CUDC-305, a Novel Synthetic HSP90 Inhibitor with Unique Pharmacologic Properties for Cancer Therapy

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Abstract

Purpose: We designed and synthesized CUDC-305, an HSP90 inhibitor of the novel imidazopyridine class. Here, we report its unique pharmacologic properties and antitumor activities in a variety of tumor types.

Experimental Design: The potency of the compound was analyzed by fluorescence polarization competition binding assay. Its antiproliferative activities were assessed in 40 human cancer cell lines. Its pharmacologic properties and antitumor activities were evaluated in a variety of tumor xenograft models.

Results: CUDC-305 shows high affinity for HSP90α/β (IC50, ~100 nmol/L) and HSP90 complex derived from cancer cells (IC50, 48.8 nmol/L). It displays potent antiproliferative activity against a broad range of cancer cell lines (mean IC50, 220 nmol/L). CUDC-305 exhibits high oral bioavailability (96.0%) and selective retention in tumor (half-life, 20.4 hours) compared with normal tissues. Furthermore, CUDC-305 can cross blood-brain barrier and reach therapeutic levels in brain tissue. CUDC-305 exhibits dose-dependent antitumor activity in an s.c. xenograft model of U87MG glioblastoma and significantly prolongs animal survival in U87MG orthotopic model. CUDC-305 also displays potent antitumor activity in animal models of erlotinib-resistant non–small cell lung cancer and induces tumor regression in animal models of MDA-MB-468 breast cancer and MV4-11 acute myelogenous leukemia. Correlating with its efficacy in these various tumor models, CUDC-305 robustly inhibits multiple signaling pathways, including PI3K/AKT and RAF/MEK/ERK, and induces apoptosis. In combination studies, CUDC-305 enhances the antitumor activity of standard-of-care agents in breast and colorectal tumor models.

Conclusion: CUDC-305 is a promising drug candidate for the treatment of a variety of cancers, including brain malignancies.

Recent advances in understanding the molecular biology of cancer have resulted in the development of drugs that target known molecular pathways (1). For a limited number of cancer subtypes, these drugs exploit the dependence of the tumor on dysregulated signaling pathways to achieve therapeutic selectivity for cancer over normal cells. Molecularly targeted agents that have been approved for clinical use include imatinib (Gleevec), a small-molecule inhibitor of BCR/ABL kinase in chronic myelogenous leukemia; trastuzumab (Herceptin), an antibody against ERBB2 (HER2) in breast cancer; bevacizumab (Avastin), an antibody against vascular endothelial growth factor in solid tumors; and erlotinib (Tarceva), a small-molecule inhibitor of epidermal growth factor receptor (EGFR) in non–small cell lung cancer (NSCLC; refs. 2–5).

However, it has been increasingly recognized that, in each individual tumor, there are a large number of mutated genes that disrupt multiple pathways, which normally exhibit extensive biological cross-talk and redundancy (6). Therefore, interfering with a single target and/or pathway may not abrogate the malignant phenotype of most tumors. For example, among the roughly 20% of breast cancer patients with HER2 overexpression, only one third respond to trastuzumab treatment. The remaining two thirds of patients fail to respond, which is likely due to other distinct molecular abnormalities within their tumors, such as activation of the insulin-like growth factor signaling pathway (7). Furthermore, resistance to molecularly targeted agents can develop through secondary target gene mutation or compensatory activation of alternative pathways, so-called "oncogenic switching."

This problem is exemplified by erlotinib, an EGFR inhibitor that has been approved for use in NSCLC. An activating mutation in EGFR (exon 19 deletions or exon 21 point mutation...
ATPase activity. HSP90 inhibitors compete with ATP at the NH2–esis and promoting apoptosis (12) and/or activation of parallel signaling pathways, including epidermal growth factor receptor inhibitor–resistant, non–small cell lung cancer, glioblastoma, triple-negative breast cancer, and acute myelogenous leukemia. Remarkably, all of these tumors represent crucial unmet medical needs. Mechanistically, CUDC-305 robustly inhibits multiple signaling pathways and induces apoptosis in cancer cells. Taken together, these original findings may have a significant effect on future clinical practice in cancer therapy, particularly in the above-mentioned cancer types.

L858R) renders cancer cells sensitive to the EGFR inhibitor. However, subsequent resistance to erlotinib emerges as a result of an additional EGFR mutation (T790M) or amplification and/or activation of parallel signaling pathways, including ERBB3 (HER3), insulin-like growth factor receptor, and c-MET (8–10). A promising strategy for mitigating such acquired drug resistance is to simultaneously inhibit multiple molecular pathways, either by using several agents in combination or by using a single agent that concurrently blocks multiple targets or pathways.

