Targeting heat shock protein 90 with CUDC-305 overcomes erlotinib resistance in non–small cell lung cancer

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Abstract

CUDC-305 is a heat shock protein 90 (HSP90) inhibitor of the novel imidazopyridine class. Here, we report its activities in non–small cell lung cancer (NSCLC) cell lines with gene deregulations conferring primary or secondary resistance to epidermal growth factor receptor (EGFR) inhibitors. We show that CUDC-305 binds strongly to HSP90 extracted from erlotinib-resistant NSCLC cells (IC50 70 nmol/L). This result correlates well with the potent anti-proliferative activity in erlotinib-resistant NSCLC cell lines (IC50 120–700 nmol/L) reported previously. Furthermore, it exhibits durable inhibition of multiple oncoproteins and induction of apoptosis in erlotinib-resistant NSCLC cells. CUDC-305 potently inhibits tumor growth in subcutaneous xenograft models of H1975 and A549, which harbor EGFR T790M mutation or K-ras mutations conferring acquired and primary erlotinib resistance, respectively. In addition, CUDC-305 significantly prolongs animal survival in orthotopic lung tumor models of H1975 and A549, which may be partially attributed to its preferential exposure in lung tissue. Furthermore, CUDC-305 is able to extend animal survival in a brain metastatic model of H1975, further confirming its ability to cross the blood-brain barrier. Correlating with its effects in various tumor models, CUDC-305 induces degradation of receptor tyrosine kinases and downstream signaling molecules of the PI3K/AKT and RAF/MEK/ERK pathways simultaneously, with concurrent induction of apoptosis in vivo. In a combination study, CUDC-305 enhanced the antitumor activity of a standard-of-care agent in the H1975 tumor model. These results suggest that CUDC-305 holds promise for the treatment of NSCLC with primary or acquired resistance to EGFR inhibitor therapy.

Introduction

Lung cancer is the leading cause of cancer mortality in the United States, accounting for one third of all cancer deaths (1). Chemotherapy alone or in combination with other agents can modestly improve these outcomes but at the cost of significant toxicity to the patient. In recent years, molecularly targeted therapies, such as epidermal growth factor receptor (EGFR) inhibitors, have gained tremendous attention for their potential to improve patient survival and reduce toxic side effects. Yet, early clinical trials of these inhibitors, such as gefitinib and erlotinib, were modestly encouraging, with a response in only ∼10% of patients who carry genetic mutations of EGFR (2–7). In addition, resistance almost invariably develops in these non–small cell lung cancer (NSCLC) patients although they respond to these receptor tyrosine kinase (RTK) inhibitors initially.

Of these instances of so-called “acquired” resistance, it is estimated that ∼50% are due to the emergence of an additional EGFR mutation in exon 20 (EGFR T790M), the “gatekeeper” residue within the kinase domain (8–10). Structural analysis suggests that the T790M mutation sterically hinders the binding of erlotinib to the EGFR kinase domain by introducing a bulky methionine residue, thereby conferring erlotinib resistance (8, 9). There is also evidence to suggest that T790M mutation causes drug resistance by increasing the affinity of EGFR for ATP (11). To overcome such EGFR T790M-mediated resistance, several irreversible EGFR inhibitors able to form covalent bonds with Cys-797 at the edge of the ATP binding site are actively being tested in clinical trials (12–16). However, only modest efficacy has been reported, believed to be in part due to persistent PI3K/AKT/mTOR signaling following treatment (17). Importantly, combined treatment with a mTOR inhibitor, rapamycin, can markedly enhance the cytotoxicity of irreversible EGFR inhibitors in EGFR T790M transgenic animal models (18). These results indicate that single-agent, irreversible EGFR inhibition may not overcome EGFR T790M-mediated resistance and that a combinatorial approach that blocks both EGFR and downstream signaling is likely required.

Another mechanism underlying acquired RTK inhibitor resistance is the activation of alternative RTKs (MET, IGF, PDGFR, etc.) that are able to bypass EGFR signaling. For example, MET amplification occurs in ∼20% of NSCLC patients with acquired resistance to EGFR inhibitors (19, 20). MET activation strongly increases cell proliferation, survival, and angiogenesis, thereby bypassing EGFR signals. It has been reported that a MET inhibitor is more effective than...
either reversible or irreversible EGFR inhibitors in inhibiting NSCLC cells harboring both $EGFR^{790M}$ mutation and $MET$ amplification (20). Hence, simultaneously blocking both EGFR and parallel signaling pathways is presumably a preferred strategy to overcome the acquired resistance mediated by alternative RTK activation.

