

Cancer Network Disruption by a Single Molecule Inhibitor Targeting Both Histone Deacetylase Activity and Phosphatidylinositol 3-Kinase Signaling

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Abstract

Purpose: Given that histone deacetylase (HDAC) inhibitors are known to induce multiple epigenetic modifications affecting signaling networks and act synergistically with phosphatidylinositol 3-kinase (PI3K) inhibitors, we developed a strategy to simultaneously inhibit HDACs and PI3K in cancer cells.

Experimental Design: We constructed dual-acting inhibitors by incorporating HDAC inhibitory functionality into a PI3K inhibitor pharmacophore. CUDC-907, a development candidate selected from these dual inhibitors, was evaluated *in vitro* and *in vivo* to determine its pharmacologic properties, anticancer activity, and mechanism of action.

Results: CUDC-907 potently inhibits class I PI3Ks as well as classes I and II HDAC enzymes. Through its integrated HDAC inhibitory activity, CUDC-907 durably inhibits the PI3K-AKT-mTOR pathway and compensatory signaling molecules such as RAF, MEK, MAPK, and STAT-3, as well as upstream receptor tyrosine kinases. CUDC-907 shows greater growth inhibition and proapoptotic activity than single-target PI3K or HDAC inhibitors in both cultured and implanted cancer cells.

Conclusions: CUDC-907 may offer improved therapeutic benefits through simultaneous, sustained disruption of multiple oncogenic signaling networks. *Clin Cancer Res*; 18(15); 4104–13. ©2012 AACR.

Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway plays an important role in cancer cell initiation, growth, proliferation, and survival. The PI3 kinases are frequently activated through mutation or receptor tyrosine kinases in many cancers (1, 2). More than 20 agents targeting the PI3K/AKT pathway are currently being evaluated in clinical trials and several of these agents have shown single-agent activity in cancers with phosphatidylinositol 3-kinase catalytic subunit (*PIK3CA*) mutations or *PTEN* deletion/mutations (3–6). However, the efficacy of PI3K inhibitors is limited by concurrent activation of other survival- and growth-related pathways (5, 7–9).

A potential strategy to overcome these limitations is to combine PI3K and MEK inhibitors to achieve synergistic antitumor activity. Preclinical studies have showed that this combination significantly improves efficacy in xenograft

tumor models that carry various genetic alterations, such as *KRAS* mutations, *HER2* amplification, or *PTEN* deletion/mutations (9–12). Of note, several phase I clinical trials testing combinations of PI3K and MEK pathway inhibitors are in progress (13).

Another promising strategy to overcome potential limitations of targeting the PI3K pathway is to disrupt multiple pathways through histone deacetylase (HDAC) inhibition (14, 15). By regulating both histone and nonhistone substrates, HDAC inhibitors can affect a variety of cell functions and synergize with PI3K inhibitors (16, 17).

In this report, we synthesized a novel series of dual-acting PI3K and HDAC inhibitors by incorporating HDAC inhibitory functionality into a PI3K inhibitor pharmacophore. We show the unique pharmacologic properties of one of the best of the series, CUDC-907, and its potent anticancer activity through oncogenic signaling network disruption.

Materials and Methods

Reagents

CUDC-907, vorinostat [suberoylanilide hydroxamic acid (SAHA)], panobinostat (LBH-589), GDC-0941, and BEZ-235 were synthesized in-house. CAL-101 was purchased from Active Biochem (Maplewood, NJ). For *in vitro* assays, compounds were dissolved in dimethyl sulfoxide (DMSO) as stock and stored at –80°C. For *in vivo* studies, CUDC-907 was formulated in 30% Captisol (Cydex Pharmaceuticals, Inc.).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Several phosphatidylinositol 3-kinase (PI3K) inhibitors are being evaluated in clinical trials for the treatment of various cancers, but single target agents often display limited clinical activity because of the genetic, epigenetic, and phenotypic heterogeneity and adaptive nature of cancer cells. Simultaneous blockade of multiple signaling nodes to thwart adaptive and evolutionary mechanisms of cancer cells offers the potential to overcome the limited activity and acquired resistance to PI3K inhibitor monotherapy. Accordingly, we designed and synthesized a novel molecule, CUDC-907, that inhibits not only PI3K directly but also other oncogenic signaling pathways indirectly through histone deacetylase (HDAC) inhibition. We report here that CUDC-907 displays potent anticancer activity in both cultured cancer cells and xenograft models and may offer therapeutic benefits in multiple cancers through broad signaling network disruption. Importantly, our work provides a framework to generate a multitarget inhibitor that can potentially overcome the dynamic and adaptive natures of tumor cells.

HDAC activity and PI3K kinase assays

The activities of classes I and II HDACs were measured using the Color-de-Lys assay system (Biomol International). The activity of PI3K was measured using the ADP-Glo luminescent kinase assay (Promega). Recombinant PI3K protein, a complex of N-terminal GST-tagged recombinant full-length human p110 and untagged recombinant full-length human p85, was coexpressed in a baculovirus-infected Sf9 cell expression system.

Cancer cell growth inhibition assay

Human cancer cell lines were purchased from American Type Culture Collection (Manassas, VA) and plated at densities of 5,000 to 10,000 per well in 96-well flat-bottomed plates with the recommended culture medium. The cells were then incubated with compounds at various concentrations for 72 hours in culture medium supplemented with 0.5% (v/v) FBS. Growth inhibition was assessed by assay of cellular ATP content using the Perkin-Elmer ATPlite kit.

Western blot analysis and immunocytochemistry

Cells grown in monolayer culture were treated as indicated. Cell lysates were resolved on polyacrylamide gels and transferred to nitrocellulose filter (Invitrogen). Immunoblotting was done using standard procedures with blocking solution (Li-Cor Bioscience) containing primary and IRDye 680- or 800CW-conjugated secondary antibodies. Immunocytochemistry was conducted on cells grown in monolayer culture that were treated as indicated in figure legends and then fixed in 4% (w/v) paraformaldehyde.