One approach to inhibiting multiple pathways with one single agent is to target the heat shock protein HSP90. Among the “client proteins” that HSP90 chaperones are many oncogenic proteins, such as the estrogen receptor, androgen receptor, HER2, ERBB1 (EGFR), MEK, c-MET, AKT, MAPK (ERK), CDK, RAF, BCR/ABL, HIF1-α, and hTERT (11). These oncoproteins, ranging from transcription factors and kinases to antiapoptotic molecules, are involved in cancer cell proliferation, survival, invasion, metastasis, and angiogenesis (11). It has been shown that pharmacologic inhibition of HSP90 function can trigger proteasomal degradation of multiple oncoproteins, thereby reducing cancer cell proliferation/survival and tumor angiogenesis and promoting apoptosis (12–14).

The chaperone function of HSP90s is highly dependent on its ATPase activity. HSP90 inhibitors compete with ATP at the NH2-terminal nucleotide-binding site to neutralize the intrinsic ATPase activity of the protein. In preclinical tumor models, HSP90 inhibitors have been shown to deplete oncoproteins and inhibit tumor growth (15). The most advanced class of HSP90 inhibitors, including tanespimycin and other 17-AAG derivatives, are now in phase II/phase III clinical trials for solid and hematologic malignancies. A combination of tanespimycin and trastuzumab has shown encouraging results in a phase II trial for trastuzumab refractory breast cancer (16). However, significant clinical limitations of these 17-AAG derivatives have been reported, including poor solubility, potential liver toxicity, substrate for the P-glycoprotein multidrug resistance efflux pump, quinine reductase NQO1 dependence, and limited oral bioavailability (17–19).

To overcome the limitations of the 17-AAG class of HSP90 inhibitors, several synthetic HSP90 inhibitors have recently been discovered (20–22) and are now being tested in phase I/phase II clinical trials. Importantly, these synthetic HSP90 inhibitors, including purine (BIIB-021), isoxazole (VER-52296, NVP-AUY922), and indazole (SNX-5422) classes exhibit more favorable pharmacologic properties than the 17-AAG class inhibitors (20–22). Here, we describe CUDC-305, a leading HSP90 inhibitor of the imidazopyridine class. In addition to potent antitumor efficacy against a broad range of cancers in preclinical tumor models, we report that CUDC-305 exhibits enhanced pharmacologic features in several areas, including high oral bioavailability, selectivity, blood-brain barrier penetration, and extended tumor retention.

### Materials and Methods

**Reagents and chemicals.** CUDC-305 and other reference HSP90 inhibitors were synthesized in house. For in vitro assays, compounds were dissolved in DMSO as stock and stored at −20°C. For in vivo studies, CUDC-305 was formulated in 30% Captisol (Cydex Pharmaceuticals, Inc.) with 2 molar equivalents of HCl. Paclitaxel (Taxol, 6 mg/mL) was purchased from Mayne Pharma, Inc. Camptothecin-11 (20 mg/mL) was purchased from Pfizer, Inc.

All other reagents including culture medium, unless otherwise stated, were purchased from Invitrogen.

**Assay for HSP90 binding.** COOH terminal His-tagged human HSP90α and HSP90β proteins were expressed in Escherichia coli. Fluorescence polarization competition binding assays were done with purified HSP90α or HSP90β and FITC-labeled geldanamycin (InvivoGen) in the presence of different concentrations of test articles. The final action contained 10 and 50 nmol/L of labeled geldanamycin and purified HSP90 protein, respectively. The assay buffer contained 20 mmol/L HEPES (pH 7.3), 50 mmol/L KCl, 1 mmol/L DTT, 50 mmol/L MgCl2, 20 mmol/L Na2MoO4, and 0.01% NP40 with 0.1 mg/mL bovine γ-globulin. Polarization degree (mP) values were determined using a Synergy II plate reader (BioTek Instruments, Inc.) with background subtraction after 24 h of incubation at 4°C.

For binding assay with HSP90 complex from cancer, cancer cell lines were cultured in flasks. Total protein was extracted with radiolabeled precipitation assay buffer (Sigma-Aldrich Corp.) following manufacturer’s instructions. Fluorescence polarization competition binding assay was done as described above. Final protein concentration was adjusted to achieve the same FITC-geldanamycin binding level as with purified HSP90α or HSP90β without test articles.

**Cell growth and viability assay.** Human cancer cell lines were purchased from American Type Culture Collection and plated at 5,000 to 10,000 per well in 96-well plates with culture medium, as suggested by the provider. The cells were then incubated with compounds at various concentrations for 120 h. Growth inhibition was assessed by ATP content assay using the Perkin-Elmer ATPLite kit. Briefly, a 25-μL cell lysis solution was added to the 50-μL phenol red–free culture medium per well to lyse cells and stabilize ATP. Then 25-μL substrate solutions were added to the wells, and subsequently, luminescence was measured with a TopCount liquid scintillation analyzer (Perkin-Elmer). Values were expressed as a percentage relative to those obtained in untreated controls. IC50 values were calculated using PRISM software (GraphPad Software) with sigmoidal dose-response curve fitting.

