breast cancer. As presented in Fig. 5D, PIK-75 displayed profound antiproliferative activity against most breast cancer cells tested. DW09849 displayed preferential

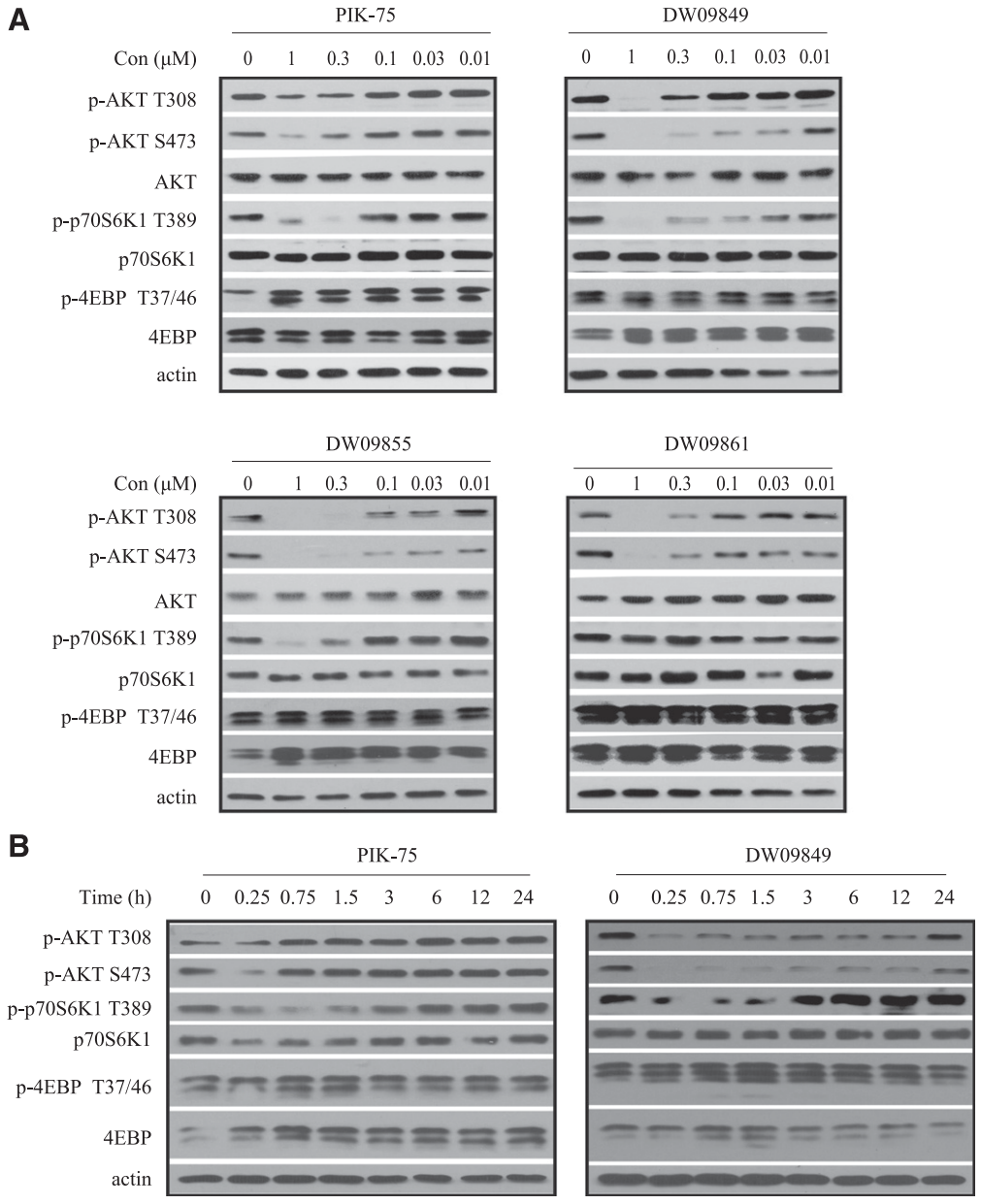


Fig. 3. DW compounds dose-dependently and persistently blocked the PI3K/mTOR signaling pathway in RH30 cells. (A) DW compounds dose-dependently inhibited PI3K signaling. RH30 cells were treated with DW09849, DW09855, DW09861, or PIK-75 at various concentrations (1, 0.3, 0.1, 0.03, and 0.01 μM) for 1 hour. (B) DW09849 persistently inhibited PI3K signaling. RH30 cells treated with 0.1 μM DW09849 or PIK-75 were harvested at indicated times (0, 0.25, 0.75, 1.5, 3, 6, 12, and 24 hours). Western blot analyses were conducted to detect the indicated proteins. Data shown are representative of three independent experiments. Con, concentration.

activity against breast cancer cells harboring mutated PIK3CA cell lines (T47D, MCF-7, BT20, BT474, MDA-MB-361) compared with other PIK3CA wild-type cell lines (MDA-MB-468, MDA-MB-231, MDA-MB-435, ZR-75-30, HCC-1937, BT549, SK-BR-3).

Discussion

In this study, we found that DW compounds, especially DW09849, among a series of imidazopyridine analogs, stood out for their potent activity against the PI3K/mTOR axis; we also confirmed the cellular target of DW compounds and characterized their mechanism of action. As DW compounds were designed based on a PI3Kα-specific inhibitor, they potently inhibited the kinase activity of PI3Kα, with less activity against PIKK family members and tyrosine kinases. In cellular context, DW09849 displayed better potency and persistency than PIK-75 in inhibiting the PI3K-Akt-mTOR signaling pathway, as indicated by a decrease in AKT phosphorylation, which consequently resulted in the G_1-phase arrest. DW09849 was further noted for its selective activity against proliferation and clonogenesis of transformed cells expressing the p110α H1047R mutant.

There is continuous interest in discovering new inhibitors with the aim of developing anticancer therapeutics as well as probing the role of PI3K in tumors and other diseases (Vadas et al., 2011). Optimization of the structure of the existing PI3K inhibitor is an efficient way to discover new compounds with better potency or selectivity. The first synthesized PI3K