Assessment of apoptosis

Apoptosis was assessed by measuring the activities of caspase-3 and -7 using the Apo-ONE Homogeneous Assay Kit (Promega).

Cell-cycle analysis

Cell-cycle arrest was measured by Accuri C6 Flow Cytometry using a Cell Cycle Phase Determination kit (Cayman Chemical Co.).

Efficacy study in a human cancer xenograft model

Six- to 8-week-old female athymic (nude *nu/nu* CD-1) or severe combined immunodeficient (SCID) mice obtained from Charles River Laboratories were injected subcutaneously with 3 to 20×10^6 cells in a medium suspension of 100 to 200 μ L into the right hind flank region. Varying doses of CUDC-907, standard anticancer agents, or vehicle were administered orally or via tail vein injection as indicated.

Results

HDAC and PI3K inhibitors are synergistic

To confirm the potential synergistic effect of PI3K and HDAC inhibition, we assessed the growth inhibition effect of 2 reference compounds, the HDAC inhibitor vorinostat and the PI3K inhibitor GDC-0941, on the human PC-3 prostate cancer cell line. The combined effect of these 2 compounds was then compared with the effect observed after treatment with each single compound and then analyzed using the median effect analysis (18). The combination index was significantly less than 1, which indicates that the 2 inhibitors given together act synergistically to suppress cancer cell growth (Supplementary Fig. S1) thus providing the rationale for the development of a dual PI3K and HDAC inhibitor.

CUDC-907 is a potent inhibitor of both HDAC and PI3K

We thus designed and synthesized a multitarget inhibitor, CUDC-907, which integrates HDAC inhibitory functionality (hydroxamic acid) into a core structure scaffold (morpholinopyrimidine) shared by several PI3K inhibitors (Fig. 1A). We first tested whether CUDC-907 is a potent pan-inhibitor of HDAC classes I and II enzymes and observed that its potency against class I HDACs was similar to that of panobinostat and greater than that of vorinostat (Table 1). We next tested the effect of CUDC-907 on PI3K activity and found that CUDC-907 is also a potent inhibitor of class I PI3K kinases with an IC_{50} of 19, 54, and 39 nmol/L for PI3K α , PI3K β , and PI3K δ , respectively. This activity is similar to that of a known PI3K inhibitor, GDC-0941 (Table 1).

The potent HDAC and PI3K inhibitory activities of CUDC-907 were further evaluated in cancer cells. CUDC-907 treatment resulted in an increase of acetylated histones as well as non-histone proteins such as tubulin and p53 (Fig. 1B). Similar to other HDAC inhibitors, CUDC-907 markedly induced p21 protein in H460, a non-small cell lung cancer (NSCLC) cell line (Fig. 1B). To confirm the specificity of the HDAC inhibition, we further showed that CUDC-907 induced the accumulation of acetylated histone

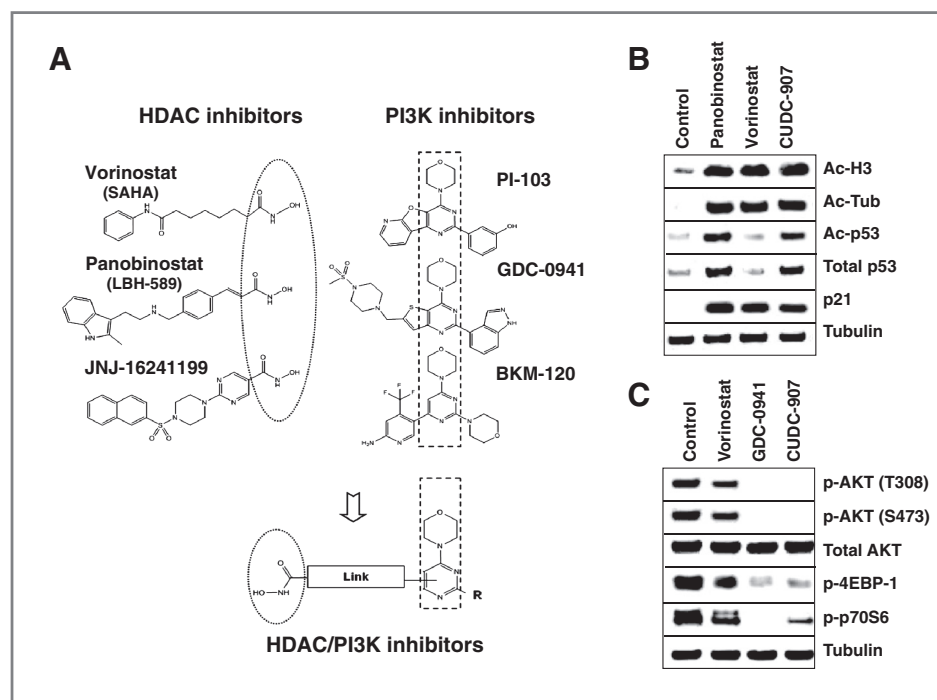


Figure 1. CUDC-907 design and its potency against PI3K and HDAC. A, schematic representation of the design for dual HDAC and PI3K inhibitors and the core chemical structure of CUDC-907. The compound was designed as a single molecule simultaneously targeting HDAC and PI3K kinase by integration of HDAC inhibitory functionality (hydroxamate moiety) into a PI3K inhibitor skeleton. B, H460 NSCLC cells were treated with DMSO, 1 $\mu\text{mol/L}$ of CUDC-907, 1 $\mu\text{mol/L}$ of panobinostat, or 5 $\mu\text{mol/L}$ of vorinostat for 1 hour. Western blot analysis indicates that CUDC-907 increases the levels of acetylated histone H3 (Ac-H3), tubulin (Ac-Tub), p53 (Ac-p53), total p53, and p21. C, H460 NSCLC cells were treated with DMSO, 1 $\mu\text{mol/L}$ of CUDC-907, 1 $\mu\text{mol/L}$ of GDC-0941, or 5 $\mu\text{mol/L}$ of vorinostat for 1 hour. Western blot analysis reveals that CUDC-907 inhibits AKT phosphorylation on both the T308 and S473 sites. Decreased phosphorylation of downstream 4EBP-1 and p70S6 was also detected.