**Western blot analysis of cells in culture.** Cancer cells grown in culture were treated with compounds at 1 μmol/L for 24 h and then harvested in 1× sample loading buffer (Sigma-Aldrich Corp.). Cell lysates were resolved on NuPAGE Novex 4-12% bis-Tris gels (Invitrogen) and then stained with antibodies against client proteins of the heat shock protein HSP90, respectively. The assay buffer contained 20 mmol/L Na2MoO4, and 0.01% NP40 with 0.1 mg/mL bovine γ-globulin. Polarization degree (mP) values were determined using a Synergy II plate reader (BioTek Instruments, Inc.) with background subtraction after 24 h of incubation at 4°C.
transferred to nitrocellulose membranes (Bio-Rad Laboratories). The blots were incubated first with a primary antibody overnight at 4°C. Antibodies to detect HSP70, AKT, phosphorylated AKT (p-AKT), c-MET, phosphorylated MET, EGFR, FLT3, phosphorylated FLT3, ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), HER2, phosphorylated HER2, phosphorylated HER3, estrogen receptor α, androgen receptor, CDK4, MEK1, survivin, activated CDC42-associated kinase, signal transducers and activators of transcription 5 (STAT5), cleaved poly(ADP-ribose) polymerase, and c-RAF (1:1,000-2,000) were obtained from Cell Signaling Technology. Antibody against cyclin D1 (1:2,000) was obtained from Santa Cruz Biotechnology. Glyceraldehyde-3-phosphate dehydrogenase (1:30,000; Abcam) or tubulin (1:5,000; Sigma-Aldrich) was used as an internal control for each assay. Membranes were then incubated with an IR-labeled secondary antibody (1:10,000): conjugated IR Dye-800 (Rockland Immunochemicals, Inc.) or conjugated Alexa Fluor-680 (Invitrogen). Membranes were imaged with the Odyssey IR Imaging System (Li-Cor Biotechnology).

**Animals and tumor implantation.** Female athymic nude (CD-1 nu/nu) or severe combined immunodeficient mice (6-8 wk of age) were obtained from Charles River Laboratories for in-house studies. For efficacy studies conducted in Crown Biosciences, Inc., BALB/c nu/nu mice were used. Animals were housed in ventilated microisolator cages in the animal facilities conditioned at a temperature of 23 ± 1°C, humidity of 50% to 70%, and a 12-h light/12-h dark cycle. The mice were provided with sterile laboratory rodent diet and water ad libitum. The animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of Curis and Crown Biosciences, Inc., respectively.

Before tumor implantation, various cancer cell lines of human origin were cultured in the medium suggested by the provider. When cultured cells reached ~70% to 90% confluence. They were harvested by treatment with trypsin-EDTA (0.25% trypsin, 1 mmol/L EDTA). The cell pellet was suspended in HBSS for implantation after medium was removed.

For s.c. tumor implantation, various numbers (3-20 × 10⁶) of cancer cells were injected into the right hind flank region of each mouse. For orthotopic implantation of breast cancer, a small incision (5 mm) was made in the skin over the lateral thorax to expose the mammary fat pad. MDA-MB-468 cancer cells (20 × 10⁶) suspended in 100 μL HBSS were injected into the mammary fat pad. Tumor size was measured with an electronic caliper. The following formula was used to calculate the tumor volume (23):

\[
\text{Tumor volume} = \frac{\text{length} \times \text{width}^2}{2}\.
\]

For intracranial implantation into nude mice, a 2- to 3-mm incision was made in the skin along the cranial midline. The injection needle was inserted 2.0 mm to the right and 0.5 mm anterior of the bregma. U87MG tumor cells (6 × 10⁵) were then injected to a depth of 3.5 mm in the right frontal lobe of brain.

**Pharmacokinetic studies.** Tumor-bearing nude mice were used for pharmacokinetic studies. CUDC-305 formulated in 30% Captisol was dissolved in saline and was administered intravenously (i.v.) at a dose of 10 mg/kg at various time points.
as a clear solution (20 mg/mL) was injected i.v. through tail vein (10 mg/kg) or orally via gavage (30 or 160 mg/kg) to each animal based on its body weight. At various time points after compound administration, three mice per time point were euthanized with CO₂, and blood and tissues were collected. Blood was collected into tubes containing sodium heparin. The plasma was separated via centrifugation. Plasma and tissues were stored at –80°C for later analysis. Analyte in storage condition was confirmed to be stable.

To prepare plasma sample for liquid chromatographic mass spectrometric (LC-MS/MS) analysis, 50 μL plasma added to 5 μL internal standard were mixed with 150 μL acetonitrile. The samples were vortexed, and then centrifuged for 5 min at 1,000 g. The supernatant was collected for LC-MS/MS analysis.

To prepare tissue samples, including tumors for LC-MS/MS analysis, tissues were homogenized in 150 μL water with 5 μL internal standard in acetonitrile. The homogenates were extracted thrice with 1.2 mL ethyl acetate. After evaporation, the residual was reconstituted in 0.1 mL acetonitrile for LC-MS/MS analysis.