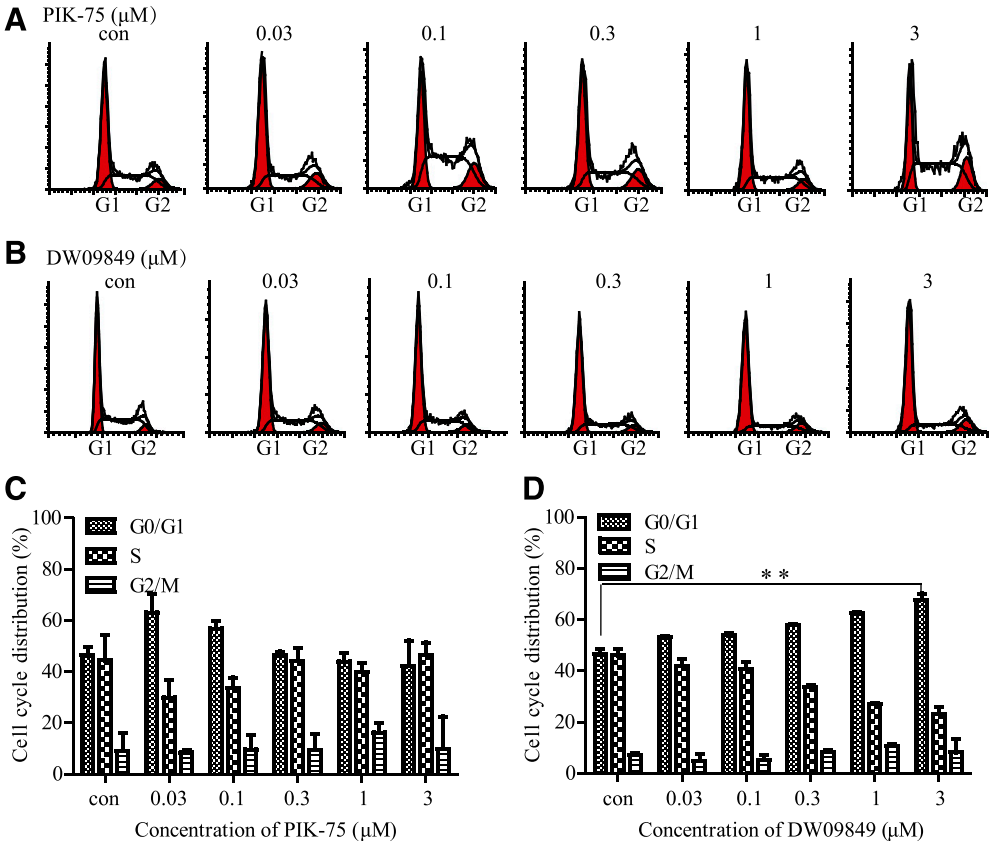


Fig. 4. DW09849 induces G_1-phase arrest in RH30 cells. RH30 cells were treated with PIK-75 or DW09849 at indicated concentrations for 24 hours, and then cells were harvested. The cell cycle distribution was analyzed by flow cytometric analysis. The percentage of cell population in G_0/G_1, S, and G_2/M phases of the cell cycle are shown. A and C, The cell cycle distribution after PIK-75 treatment. B and D, The cell cycle distribution after DW09849 treatment. Columns, mean of three independent experiments. Bars, standard error. $^{**}P < 0.01$. Con, concentration.

inhibitor, LY294002, was reported in 1994, which is based on the structure of a fungal natural product wortmannin (Vlahos et al., 1994). These two compounds were used as templates for modification and optimization in an effort to overcome their drawbacks in potency, selectivity, and pharmaceutical properties in the early years of searching for PI3K inhibitors. As a result, a number of PI3K inhibitors have emerged, and some of them entered clinical trials (Shuttleworth et al., 2011). PIK-75 was reported to highly selectively inhibit p110α among class I PI3K isoforms (Hayakawa et al., 2007; Zheng et al., 2012). Isoform-selective PI3K inhibitors are supposed to possess reduced side effects compared with pan-PI3K inhibitors and have been validated as a promising strategy for cancer therapy (Ward and Finan, 2003; Ciraolo et al., 2011). As PIK-75 exhibited intolerant off-target effects (Knight et al., 2006; Jamieson et al., 2011), DW series compounds were designed and synthesized with the aim of discovering new compounds with better biologic properties. Results presented in this study demonstrated that DW compounds, especially DW09849, are potent candidates for PI3K inhibition with improved selectivity. DW compounds displayed comparable activity with PIK-75 in inhibiting the kinase activity of PI3Kα with IC_{50} values in the nanomolar range, whereas they were less active than PIK-75 and displayed little activity against other PIKK family members as well as a panel of tyrosine kinases at the same concentration range. Moreover, DW compounds were more potent than PIK-75 in inhibiting the PI3K signaling in tumor cells, which is indicated by reduced AKT phosphorylation at Ser473 and Thr308 as well as decreased phosphorylation of p70-S6K1 at Thr389. In particular, DW09849 not only displayed higher potency than PIK-75 in inhibiting the PI3K signaling pathway, but also more persistently inhibited this process. In accordance with the aforementioned facts, DW09849 treatment resulted in G_0/G_1 cell cycle arrest, which has been recognized as one of the characteristics of selective PI3K inhibition. However, such phenomena failed to be observed in the case of PIK-75, further supporting the notion that DW09849 is a potent PI3K inhibitor with better selectivity than PIK-75. Therefore, although DW09849 and PIK-75 exhibited similar selectivity against p110α among class I PI3Ks, DW09849 is more specific for PI3K inhibition. It should be noted that DW09849 was less potent than PIK-75 in their activities to inhibit the proliferation of RH30 cells and RK3E cells, irrespective of its greater activity against PI3K signaling. This inconsistency indicates that unrecognized targeting by PIK-75 might contribute to its antiproliferative activity, whereas DW09849 was more dependent on PI3K inhibition to execute its activity.