H3, tubulin, and p53, as well as p21 expression in a dose-dependent manner (Supplementary Figs. S2–S5). Similarly, we observed that CUDC-907 inhibited the PI3K pathway, as indicated by the dose-dependent decreases in phosphorylation of AKT and its downstream targets, 4EBP-1 and p70S6, in H460 cells (Fig. 1C).

CUDC-907 durably inhibits AKT activation

One potential mechanism to explain how tumor cells evade single-target kinase inhibitors is the reactivation of downstream signaling through multiple compensatory

mechanisms. Consistently, we observed that although GDC-0941 more potently inhibited AKT than CUDC-907 at early time points (at 1 hour after treatment), CUDC-907 led to sustained AKT inhibition and suppressed AKT more potently than GDC-0941 at 16 hours after treatment (Fig. 2A). The greater PI3K-inhibiting activity observed at later time points likely resulted from the prevention of compensatory reactivation of AKT through CUDC-907 induced HDAC inhibition, as we observed similar, although less potent effects with the other HDAC inhibitors vorinostat and panobinostat (Fig. 2A).

Table 1. Inhibition of the enzymatic activities of HDACs and PI3Ks by CUDC-101 and reference compounds

Compound	IC ₅₀ (nmol/L) in enzyme assays														
	HDAC1	HDAC2	HDAC3	HDAC8	HDAC4	HDAC5	HDAC6	HDAC7	HDAC9	HDAC10	HDAC11	PI3K α	PI3K β	PI3K δ	PI3K γ
Vorinostat	42.5	156	33.1	113	NA	NA	21.6	NA	NA	68.4	51.3	NA	NA	NA	NA
Panobinostat	1.4	6.8	1.5	26.7	195.6	123.8	8.2	1864	922.2	2.1	1.6	NA	NA	NA	NA
GDC-0941	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	8	31	4	55
Cal-101	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	354	108	18	31
CUDC-907	1.7	5.0	1.8	191	409	674	27	426	554	2.8	5.4	19	54	39	311

Abbreviation: NA, not active.