A PE Sciex API-3000 LC-MS/MS system (Applied Biosystems, Inc.) was used to analyze compound concentrations in plasma and various tissues. Five microliters of each sample were injected; the flow rate was 200 μL/min. The limit of detection of analyte was 1 ng/mL in plasma and 2 ng/g in tissues. The assay was linear in the concentration range of 1 to 2,000 ng/mL in plasma and 4 to 4,000 ng/g in tissues. The recovery rate over above concentration range was >85.6% in plasma and >81.2% in tissues. The assay was linear in the concentration range of 200 μg/mL for LC-MS/MS analysis.

For immunohistochemical analysis, tumor tissues were collected, placed in dry ice until transferred to –80°C. Protein was extracted using Tissue lyser (Qiagen) and T-PER tissue protein extraction reagents plus Halt protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) following manufacturers' instructions. Protein (30 μg) was routinely used for Western blot analysis as described above.

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**Pharmacodynamic studies.** A single-dose pharmacodynamic (PD) study was routinely conducted to evaluate the biological effects of the compound at molecular levels in established tumor model before efficacy study was initiated. For single-dose PD study, three mice per time point were sacrificed and tumors were collected for Western blot analysis after a single oral dosing of CUDC-305. In the case of efficacy study in U87MG s.c. tumor model, tumors were also collected at the end of the efficacy study for Western blot and immunohistochemical analyses to assess the correlation between PD and efficacy results.

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CUDC-305 inhibits cancer cell proliferation in vitro. To further confirm its anticancer activity, we tested the growth inhibitory effects of CUDC-305 against a total of 40 human cancer cell lines, including 34 solid and 6 hematologic tumor–derived lines (Table 1). CUDC-305 inhibited the proliferation of these cancer cell lines with an IC₅₀ ranging from 40 to 900 nmol/L (mean IC₅₀, 220 nmol/L).

Interestingly, we noticed that cancer cell lines with HER2 overexpression and/or amplification, such as BT-474 (breast cancer; IC₅₀, 40 nmol/L), were quite sensitive to CUDC-305, as was an AML cell line, MV4-11, carrying a FLT3 internal tandem duplication mutation (IC₅₀, 100 nmol/L). These observations are consistent with the notion that CUDC-305 can down-regulate key mediators of receptor tyrosine kinase signaling and provide further support for HSP90 inhibition as an important therapeutic approach to receptor tyrosine kinase–dependent tumors.

Notably, among the NSCLC cell lines we tested, CUDC-305 was able to effectively inhibit the proliferation of cells that are resistant to conventional EGFR inhibitors, including H1975 (EGFR secondary mutation, T790M), Calu-6 (K-ras mutation), H1993 (c-MET amplification), and H460 (K-ras and PI3K mutations). These results strongly suggest that HSP90 inhibition is an effective therapeutic strategy to overcome resistance to traditional receptor tyrosine kinase inhibitors.

Estrogen receptor, progesterone receptor, and HER2 triple-negative breast cancer tumors have an unfavorable prognosis, and chemotherapy is currently the sole option for treatment. Notably, CUDC-305 inhibited the proliferation of MDA-MB-468, a triple-negative breast cancer cell line, with a low IC₅₀ of 1124.4 nmol/L. This result suggests that, due to its effects on many oncogenic proteins, our HSP90 inhibitor may bring potential therapeutic benefits to important areas with unmet medical needs.

CUDC-305 exhibits favorable pharmacokinetic profiles in tumor-bearing nude mice. The oral bioavailability in mice was determined by calculating percentage of plasma area under concentration–time curve (AUC) after oral administration versus plasma AUC achieved after i.v. administration of compound. The AUC values after oral (30 mg/kg) and i.v. (10 mg/kg) administration of CUDC-305 were 6.2 and 2.2 μmol/L h, respectively. Hence, oral bioavailability (F) of CUDC-305 in mice is 96.0%. In addition, CUDC-305 tends to be retained in tumor longer than in normal tissues after oral administration. As shown in Fig. 2A, CUDC-305 dosed orally at 30 mg/kg exhibited a maximum concentration (Cmax) of 1.3 μmol/L and a half-life of 7.8 hours in plasma, yet it reached a much higher concentration (Cmax, 4.7 μmol/L) with a much longer half-life (T½, 20.4 hours) in tumors. The AUC values for tumor and plasma were 114.9 and 6.2 μmol/L hours, respectively, with a ratio of 18.5:1, indicating a very high and durable distribution of CUDC-305 in tumor tissue after oral administration. Furthermore, CUDC-305 displayed a much longer half-life in tumor (20.4 hours) than in normal tissues (2.7 hours). For example, CUDC-305 was cleared quickly from liver tissue with a half-life of only 2.6 hours, although it reached a very high concentration after oral administration (Fig. 2A). Of particular note, CUDC-305 exhibited a therapeutic exposure in brain tissue (Cmax, 3.1 μmol/L; AUC, 23.7 μmol/L h) and in the brain/AUC plasma, 3.8:1, with a half-life of 4.0 hours (Fig. 2A). This result indicates that CUDC-305 can penetrate the blood–brain barrier, which may be therapeutically beneficial in the clinic.