The PI3K pathway is frequently deregulated in a wide range of tumor types as a result of genetic and epigenetic aberrations. Oncogenic mutations in PIK3CA itself account for a large part of hyperactivation of the PI3K pathway (Kan et al., 2010). Among mutations occurring in PIK3CA, the H1047R mutation is one of the most frequent that results in a significant increase in enzymatic activity (Samuels et al., 2004) and is able to transform normal cells (Zhao et al., 2005). Selective targeting of oncogenic mutation provides an ideal strategy for cancer therapy, which is expected to largely reduce side effects (Vanhaesebroeck et al., 2010). It is intriguing to find that DW09849 displayed selective activity against cells harboring p110α H1047R, demonstrated by its better potency to inhibit the proliferation of RK3E/HR cells

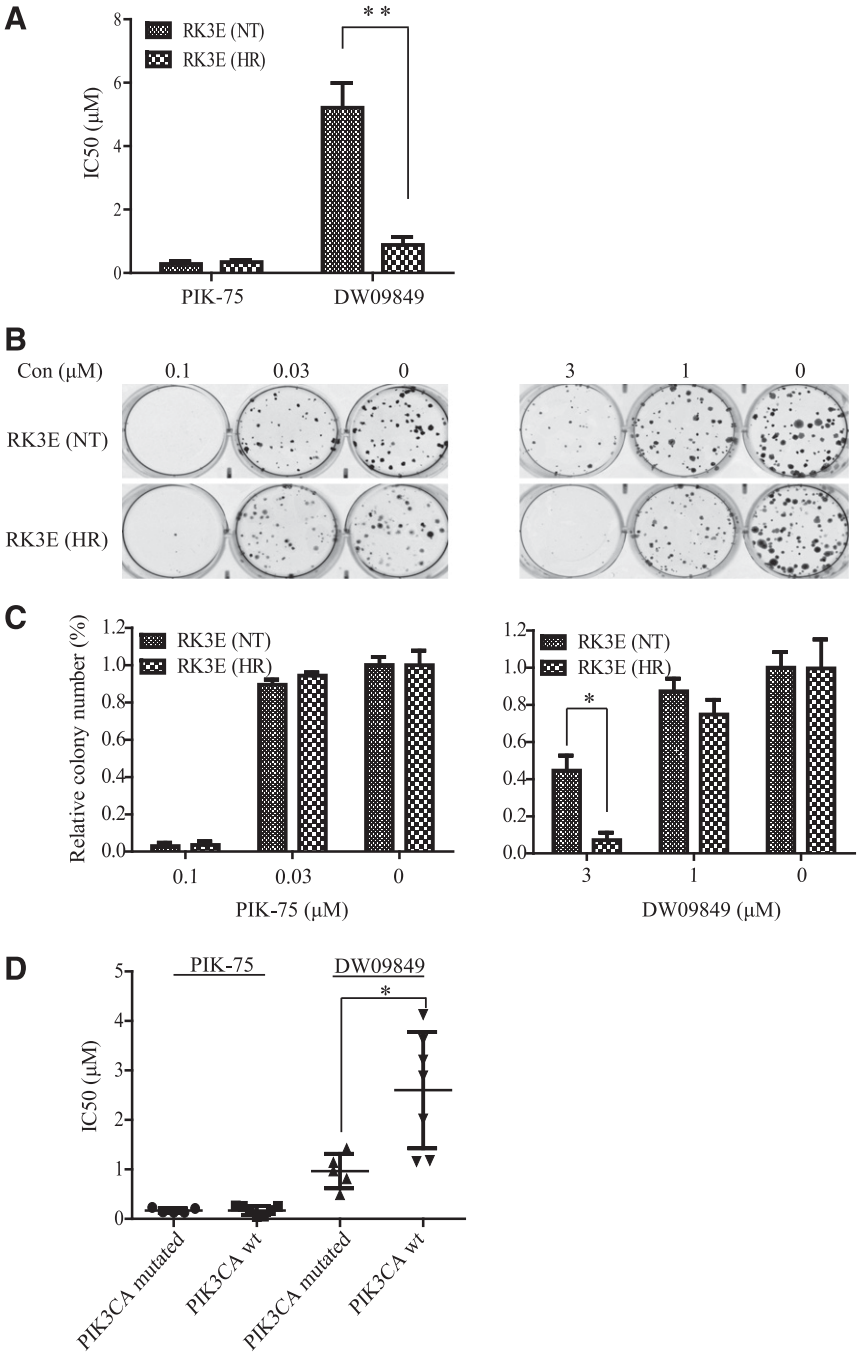


Fig. 5. DW09849 selectively inhibits proliferation and clonogenesis of p110α H1047R mutated RK3E/HR cells. (A) DW09849 preferentially inhibited proliferation of RK3E/HR cells. RK3E/NT and RK3E/HR were plated in 96-well plates and incubated with various concentrations of PIK-75 or DW09849 for 72 hours, and SRB assay was used to determine cell proliferation. Columns, mean of three independent experiments. Bars, standard error. $^{**}P < 0.01$. (B) DW09849 selectively inhibited clonogenesis of RK3E/HR cells. Cells seeded in 6-well plates were treated with various concentrations of DW09849 or PIK-75 for 7 days, and cells were stained with 0.1% violet and photographed subsequently. Representative images of three independent experiments are shown. (C) Visible colonies ($\geq$50 cells) were counted