Although much is known about the molecular underpinnings of acquired resistance to EGFR inhibitors, the mechanisms of primary resistance, which occur in the majority of NSCLC cases, are not fully understood. Constitutive activation of signaling pathways downstream of EGFR due to genetic or epigenetic alterations may play a role. For example, $K$-ras mutations at codon 12/13 account for roughly one third of all primary resistance in NSCLC and are mutually exclusive with EGFR activating mutations (21–23). EGFR inhibitors are ineffective because mutant $K$-ras constitutively activates downstream RAF/MAPK, bypassing EGFR inhibition. As a result, $K$-ras mutations are considered negative predictors for EGFR inhibitor therapy in NSCLC (21, 22). Other mechanisms of primary resistance to EGFR inhibitors have also been suggested. For example, hyperactivation of the PI3K/AKT pathway due to gene deletion or promoter methylation of $PTEN$ is a major mechanism of erlotinib resistance (24). In addition, preexisting redundant signaling through RTKs IGF, PDGFR, HER3, c-MET, and HER2 maintains activation of critical cell survival pathways in the presence of EGFR inhibition (25). Therefore, to overcome primary resistance to EGFR inhibitors, an approach that blocks multiple signaling mediators of the same and/or parallel pathways is preferable.

Based on their ability to simultaneously inhibit multiple proteins that regulate cancer cell proliferation, survival, cell cycle progression, invasion, and metastasis (26, 27), heat shock protein 90 (HSP90) inhibitors are a promising alternative for overcoming primary and/or acquired resistance to EGFR inhibitors in NSCLC. As we have previously reported, CUDC-305 is a novel HSP90 inhibitor of the imidazo-pyridine class that displays unique pharmacologic properties, including high oral bioavailability (96.0% in mice), sustained tumor retention (half-life 20.4 hours in mice), and blood-brain barrier penetration (28). It shows high affinity for HSP90 (IC$_{50}$ $\sim$100 nmol/L) and HSP90 complex derived from cancer cells (IC$_{50}$ 48.8 nmol/L). In addition, it displays potent antitumor activity against a variety of tumor types in vitro (mean IC$_{50}$ 220 nmol/L) and in vivo, including glioblastoma, triple-negative breast cancer, acute myelogenous leukemia, and colorectal cancer. Correlating with its efficacy in these various tumor models, CUDC-305 robustly inhibits multiple signaling pathways and induces apoptosis in these cancer cells (28).

Another encouraging finding is that CUDC-305 was able to effectively inhibit the proliferation of NSCLC cells that become resistant to conventional EGFR inhibitors, including H1975 (EGFR secondary mutation, T790M), Calu-6, H460, A549, H2122 (K-ras mutation), and H1993 (c-MET amplification). Importantly, CUDC-305 inhibited proliferation of these erlotinib-resistant NSCLC cell lines with an IC$_{50}$ similar to that observed in H292 and A358, erlotinib-sensitive NSCL cell lines. In preliminary in vivo studies, CUDC-305 inhibited subcutaneous tumor growth and induced degradation of multiple HSP90 client proteins, including mutant EGFR and key regulators of the RAF/MEK/ERK and PI3K/AKT signaling cascades in H1975 tumor model. These results strongly suggest that HSP90 inhibition by CUDC-305 may be an effective therapeutic strategy to overcome resistance to traditional RTK inhibitors in NSCLC that presents huge unmet medical needs.

In the current studies, the binding affinity of CUDC-305 to HSP90 complex derived from erlotinib-resistant NSCLC cell lines was first tested. Various in vitro “mechanism of action” studies were carried out to evaluate compound activity in erlotinib-resistant NSCLC cell lines. Antitumor activity was then tested in subcutaneous tumor models of A549, which harbors K-ras mutation, a major mutation responsible for primary resistance to erlotinib in NSCLC. Antitumor activity was also tested in subcutaneous tumor models of H1975, which carries a secondary EGFR mutation responsible for acquired resistance to erlotinib in NSCLC. Due to preferential exposure of CUDC-305 in lung and therapeutic exposure in brain tissues, antitumor activity was also evaluated in orthotopic lung cancer model and brain metastatic tumor models established from these two NSCLC cell lines resistant to erlotinib. For each individual model, pharmacodynamic studies were carried out to evaluate compound biological events at the molecular level in established tumors.