When CUDC-305 was dosed orally at its maximum tolerated dose (160 mg/kg in nude mice), a roughly dose–proportional exposure in plasma was achieved (AUC, 42.6 μmol/L h). However, higher than the dose-proportional exposure (AUC, 1124.4 μmol/L h) was observed in tumor tissues. Even at 48 hours, a high-level (9.4 μmol/L) CUDC-305 could be detected in tumor tissue, whereas compound concentrations in plasma and normal organs were negligible (data not shown).

CUDC-305 induces degradation of HSP90 client proteins, inhibits tumor growth, and prolongs survival in various animal models of U87MG glioblastoma. Given the therapeutically relevant exposure of CUDC-305 in brain tissue, we decided to test the efficacy of the compound in the U87MG glioblastoma tumor models. A single-dose PD study was first conducted to evaluate
the duration of its biological effects in tumor xenografts implanted s.c. After a single oral dosing of CUDC-305 at 160 mg/kg, U87MG tumors were collected at various time points over a 48-hour period and subjected to Western blot analysis. As shown in Fig. 2B, HSP70 was induced from 3 to 48 hours after compound administration, correlating with our earlier findings of extended tumor exposure (9.4 μmol/L at 48 hours, 20-fold above IC₅₀ in U87MG cells) after a single oral dosing of CUDC-305 at 160 mg/kg. Therefore, an every-other-day (q2d) dosing regimen was adopted for most efficacy studies. HSP90 client proteins, including p-AKT, and c-RAF were shown to be down-regulated, along with the induction of apoptosis as measured by poly(ADP-ribose) polymerase cleavage (Fig. 2B). Note that although minimal or no inhibition was observed for c-MET or total AKT in this single-dose PD study, inhibition of these HSP90 client proteins was achieved after multiple oral doses, as shown in our subsequent efficacy-PD study in the same tumor model.

Next, we evaluated the antitumor efficacy of the compound in the same U87MG s.c. tumor model. CUDC-305 was delivered orally at three dosage levels (40, 80, or 160 mg/kg, orally, q2d) when tumors reached an average volume of 122 mm³. During treatment period, tumor volume and body weight were measured twice weekly to determine antitumor activity.
and toxicity of compound. In addition, tumors from each group were collected at the end of the efficacy study for both Western blot and immunohistochemical analyses.

As shown in Fig. 2C, CUDC-305 displayed dose-dependent inhibition of tumor growth in the U87MG s.c. tumor model. The T/C values were 51.1% (*P* < 0.001) for the 40 mg/kg group and 19.6% (*P* < 0.001) for the 80 mg/kg group. Tumor regression was observed in the group treated at 160 mg/kg (regression of 37.9%, *P* < 0.001). No loss of body weight or other side effects were observed in any of the treatment groups.

Four tumors from each treatment group were collected at the end of the efficacy study and subjected to Western blot analysis. HSP70 was strongly induced in a dose-dependent manner (Fig. 2D), in agreement with the efficacy results (Fig. 2C). HSP90 client proteins, including c-MET, p-AKT, AKT, c-RAF, and cyclin D1, were potently inhibited in the high-dose group (160 mg/kg). It was noticeable that, in the 2 low-dose groups (40 and 80 mg/kg), minimal inhibition of these client proteins was observed (Fig. 2D). Because all tumors were collected 12 hours after the last dosing, it is possible that lower doses of compounds have resulted in a shorter period of inhibition of these proteins, which could not be captured at a later time point.

Three tumors from each treatment group were also collected at the end of the efficacy study and subjected to immunohistochemical analysis. Cell proliferation, as measured by Ki-67 staining, was inhibited in a dose-dependent manner (Fig. 3A). This result is in accordance with the suppression of cell proliferation and survival pathways by CUDC-305 observed in Western blot analysis (Fig. 2D). Microvessel density, as measured by CD34 staining, showed a dose-dependent reduction (Fig. 3B), suggesting that CUDC-305 has antiangiogenic effects.

To further assess the ability of CUDC-305 to cross blood-brain barrier and inhibit orthotopic tumor growth in brain, U87MG glioblastoma cells were implanted intracranially into nude mice. Starting 5 days after tumor implantation, mice were treated with CUDC-305 (120 mg/kg, orally, q2d) or vehicle control. As shown in Fig. 3C, treatment with CUDC-305 significantly prolonged survival of intracranial tumor-bearing mice (*P* < 0.01, Mantel-Cox log-rank test).