and plotted. Columns, mean of three independent experiments. Bars, standard error. $^{*}P < 0.05$. (D) DW09849 selectively inhibited proliferation of PIK3CA mutated breast cancer cell lines. Breast cancer cells were plated in 96-well plates and incubated with various concentrations of PIK-75 or DW compounds for 72 hours, and SRB assay was used to determine cell proliferation. Data shown are the mean of three separate experiments performed in triplicate. $^{*}P < 0.05$. Con, concentration; wt, wild type.

than RK3E/NT cells. In accordance with this result, DW09849 inhibited the clonogenic activity of RK3E/HR cells at lower concentrations than those required to inhibit RK3E cells. These results provide a hint that DW09849 may be capable of selectively inhibiting p110α mutated cells. This selective activity was also observed in PIK3CA mutated breast cancer cell lines. It is plausible that proliferation of RK3E/HR and PIK3CA mutated cells is "addicted" to constitutive activation of PI3K signaling, resulting from oncogenic p110α mutation. Thus, these cells are more sensitive to PI3K inhibitors. These results also suggested that DW09849 was able to inhibit the kinase activity of mutated p110α, which is supported by the results from the molecular modeling of DW09849 with wild-type PI3Kα and the H1047R mutant, where DW09849 displayed a potentially higher affinity with H1047R mutant. So far, compounds specifically targeting mutated p110α have not been reported. Better understanding the interaction of the compound and PI3Kα will be helpful to discover inhibitors that specifically targeting mutated p110α. DW09849 could be used as a probe for this purpose.