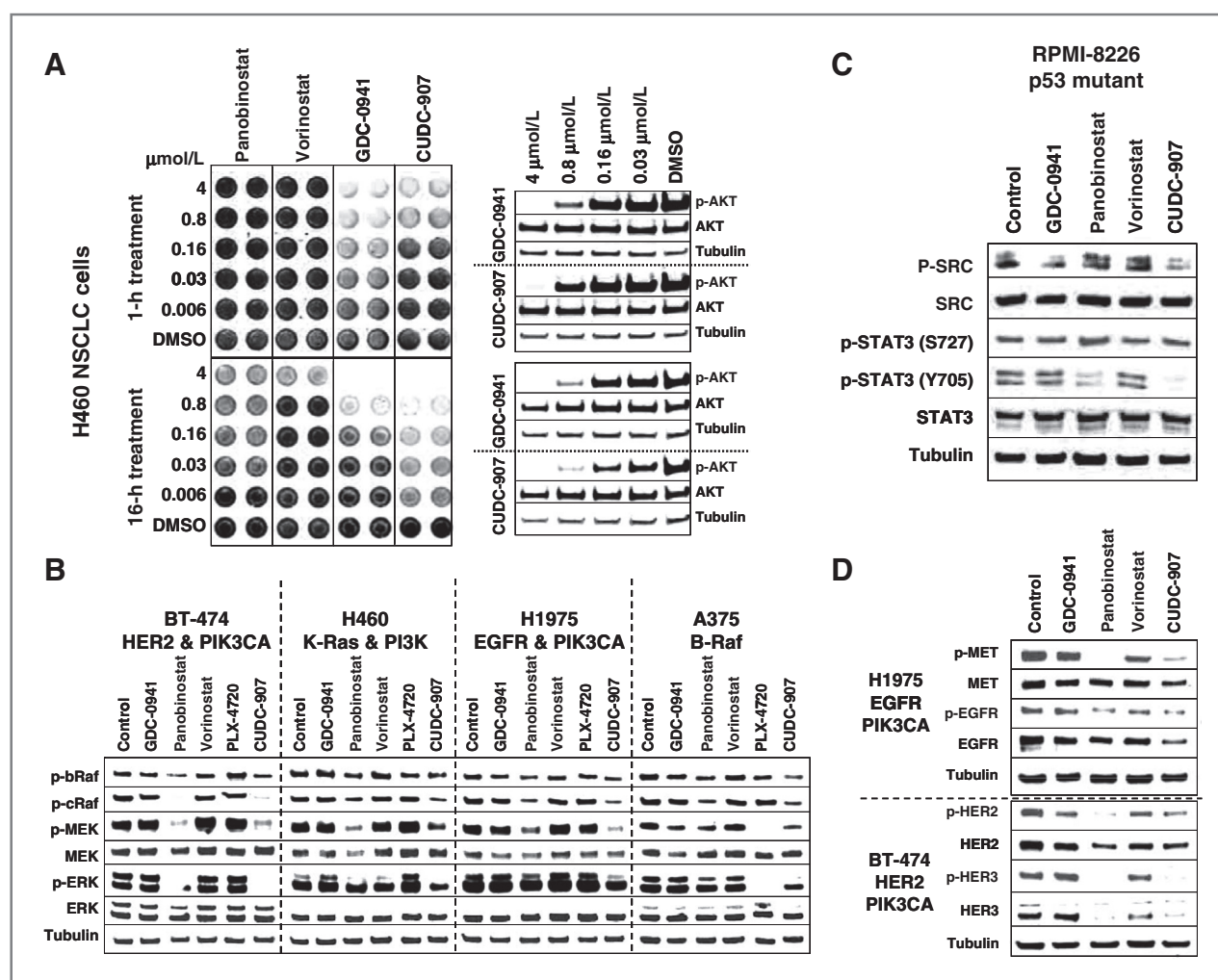


Figure 2. CUDC-907 durably suppresses activation of AKT and modulates receptor tyrosine kinase, RAF-MEK-MAPK and SRC/STAT signaling. **A**, as indicated, H460 NSCLC cells were cultured in the presence of 10 μg/mL of insulin and treated with DMSO, CUDC-907, GDC-0941, panobinostat, or vorinostat with various concentrations as indicated for 1 or 16 hours (left). Immunocytochemical analyses indicate that GDC-0941 potentially inhibits AKT phosphorylation at 1 hour; however, AKT was reactivated in cells treated with the compound at lower concentrations at 16 hours. In contrast, CUDC-907 inhibited AKT phosphorylation even at 16 hours after treatment and was more potent than GDC-0941 in reducing AKT phosphorylation at this time point. Western blot analysis (right) further confirmed the immunocytochemical results shown in the left. **B**, BT-474 breast, H460 NSCLC, H1975 NSCLC, and A375 melanoma cells were treated with DMSO, 0.1 μmol/L of CUDC-907, 0.1 μmol/L of GDC-0941, 0.1 μmol/L of PLX-4720, 0.1 μmol/L of panobinostat, or 0.5 μmol/L of vorinostat for 16 hours as indicated. Western blot analyses indicate that CUDC-907 inhibits the phosphorylation of CRAF, MEK, and MAPK. **C**, as indicated, RPMI-8226 multiple myeloma cells were treated with DMSO, 0.1 μmol/L of CUDC-907, 0.1 μmol/L of GDC-0941, 0.1 μmol/L of panobinostat, or 0.5 μmol/L of vorinostat for 16 hours. Western blot analyses indicate that CUDC-907 suppresses SRC and STAT3 phosphorylation. **D**, H1975 NSCLC and BT-474 breast cancer cells were treated with DMSO, 0.1 μmol/L of CUDC-907, 0.1 μmol/L of GDC-0941, 0.1 μmol/L of panobinostat, or 0.5 μmol/L of vorinostat for 16 hours. Western blot analyses indicate that CUDC-907 reduces both phosphorylated and total protein levels of the tyrosine receptor kinases MET, EGFR, HER2, and HER3 in H1975 and BT-474 tumor cells. Similar reductions in levels of these receptor kinases were also observed after panobinostat treatment.

CUDC-907 suppresses other signaling pathways via HDAC inhibition

We sought to evaluate whether CUDC-907 prevents cancer cells from using other growth and survival pathways via feedback mechanisms. In *BRAF*-mutant tumor cells (e.g., A375 melanoma cells), activation of downstream MEK sensitizes cells to treatment with the *BRAF* inhibitor PLX-04720, but not the single-target PI3K inhibitor GDC-0941. CUDC-907 and panobinostat were both able to inhibit the activation of MEK (Fig. 2B). In cancer cell lines with mutations other than those resulting in constitutive acti-

vation of PI3K, such as NSCLC H460 cells with *KRAS* mutation, breast cancer BT-474 cells with *HER2* amplification, and NSCLC H1975 cells with *EGFR* mutations conferring drug resistance, the RAF-MEK-MAPK signaling pathway was active and insensitive to treatment with GDC-0941. In contrast, CUDC-907 and panobinostat suppressed phosphorylation of CRAF, MEK, and MAPK in these cancer cells harboring different mutations (Fig. 2B). Together, these results suggest that HDAC inhibition by CUDC-907 effectively suppresses the RAF-MEK-MAPK signaling pathway.