CUDC-305 causes degradation of oncoproteins and induces tumor regression in the animal models of MV4-11 AML. Our earlier *in vitro* studies showed that CUDC-305 can potently inhibit mutant oncoprotein FLT3 and its downstream signaling molecule STAT5 (Fig. 1C), leading to potent antiproliferative activity.
in MV4-11 AML cell line with a low IC_{50} (100 nmol/L; Table 1). Based on these results, we decided to further evaluate the antitumor efficacy of CUDC-305 in MV4-11 tumor xenografts implanted s.c. into severe combined immunodeficient mice. A single-dose PD study was first conducted to evaluate the biological effects of the compound in vivo. As shown in Fig. 4A, a single oral dosing (160 mg/kg) of CUDC-305 was able to down-regulate mutant FLT3 and downstream signaling molecules p-AKT and STAT5 with concurrent induction of HSP70. In the same tumor xenograft model, a 3-week treatment with CUDC-305 (160 mg/kg, orally, q2d) induced complete tumor regression (P < 0.001) in mice with small-sized pretreatment tumors (146 mm³), whereas tumors in the control group grew exponentially, reaching 2,200 mm³ by the end of study (Fig. 4B). However, CUDC-305 delivered on a different dosing schedule (90 mg/kg, 2-1-2, on-off-on) was much less efficacious. Furthermore, CUDC-305 treatment at 160 and 200 mg/kg (orally, q2d) induced complete tumor regression in mice with large-sized pretreatment tumors (380 and 835 mm³, respectively, P < 0.001; Fig. 4C and D). Thus, CUDC-305 is able to induce degradation of both mutant transforming oncoprotein FLT3 and downstream signaling mediators of the FLT3 signaling cascade in the MV4-11 AML model, leading to complete tumor regression.

CUDC-305 induces degradation of multiple signaling proteins and inhibits growth of a NSCLC tumor xenograft resistant to EGFR inhibitor therapy. In vitro antiproliferation studies revealed that H1975 cell line, a type of NSCLC that carries a secondary EGFR mutation and is resistant to EGFR inhibitors, is sensitive to CUDC-305 treatment (IC_{50}, 140 nmol/L; Table 1). To further evaluate the activity of the compound in vivo, we did a single-dose PD study (80 mg/kg, orally) in established H1975 s.c. tumors. As shown in Fig. 5A, we observed potent inhibition of multiple HSP90 client proteins, including mutant EGFR and downstream key regulators of the cell proliferation (c-RAF, p-MEK, phosphorylated ERK), survival (AKT, p-AKT),
and cell cycle progression (CDK4) pathways, as well as increase of HSP70 and induction of apoptosis as measured by poly (ADP-ribose) polymerase cleavage at 8 hours after CUDC-305 treatment (Fig. 5A).

An efficacy study in the same H1975 s.c. tumor model was conducted next. As shown in Fig. 5B, CUDC-305 treatment significantly inhibited H1975 s.c. tumor growth, with a T/C value of 15.4% compared with the control group ($P < 0.001$).

CUDC-305 alone or in combination with standard-of-care agents inhibits tumor growth in other cancer types. In addition to the cancer types described above, CUDC-305 also displayed antitumor efficacy in animal models of various other tumor types. Of particular interest is its shown efficacy in the N87, a HER2 overexpressing gastric tumor model. At a daily dosing of 80 mg/kg, CUDC-305 caused depletion of HER2 protein in tumors analyzed by Western blot and induced tumor regression by 11.3% ($P < 0.001$; data not shown) in the N87 s.c. tumor model. In the MDA-MB-468 breast cancer orthotopic model, potent antitumor activity was also observed. This model belongs to the triple-negative breast cancer subtype, which accounts for 15% of all breast cancers with poor prognoses. When delivered as a single agent at 120 mg/kg for 2 weeks,
CUDC-305 induced 3.4% tumor regression ($P < 0.001$; Fig. 5C). In combination with paclitaxel, a standard-of-care agent in breast cancer, CUDC-305 significantly enhanced the antitumor activity of paclitaxel (tumor regression of 36.6%, $P < 0.001$; Fig. 5C). A combination study was also conducted in animal model of Colo205 colorectal cancer, revealing that CUDC-305 significantly enhanced the antitumor activity of camptothecin-11, standard-of-care agent for colorectal cancer therapy ($P < 0.05$; Fig. 5D).

**Discussion**

Our results show that CUDC-305 is a potent HSP90 inhibitor with optimal pharmacologic properties, including high oral bioavailability, sustained tumor retention, and a potentially favorable therapeutic window. Moreover, its HSP90 binding activity is similar to those of other leading synthetic HSP90 inhibitors.

Pharmacokinetic studies in mice show that CUDC-305 has high oral bioavailability (96.0%), making chronic oral dosing possible. This may represent a differentiating factor compared with other synthetic HSP90 inhibitors, including compounds in the isoxazole class, which have limited bioavailability (21).

Notably, CUDC-305 exhibits a sustained exposure in tumor tissues (Fig. 2A). This favorable pharmacologic property is in contrast to the isoxazole class compounds, which have a reported tumor/plasma AUC ratio of 4.0 after i.v. administration (21), and the purine class compounds, which have shorter half-lives (27).