In summary, DW compounds were synthesized and verified as selective p110α inhibitors. DW09849, one of the most potent compounds among them, showed selective antiproliferative activity against p110α mutated (especially H1047R mutated) cells. However, structure refining is warranted to obtain inhibitors with more specificity and selectivity. In addtion, the mode of action with PI3Kα and its mutant as well as the anticancer activity of DW09849 should be further investigated to discover inhibitors with improved activity and selectivity.

Authorship Contributions

Participated in research design: Liu, Duan, Ding, Meng.

Conducted experiments: Liu, Gao, Zhang, Guo, Tong.

Contributed new reagents or analytic tools: Liu, Gao, Wang, Cao.

Performed data analysis: Liu, Zhang, Meng.

Wrote or contributed to the writing of the manuscript: Liu, Duan, Meng.

References

Bader AG, Kang S, Zhao L, and Vogt PK (2005) Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* **5**:921–929.

Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science* **296**:1655–1657.

Chen SM, Liu JL, Wang X, Liang C, Ding J, and Meng LH (2012) Inhibition of tumor cell growth, proliferation and migration by X-387, a novel active-site inhibitor of mTOR. *Biochem Pharmacol* **83**:1183–1194.

Ciraolo E, Morello F, and Hirsch E (2011) Present and future of PI3K pathway inhibition in cancer: perspectives and limitations. *Curr Med Chem* **18**:2674–2685.

Engelman JA (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* **9**:550–562.

Fu SL, Huang YJ, Liang FP, Huang YF, Chuang CF, Wang SW, and Yao JW (2005) Malignant transformation of an epithelial cell by v-Src via tv-a-mediated retroviral infection: a new cell model for studying carcinogenesis. *Biochem Biophys Res Commun* **338**:830–838.

Hayakawa M, Kawaguchi K, Kaizawa H, Koizumi T, Ohishi T, Yamano M, Okada M, Ohta M, Tsukamoto S, and Raynaud FI, et al. (2007) Synthesis and biological evaluation of sulfonylhydrazone-substituted imidazo[1,2-a]pyridines as novel PI3 kinase p110alpha inhibitors. *Bioorg Med Chem* **15**:5837–5844.

Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, Pearline RV, Cantley LC, and Brugge JS (2005) Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. *Cancer Res* **65**:10992–11000.

Jamieson S, Flanagan JU, Kolekar S, Buchanan C, Kendall JD, Lee WJ, Rewcastle GW, Denny WA, Singh R, and Dickson J, et al. (2011) A drug targeting only p110α can block phosphoinositide 3-kinase signalling and tumour growth in certain cell types. *Biochem J* **438**:53–62.

Kan Z, Jaiswal BS, Stinson J, Janakiraman V, Bhatt D, Stern HM, Yue P, Haverty PM, Bourgon R, and Zheng J, et al. (2010) Diverse somatic mutation patterns and pathway alterations in human cancers. *Nature* **466**:869–873.

Knight ZA, Gonzalez B, Feldman ME, Zunder ER, Goldenberg DD, Williams O, Loewith R, Stokoe D, Balla A, and Toth B, et al. (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* **125**: 733–747.

Kok K, Geering B, and Vanhaesebroeck B (2009) Regulation of phosphoinositide 3-kinase expression in health and disease. *Trends Biochem Sci* **34**:115–127.

Kong DX and Yamori T (2010) ZSTK474, a novel phosphatidylinositol 3-kinase inhibitor identified using the JFCR39 drug discovery system. *Acta Pharmacol Sin* **31**: 1189–1197.

Leevers SJ, Vanhaesebroeck B, and Waterfield MD (1999) Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr Opin Cell Biol* **11**: 219–225.

Li T, Wang J, Wang X, Yang N, Chen SM, Tong LJ, Yang CH, Meng LH, and Ding J (2010) WJD008, a dual phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin inhibitor, prevents PI3K signaling and inhibits the proliferation of transformed cells with oncogenic PI3K mutant. *J Pharmacol Exp Ther* **334**: 830–838.

Ma XM and Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* **10**:307–318.