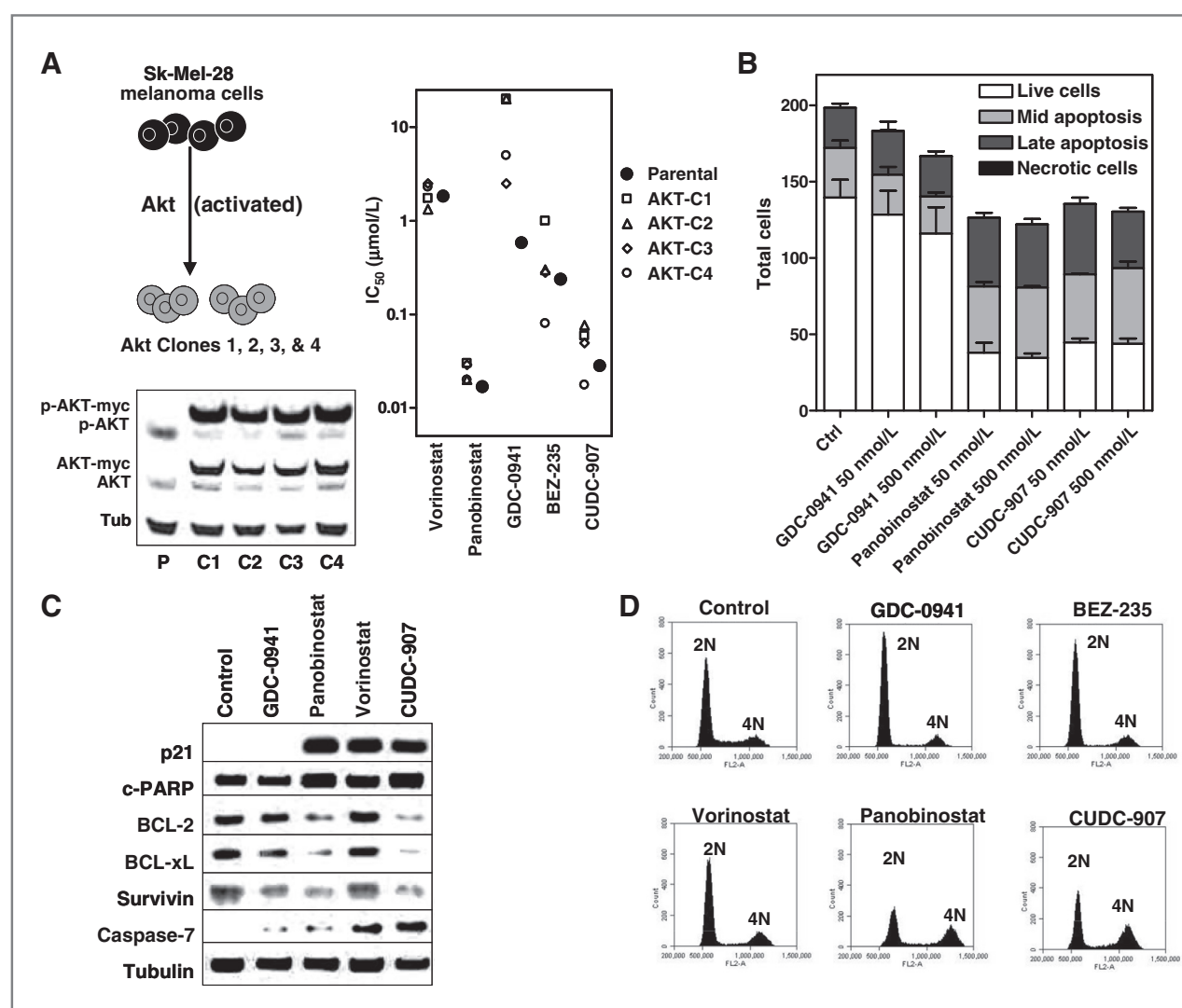


Figure 3. CUDC-907 evades drug resistance and induces apoptosis and G_2 -M phase cell-cycle arrest. **A**, Sk-Mel-28 melanoma cells were transfected with myc-tagged constitutively active (myristoylated) AKT-1, and stable clones were selected and expanded. Western blot analysis indicates that stable clones expressed high levels of myc-tagged constitutively active AKT (AKT-myc) when compared with parental cells. Growth assays reveal that clones expressing AKT-myc tolerate treatment with a single-target PI3K inhibitor but remain sensitive to treatment with CUDC-907. **B**, RPMI-8226 multiple myeloma cells were incubated with various concentrations (as indicated) of CUDC-907, vorinostat, GDC-0941, or panobinostat for 24 hours. Cytometric analysis was used to characterize live, midapoptotic, late apoptotic, and necrotic or dead cells, characterized by double-negative, Z-DEVD, double-positive, or 7-aminoactinomycin D (7-AAD) staining, respectively. Both CUDC-907 and panobinostat induce the accumulation of apoptotic cells. Ctrl, control. **C**, H460 cells were treated with GDC-0941, panobinostat, vorinostat, or CUDC-907 at $1 \mu\text{mol/L}$ for 16 hours before Western blot analysis was conducted. CUDC-907 increases the levels of activated caspase-7, p21, and cleaved PARP (c-PARP), accompanied by a reduction in levels of BCL-2, BCL-xL, and survivin. **D**, H460 cells were treated for 24 hours with $1 \mu\text{mol/L}$ of CUDC-907 and reference compounds before propidium iodide staining. Flow cytometric analyses show that CUDC-907 and panobinostat both induce the accumulation of cells at G_2 -M.

Furthermore, we were able to show that CUDC-907 caused the reduction of both p-STAT3 (Y-705) and p-SRC in RPMI-8226 multiple myeloma cells (Fig. 2C) and reduced both phosphorylated and total protein levels of MET and EGFR as well as HER2 and HER3 in H1975 NSCLC cells and BT-474 breast cancer cells, respectively (Fig. 2D). These results indicate that CUDC-907 can downregulate and suppress the activation of the SRC/STAT signaling pathway and multiple receptor tyrosine kinases, again presumably because of its HDAC inhibitory activity as we observed that panobinostat also induced a similar effect (Fig. 2C and D).

CUDC-907 may potentially evade drug resistance

To further explore the potential of CUDC-907 to thwart compensatory mechanisms, we generated clones stably expressing activated AKT-1 in Sk-Mel-28 melanoma cancer cells. Compared with parental cells, clones with constitutively active AKT became insensitive to the treatment of GDC-0941. However, those drug-resistant clones were still mainly sensitive to CUDC-907 and panobinostat (Fig. 3A). These results suggest that the incorporation of HDAC inhibitory activity within CUDC-907 prevents the development of drug resistance.

Table 2. Growth inhibition of human cancer cell lines by CUDC-907 and reference compounds