The selective retention of CUDC-305 in tumor tissues can be attributed to a few factors. First, HSP90 is likely overexpressed in cancer cells to support cancer cell transformation. Increased HSP90 expression in cancer cells has also been described as a stress response to gene mutations and metabolic dysregulation, as well as to hostile environmental conditions including hypoxia, nutrient deprivation, and acidosis (28). Second, HSP90 protein isolated from tumor tissues seems to have an enhanced affinity for HSP90 inhibitors. Indeed, it has been reported that tumor-derived HSP90 has a 100-fold higher binding affinity for 17-AAG than does HSP90 from normal cells (29). This may be a result of conformational differences in HSP90 in cancer versus normal cells. In cancer cells, HSP90 exists as an activated super-chaperone complex that is hypersensitive to HSP90 inhibition, whereas in normal cells, HSP90 is predominantly uncomplexed and less sensitive to HSP90 inhibition (30, 31). Finally, in contrast to other HSP90 inhibitors in clinical development, CUDC-305 has a relatively high lipophilicity with a $\text{Log}P$ of $\sim 4.0$. These balanced chemical properties may facilitate its penetration and retention in tumors. Therefore, the abundance of HSP90 in cancer cells, its high affinity for HSP90 inhibitors, and the high lipophilicity of CUDC-305 all contribute to the selective accumulation and retention of CUDC-305 in tumor tissues. The preferential retention of CUDC-305 in tumors may also explain its selective effects on tumor cells (mean $I_{C50} = 220 \text{ nmol/L}$) as opposed to normal human primary cells (mean $I_{C50} \sim 500 \text{ nmol/L}$) as observed in our in-house antiproliferation studies (data not shown). Isoxazole class compounds, in contrast, were reported to be active against human cancer cells, as well as nontumorigenic human prostate and breast epithelial cells (21). The selective pharmacologic action of CUDC-305 is essential to achieve enhanced efficacy and reduced toxicity in vivo.

The sustained exposure of CUDC-305 in tumor cells results in persistent biological effects, as shown in the single-dose PD study in U87MG s.c. tumors (Fig. 2B). These biological effects have been maintained for as long as 48 hours after a single dosing of CUDC-305, supporting the use of an q2d dosing regimen in efficacy studies. A 2-week pharmacokinetic simulation based on single oral dosing of CUDC-305 at 30 and 160 mg/kg shows no accumulation in normal tissues but a sustained exposure and accumulation in tumor tissues (data not shown), providing further evidence for an intermittent dosing schedule. Although nonlinear exposure was observed in tumor tissues for the two dosage levels used, in contrast to plasma exposure, which seems roughly dose-proportional, additional dose-linearity studies with more dosage levels of compound will be pursued in future.

Cell-based assays reveal that CUDC-305 potently inhibits the growth of a broad range of cancer cell lines derived from both solid and hematologic tumors (Table 1). Moreover, cancer cells with oncogenic mutations and/or amplifications (HER2, FLT, B-RAF, c-MET, etc.) seem to be relatively sensitive to CUDC-305, suggesting that these oncogenes are highly dependent on HSP90 chaperone function and that CUDC-305 can exploit the “oncogenic addiction” of some cancer cells. The sensitivity of these cancer cell lines to CUDC-305 is consistent with the potent inhibition of oncogenic proteins of the compound and downstream signaling molecules as shown in the in vitro mechanism of action studies (Fig. 1B-D).

Due to the inability of many anticancer drugs to effectively penetrate the blood-brain barrier, both primary and metastatic brain cancers represent a crucial unmet medical need. Among synthetic HSP90 inhibitors, CUDC-305 is unique in its high distribution in brain tissue. SNX-5422, for example, reportedly does not reach the brain (22). We first showed in an s.c. tumor model, that CUDC-305 can inhibit U87MG glioblastoma tumor growth in a dose-dependent manner (Fig. 2C). Then we showed that CUDC-305 is able to inhibit intracranial glioblastoma growth and prolong animal survival in an intracranial tumor model (Fig. 3C), supporting its pharmacologic activity via blood-brain barrier penetration. PD studies in U87MG s.c. tumors collected at the end of the efficacy study showed a dose-dependent inhibition of multiple HSP90 client proteins by CUDC-305 (Fig. 2D), correlating with efficacy results. These down-regulated HSP90 client proteins include ones involved in cell proliferation, as well as cell survival. The latter result is particularly noteworthy because the AKT pathway is constitutively activated in U87MG cells due to PTEN gene deletions. Roughly one third of glioblastoma patients carry such PTEN deletions, a mutation that confers primary resistance to EGFR inhibitors (32). Because EGFR and AKT are both HSP90 client proteins, HSP90 inhibitors may be a more effective therapeutic strategy in glioblastoma than EGFR inhibitors. Considering the poor prognosis of glioblastoma and other brain cancers, as well as the dearth of effective therapeutic agents, the potential of CUDC-305 as an agent for primary and metastatic brain cancers is significant. Although blood-brain barrier penetration may raise concern over central nervous system toxicity, CUDC-305 with a unique chemical structure displays no central nervous...
system–related toxicity in our GLP toxicity studies. Nonetheless, central nervous system toxicity will be further evaluated and closely monitored in future studies.