Marone R, Cmiljanovic V, Giese B, and Wymann MP (2008) Targeting phosphoinositide 3-kinase: moving towards therapy. *Biochim Biophys Acta* **1784**:159–185.

Minniti CP, Luan D, O'Grady C, Rosenfeld RG, Oh Y, and Helman LJ (1995) Insulin-like growth factor II overexpression in myoblasts induces phenotypic changes typical of the malignant phenotype. *Cell Growth Differ* **6**:263–269.

Pal I and Mandal M (2012) PI3K and Akt as molecular targets for cancer therapy: current clinical outcomes. *Acta Pharmacol Sin* **33**:1441–1458.

Parekh DB, Katso RM, Leslie NR, Downes CP, Procyk KJ, Waterfield MD, and Parker PJ (2000) Beta1-integrin and PTEN control the phosphorylation of protein kinase C. *Biochem J* **352**:425–433.

Sabbah DA, Vennerstrom JL, and Zhong H (2010) Docking studies on isoform-specific inhibition of phosphoinositide-3-kinases. *J Chem Inf Model* **50**:1887–1898.

Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, and Riggins GJ, et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**:554.

Sanner MF (1999) Python: a programming language for software integration and development. *J Mol Graph Model* **17**:57–61.

Satoh T, Hosokawa M, Atsumi R, Suzuki W, Hakusui H, and Nagai E (1994) Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1- piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol Pharm Bull* **17**:662–664.

Shuttleworth SJ, Silva FA, Cecil AR, Tomassi CD, Hill TJ, Raynaud FI, Clarke PA, and Workman P (2011) Progress in the preclinical discovery and clinical development of class I and dual class I/IV phosphoinositide 3-kinase (PI3K) inhibitors. *Curr Med Chem* **18**:2686–2714.

Vadas O, Burke JE, Zhang X, Berndt A, and Williams RL (2011) Structural basis for activation and inhibition of class I phosphoinositide 3-kinases. *Sci Signal* **4**:re2.

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, and Bilanges B (2010) The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* **11**:329–341.

Vanhaesebroeck B, Leevers SJ, Panayotou G, and Waterfield MD (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* **22**:267–272.

Vivanco I and Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* **2**:489–501.

Vlahos CJ, Matter WF, Hui KY, and Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269**:5241–5248.

Wang X, Li JP, Yang Y, Ding J, and Meng LH (2013) A pharmacological model reveals biased dependency on PI3K isoforms for tumor cell growth. *Acta Pharmacol Sin* **34**:1201–1207.

Ward SG and Finan P (2003) Isoform-specific phosphoinositide 3-kinase inhibitors as therapeutic agents. *Curr Opin Pharmacol* **3**:426–434.

Workman P, Clarke PA, Raynaud FI, and van Montfort RL (2010) Drugging the PI3 kinome: from chemical tools to drugs in the clinic. *Cancer Res* **70**:2146–2157.

Zhao JJ, Liu Z, Wang L, Shin E, Loda MF, and Roberts TM (2005) The oncogenic properties of mutant p110alpha and p110beta phosphatidylinositol 3-kinases in human mammary epithelial cells. *Proc Natl Acad Sci USA* **102**:18443–18448.

Zheng Z, Amran SI, Zhu J, Schmidt-Kittler O, Kinzler KW, Vogelstein B, Shepherd PR, Thompson PE, and Jennings IG (2012) Definition of the binding mode of a new class of phosphoinositide 3-kinase α-selective inhibitors using in vitro mutagenesis of non-conserved amino acids and kinetic analysis. *Biochem J* **444**:529–535.

Zhong L, Guo XN, Zhang XH, Wu ZX, Luo XM, Jiang HL, Lin LP, Zhang XW, and Ding J (2005) Expression and purification of the catalytic domain of human vascular endothelial growth factor receptor 2 for inhibitor screening. *Biochim Biophys Acta* **1722**:254–261.

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