		IC ₅₀ (μmol/L) in growth inhibition assay			
Cancer types	Cell lines	Vorinostat	GDC-0941	Vorinostat + GDC-0941	CUDC-907
Hematologic					
B-cell lymphoma	RL	0.94	1.34	0.15	0.0020
	DOHH2	0.57	0.03	0.01	0.0010
	Granta 519	3.02	>20	0.59	0.0070
	SU-DHL4	1.15	0.45	0.25	0.0030
	Daudi	1.23	>20	0.89	0.0150
	Raji	3.56	>20	3.90	0.0090
	Pfeiffer	2.73	0.55	0.37	0.0040
T-cell lymphoma	HH	0.18	0.18	0.13	0.0010
	MJ	0.87	>20	0.98	0.0050
	HuT78	0.09	0.15	0.05	0.0010
ALL	MOLT4	0.31	0.29	0.13	0.0010
	SUP-B15	0.44	7.61	0.28	0.0007
AML	HL60	0.42	1.39	0.19	0.0020
	U937	0.81	0.97	0.29	0.0020
	THP-1	1.12	3.39	0.38	0.0160
	MV-4-11	0.23	1.39	0.18	0.0004
CML	K562	1.48	7.85	0.89	0.0310
	MEG-01	2.79	>20	2.50	0.0060
Multiple myeloma	RPMI-8226	1.01	>20	0.69	0.0020
	OPM-2 ^c	0.43	0.05	0.11	0.0010
	ARH77	1.42	14.79	0.83	0.0050
Solid					
Colon	WiDr ^a	1.29	>20	0.75	0.0400
	HCT-116 ^{a,b}	1.45	7.53	0.45	0.0070
	SW403 ^a	2.97	>20	1.11	0.0070
	SW620 ^a	1.17	>20	0.53	0.0050
	SW116 ^a	1.72	>20	1.55	0.0060
NSCLC	T-84 ^a	3.83	2.97	0.84	0.0300
	H358 ^a	2.00	2.85	1.08	0.0090
	H292 ^a	2.79	1.68	0.77	0.0080
	H2122 ^a	1.76	1.48	0.65	0.0080
	H460 ^a	10.72	>20	2.46	0.0500
	A549 ^a	2.79	4.35	1.04	0.0100
	Calu6 ^a	3.10	>20	1.54	0.0200
Pancreas	MiaPaca2 ^a	2.39	>20	1.79	0.0200
	CaPan2 ^a	3.75	>20	0.93	0.0200
	CFPAC-1 ^a	6.22	>20	1.91	0.0500
	PANC-1 ^a	7.21	1.25	1.39	0.0500
Breast	SW1990 ^a	2.30	>20	1.64	0.0080
	HCC1500	3.21	>20	2.00	0.0060
	HCC1806	4.50	>20	1.22	0.0700
	MDA-MB-231 ^a	7.29	>20	2.67	0.1200
	SKBr3	9.84	>20	3.69	0.0500
	BT474 ^b	1.26	0.49	0.26	0.0060
	MDA-MB-361 ^b	0.68	0.14	0.06	0.0010
	UACC-893 ^b	1.96	1.15	0.53	0.0100
	MDA-MB-453 ^b	0.53	0.64	0.31	0.0070
	MCF-7 ^b	1.67	0.41	0.39	0.0080
	T47D ^b	2.88	>20	0.49	0.0200
ZR-75-1 ^c	4.43	0.90	1.28	0.0800	
MDA-MB-468 ^c	6.81	0.98	1.07	0.0900	

NOTE: For combination treatments, reference compounds vorinostat and GDC-0941 were added to the cells at a 1:1 molar ratio with the IC₅₀ values were calculated as the concentrations of single agents. All results shown in this table are representative of 2 to 3 experiments.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia.

^aCell lines with *KRAS* mutations.

^bCell lines with *PIK3CA* mutations.

^cCell lines with *PTEN* deletions/mutations.

CUDC-907 induces apoptosis and G₂-M cell-cycle arrest in cancer cells

Next, we evaluated the ability of CUDC-907 to induce apoptosis and cell-cycle arrest in cancer cells. CUDC-907 induced caspase-3 and -7 activation in HCT-116 colon cancer cells in a dose-dependent manner (Supplementary Fig. S6). We next confirmed that CUDC-907 treatment resulted in the accumulation of activated caspase-3 in RPMI-8226 myeloma and annexin V binding sites in MDA-MB-468 breast cancer cells (Fig. 3B and Supplementary Fig. S7). Furthermore, we observed the accumulation of cleaved PARP, activated caspase-7, and p21, as well as the reduction of antiapoptotic proteins including BCL-2, BCL-xL, and survivin in H460 NSCLC cells (Fig. 3C). Consistent with earlier reports of other HDAC inhibitors (15, 19–21), we observed that CUDC-907 and panobinostat induced cell-cycle arrest at G₂-M phase. In contrast, the PI3K inhibitors GDC-0941 and BEZ-235 predominantly induced G₀/G₁ cell-cycle arrest (Fig. 3D and Supplementary Fig. S8).

CUDC-907 effectively inhibits cancer cell growth

Our mechanistic studies suggest that CUDC-907 disrupts multiple oncogenic signaling pathways and induces apoptosis and cell-cycle arrest. Consistent with this observation, we found that CUDC-907 potently inhibits the growth of cancer cells derived from both hematologic and solid tumors (Table 2). Of note, in a survey of a panel of breast cancer cell lines, GDC-0941 is only effective in suppressing cells harboring a PI3K p110 α mutation, conversely, CUDC-907 potently inhibits the proliferation of cells expressing either mutant or wild-type PI3K. Similarly, cancer cells with activating *KRAS* mutations are sensitive to CUDC-907 but not to a single-target PI3K inhibitor (Table 2).

CUDC-907 inhibits targets and tumor growth in xenograft tumor models

As HDAC inhibitors have been shown to be promising therapeutic agents in hematologic cancers, we evaluated CUDC-907 in implanted Daudi non-Hodgkin lymphoma (NHL) cancer cells. CUDC-907 is bioavailable in animal models (Supplementary Fig. S9); we also showed that oral administration of CUDC-907 inhibited growth of the Daudi cancer cell xenografts in a dose-dependent manner. Tumor stasis was observed at 100 mg/kg in this model without obvious toxicity (Fig. 4A). Importantly, in the same model, CUDC-907 achieved better efficacy than GDC-0941, vorinostat, or a combination of these 2 compounds given at their maximal tolerated doses (MTD; Fig. 4A). Furthermore, CUDC-907 caused tumor regression or stasis after intravenous (50 mg/kg) or oral administration (100 mg/kg) in a xenograft tumor model of SU-DHL4 diffuse large B-cell lymphoma (DLBCL) and caused tumor stasis in *KRAS*-mutant A549 NSCLC cell xenografts (Fig. 4A). CUDC-907 was also well tolerated in treated animals, suggesting a favorable safety profile in its future development as a potential clinical drug candidate.