The activity of CUDC-305 in cancer cell lines with gene mutations was further confirmed in the MV4-11 AML tumor xenograft model. CUDC-305 is able to induce complete tumor regression in three separate studies with various pretreatment tumor sizes (Fig. 4B-D). This antitumor effect is associated with a robust inhibition of the transforming mutant oncoprotein FLT3, as well as downstream signaling molecule STAT5, as shown in both in vitro (Fig. 1C) and in vivo (Fig. 4A). These results may suggest that MV4-11 AML cells have “high-jacked” and, thus, become addicted to the hyperactivated FLT3 signaling pathway for survival, which in turn is highly dependent on HSP90 chaperone function for maintenance of the deregulated pathway components. Therefore, complete blockade of FLT3 signaling through simultaneous inhibition of the two key regulators (both FLT3 and STAT5) of the FLT3 signaling pathway by CUDC-305 treatment can abrogate malignant phenotype, leading to fast tumor regression. In humans, the FLT3 internal tandem duplication mutation causes constitutive, ligand-independent activation of the FLT3 tyrosine kinase, leading to leukemogenesis (33). Moreover, in roughly 20% to 25% of AML cases, this mutation may also contribute to an aggressive malignant phenotype and resistance to traditional therapies (34). Considering the prevalence and lethality of AML and the lack of advances in treatment in the past 35 years, the potent antitumor activity of CUDC-305 in AML as single agent is exciting. An important direction for future study will be to test CUDC-305 activity in AML cell lines with wild-type FLT3, an isoform that is overexpressed in 80% of AML cases. In addition to FLT3 mutation and/or overexpression, it has been recognized that several types of oncogenes underlie AML development. Therefore, it is conceivable that simultaneous inhibition of multiple pathways in the same leukemic cell by CUDC-305 will be a more broadly applicable therapeutic approach than the selective FLT3 inhibitors currently under development (35–38).

Perhaps, most importantly, CUDC-305 displays potent antitumor activity against H1975 NSCLC cancer cell line harboring EGRF secondary mutation responsible for acquired erlotinib resistance (Table 1; Fig. 5B). This mutation, T790M, enhances EGFR catalytic activity and confers resistance to reversible tyrosine kinase inhibitors (39). Our PD study in H1975 tumor shows that CUDC-305 is able to induce simultaneous degradation of multiple HSP90 client proteins in vivo, including mutant EGFR and key regulators of the RAF/MEK/ERK and PI3K/AKT signaling cascades downstream of EGFR (Fig. 5A). In addition, CUDC-305 exhibits activity both in vitro (Table 1) and in vivo (data not shown) against NSCLC cell lines that carry either K-ras mutations (Calu-6, H460) or c-MET amplification (H1993), genetic abnormalities thought to confer primary resistance to erlotinib. K-ras mutations constitutively activate downstream RAF/MEK/ERK signaling independent of upstream EGFR inhibition, conferring primary resistance to EGFR inhibitors (40). The activity of the compound in K-ras mutant NSCLC cell lines may be due to its ability to inhibit multiple signaling pathways, such as AKT and MAPK/ERK downstream of RAS. In light of these results, it seems reasonable to expect that targeting HSP90 may be a more efficacious approach in overcoming both primary and acquired resistance in NSCLC than the irreversible EGFR inhibitors, which caused incomplete blockade of the AKT signaling in T790M-driven tumors (41), offering the potential for significant therapeutic benefits.

MDA-MB-468, a breast cancer cell line, is resistant to the HSP90 inhibitor 17-AAG due to a mutation in the NQO1 gene, which encodes DT-Diaphorase, an enzyme that metabolizes 17-AAG to a more potent form, 17-AAGH2 (42). However, CUDC-305 showed potent antitumor activity in this cell line in vitro (Table 1) as well as in vivo (Fig. 5C), suggesting that CUDC-305 inhibits tumor growth independently of NQO1 status and may have a broader spectrum of activity than 17-AAG. An important area for future study is the mechanism of CUDC-305 sensitivity in the triple-negative breast cancer subtype, a particularly devastating form for which effective therapeutics are sorely lacking. In MDA-MD-468 breast cancer and Colo205 colorectal cancer models, we show that CUDC-305 can enhance the antitumor activity of standard-of-care agents (Fig. 5C and D), an effect that is likely due to AKT suppression as a result of HSP90 inhibition (43).

In summary, CUDC-305 is a novel, synthetic, small-molecule inhibitor of HSP90 with potent and sustainable biological effects in cancer as shown in vitro and in vivo. Its antitumor effect likely stems from its ability to simultaneously block multiple signaling pathways, effectively interrupting the interactions among signaling networks in cancer cells. In addition, it displays other optimal pharmacologic properties, including high oral bioavailability, sustained tumor retention, blood-brain barrier penetration, and a potentially more favorable therapeutic window. Furthermore, in vitro safety and toxicology studies suggest a favorable safety profile. Based on these results, CUDC-305 has been nominated as a drug candidate for further development.

Disclosure of Potential Conflicts of Interest

All authors are employees and shareholders of Curis, Inc.

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