To determine if the observed anticancer activity of CUDC-907 is target-specific, we conducted pharmacodynamic

studies in the Daudi xenograft tumor models after oral or intravenous administration of CUDC-907 (Fig. 4B). We observed that CUDC-907 inhibited HDACs as indicated by the accumulation of acetylated histone H3 (AcH3) as well as PI3K as indicated by the decrease of p-AKT. The induction of acetylated histone H3 lasted for 24 hours after oral administration as compared with 6 hours after intravenous administration. Importantly, we also observed potent inhibition of the RAF-MEK-MAPK signaling pathway as indicated by the reduction of p-MAPK after oral or intravenous administration of CUDC-907. Inhibition of multiple pathways was accompanied by the induction of apoptosis as indicated by increased PARP cleavage (Fig. 4B).

Discussion

Because imatinib was approved in 2001 as the first tyrosine kinase inhibitor indicated for the treatment of CML, more than 20 agents that target key signaling kinases have been developed for the treatment of various cancers. Generally, these drugs improve patient outcomes and are less toxic than commonly used chemotherapeutics. However, limited activity, as well as intrinsic and acquired resistance are major obstacles and represent major challenges to the use of targeted drugs in the treatment of patients with cancer. Currently, there remains a need for novel drugs with improved efficacy and reduced drug resistance.

Given the experimental evidence supporting a synergistic effect of PI3K and HDAC inhibitors on cancer cell growth, we created CUDC-907, a novel compound that combines PI3K and HDAC inhibitor functionality in a single scaffold, to improve upon single-target PI3K inhibitors. Inhibition of HDACs can lead to the acetylation of histone and nonhistone proteins. This modulation subsequently regulate the expression or activity of many cellular proteins, including p53, E2F, c-Myc, NF κ B, HIF-1 α , STAT3, androgen receptor, α -tubulin, and heat shock protein 90 (HSP90; refs. 14, 15, 19–25). Consistently, we observed that CUDC-907 treatment triggers HDAC inhibition thus disrupting multiple signaling pathway nodes.

After the approval of 2 HDAC inhibitors, vorinostat and romidepsin, for the treatment of cutaneous T-cell lymphoma, multiple preclinical studies of HDAC inhibitors alone or in combination with other anticancer drugs have shown promising results for the treatment of hematologic malignancies (15). The PI3K pathway plays a primary role in B-cell signaling and survival. The PI3K δ inhibitor CAL-101 has shown efficacy in the treatment of patients with indolent NHL and mantle cell lymphoma, subtypes of NHL (26). In addition, simultaneous inhibition of HDAC and PI3K/mTOR signaling with panobinostat and rapamycin has been shown to synergistically inhibit tumor cell growth and induce apoptosis in diffuse large B-cell lymphoma (27) as well as in solid tumor cells (28). Because of its integrated HDAC and PI3K inhibitory activities, CUDC-907 may therefore be well suited for future clinical development for various hematologic tumors as well as for other human

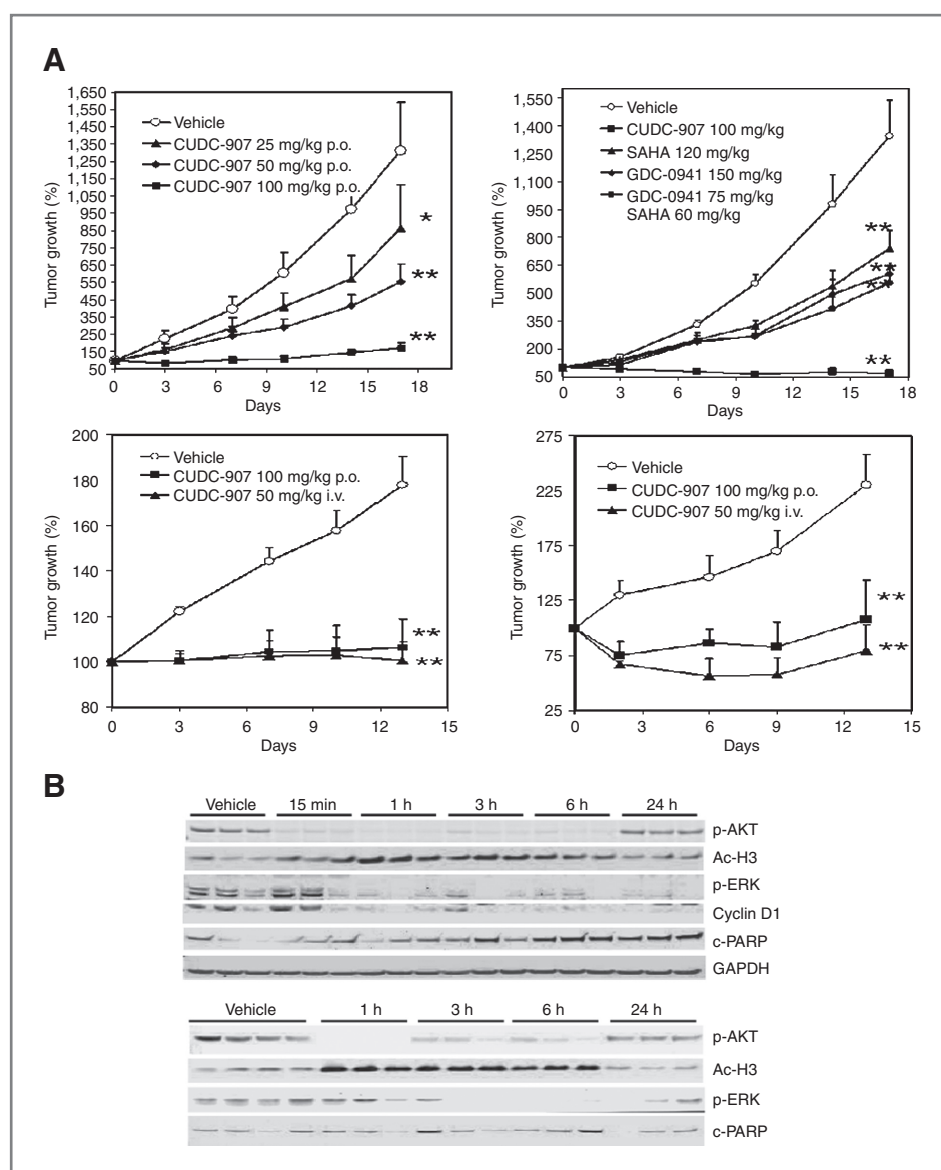


Figure 4. CUDC-907 suppresses tumor growth, inhibits HDAC activity, and blocks signaling of PI3K and MAPK pathways in xenograft models. **A**, top left, CUDC-907 was administered orally at 25, 50, and 100 mg/kg in the Daudi NHL xenograft mouse model. Pretreatment tumor size was $125 \pm 80 \text{ mm}^3$ (mean \pm SE). CUDC-907 inhibits tumor growth in a dose-dependent manner. *, $P < 0.05$; **, $P < 0.01$ (ANOVA) when compared with the vehicle control group; top right, the antitumor activity of CUDC-907 was compared with either GDC-0941 or vorinostat alone or a combination of GDC-0941 and vorinostat. All compounds and vehicle controls were dosed orally, 5 times weekly. The indicated doses are the maximum tolerated dose (MTD) of each treatment, and the pretreatment tumor size was $157 \pm 65 \text{ mm}^3$ (mean \pm SE). Data show that CUDC-907 is more efficacious than vorinostat, GDC-0941, or a combination of both; bottom left, CUDC-907 causes tumor stasis in the KRAS-mutant and EGFR inhibitor-resistant A549 NSCLC xenograft model after intravenous or oral administration. The pretreatment tumor size was $99.5 \pm 23.4 \text{ mm}^3$; bottom right, CUDC-907 strongly inhibits tumor growth in the SU-DHL4 diffuse large B-cell lymphoma xenograft model after intravenous (50 mg/kg) or oral administration (100 mg/kg). The pretreatment tumor size was $147 \pm 21 \text{ mm}^3$. **B**, pharmacodynamic studies of CUDC-907 after 100 mg/kg oral administration (p.o.; top) or 50 mg/kg intravenous administration (bottom) in the Daudi xenograft tumor model. CUDC-907 increases the levels of acetylated histone H3 (Ac-H3) and cleaved PARP and reduces the levels of p-AKT, p-MAPK, and cyclin D1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cancers exhibiting both aberrant PI3K signaling and deregulated HDAC activity.

Intrinsic and acquired drug resistance to single-target PI3K inhibitors occurs through reactivation of alternative or downstream signaling pathways. Drug-resistant mutations in the oncogenic isoform of PI3K p110 α have been

predicted using yeast genetic screening (29), although point mutations in p110 conferring drug resistance have yet to be found in clinical samples. CUDC-907 could potentially overcome drug resistance mechanisms through its ability to simultaneously inhibit PI3K activity and broadly regulate other signaling proteins through HDAC inhibition. We

found that CUDC-907 effectively inhibits the growth of cancer cells transfected with constitutively active AKT. These results suggest that the incorporation of HDAC inhibitory activity within CUDC-907 may prevent the development of drug resistance because of downstream activation of PI3K. Future studies will test whether PI3K inhibitor-resistant clones generated from cancer cell lines remain sensitive to CUDC-907.

Although animal studies have showed that hydroxamate HDAC inhibitors such as vorinostat and panobinostat have low oral bioavailability and short half-life in plasma (30–33), mouse pharmacokinetic studies show that CUDC-907 has an oral bioavailability approximately 2-fold higher than vorinostat and other HDAC inhibitors (Supplementary Fig. S8). Notably, we also observed that CUDC-907 tends to accumulate in tumor tissue with an AUC (area under the plasma concentration time curve) approximately 2-fold greater than in plasma (Supplementary Fig. S8). These results suggest that the higher intratumoral accumulation and antitumor activity of CUDC-907 compared with other HDAC inhibitors may potentially result in improved clinical activity.

Inhibition of multiple targets can be achieved by combining several drugs that each suppresses a specific target. However, a single dual-targeted inhibitor may have several advantages such as pharmacokinetic characteristics, reduced toxicity, patient compliance and ultimately, a more efficient clinical development over combination therapy. On the basis of this concept, we previously created CUDC-101, which integrates HDAC inhibitory functionality into a pharmacophore of the receptor tyrosine kinases EGFR and HER2 (34, 35). The first-in-human phase I study of CUDC-101 has provided the first evidence of the tolerability of a single multitarget inhibitor (36). These results further sup-

port the feasibility of multitarget inhibitors as anticancer therapeutics.

In summary, CUDC-907, a novel compound that combines PI3K and HDAC inhibition functionality into a single scaffold, disrupts cancer networks via potent inhibition of the PI3K pathway and epigenetic effects of HDACs. It may offer greater therapeutic benefit than other anticancer drugs currently used in the clinic.

Disclosure of Potential Conflicts of Interest

C. Qian is a consultant/advisory board member for Curis, Inc. M. Voi has stock options in Curis, Inc. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Qian, R. Bao, D.-G. Wang, J. Wang, G.-X. Xu, X. Cai

Writing, review, and/or revision of the manuscript: C. Qian, R. Bao, X. Cai, M. Voi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Wang

Study supervision: C. Qian, R. Bao, J. Wang

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