**Materials and Methods**

**Reagents and Chemicals**

The HSP90 inhibitor CUDC-305 and EGFR inhibitors (lapatinib, erlotinib) were synthesized in house. Paclitaxel (6 mg/mL paclitaxel, 527 mg Cremophor EL, 49.7% alcohol, 2 mg citric acid) was purchased from Mayne Pharma, Inc. All other reagents, including the culture medium, unless otherwise stated, were purchased from Invitrogen.

**Assay for HSP90 Binding**

For binding assay with HSP90 complex from cancer, two erlotinib-resistant NSCLC cell lines, H1975 and H1993, were cultured in flasks. Total protein was extracted with radiionmunoprecipitation assay buffer (Sigma-Aldrich Corp.) following the manufacturer’s instructions. Fluorescence polarization binding assays were done using cancer cell extracts with competition from fluorescein isothiocyanate-labeled geldanamycin (InvivoGen), in the presence of varying concentrations of the compound as described before (28).

**Western Blot Analysis, Apoptosis, and Cell Proliferation Assays in Cells**

Various NSCLC cell lines of human origin were purchased from the American Type Culture Collection and plated at 5,000 to 10,000 per well in 96-well plates with the culture medium as suggested by the provider. Cancer cells in culture were then treated with CUDC-305 at 1 μmol/L for varying periods and harvested in a sample loading buffer (Sigma-Aldrich Corp.). Cell lysates were subjected to Western blot analysis as described (28). Glyceraldehyde 3-phosphate dehydrogenase (1/30,000, Abcam) was

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used as an internal control. All other primary antibodies were obtained from Cell Signaling Technology.

To evaluate apoptosis induction and antiproliferation of CUDC-305 in H1975 cells, the cells were first incubated with CUDC-305 for 7 h and then cultured in compound-free medium for additional 24, 48, and 96 h, for poly(ADP-ribose) polymerase (PARP), caspase-3/7, and antiproliferation assays, respectively. The level of cleaved PARP was detected using Cleaved PARP Activation Kit and quantified with Cellomic ArrayScan automated microscope (Thermo Scientific). Caspase-3/7 activity was evaluated using kits from Promega Corporation following the manufacturer's instructions. Antiproliferation was assessed by ATP content assay using the ATPlite kit (Perkin-Elmer) as described elsewhere (28).

Animals and Tumor Implantation

Female athymic nude (CD-1 nu/nu) mice at 5 to 8 wk of age were obtained from Charles River Laboratories. For survival studies conducted at Crown Biosciences, Inc., Balb/C nu/nu mice were used. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Curis or Crown Biosciences, Inc., respectively.

To establish subcutaneous tumors in nude mice, various numbers (3 × 10⁸ to 5 × 10⁹) of cultured cells, depending on the cell lines, were suspended in 100 to 200 μL Hank’s balanced salt solution (HBSS) and injected into the right hind flank region of each mouse. Tumor size was measured with an electronic caliper. The following formula was used to calculate the tumor volume (29):

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

To establish orthotopic lung tumors, nude mice were anesthetized by i.p. injection of pentobarbital sodium. A 1.0-cm incision was made in the skin of the left chest at the dorsal mid-axillary line just below the inferior border of the scapula. Subcutaneous fat and muscles were separated to expose the second intercostal space. On observing the left lung motion through the pleura, A549 (1 × 10⁶) or H1975 (2.0 × 10⁶) cells suspended in 10 μL HBSS/Matrigel (BD Biosciences) were then injected into the left lung parenchyma to a depth of 4 mm using a 27-gauge needle attached to a Hamilton syringe. After inoculation, the skin incision was closed with a suture.

To establish H1975 intracranial tumors mimicking brain metastases from lung cancer, a 2- to 3-mm incision was made in the skin along the cranial midline after mice were anesthetized with pentobarbital sodium. A 30-gauge needle attached to a Hamilton syringe was inserted 2.0 mm to the right and 0.5 mm anterior of the bregma, to a depth of 3.5 mm in the right frontal lobe of the brain. H1975 cells (5 × 10⁶) suspended in 2 μL HBSS were then slowly injected into the brain. The skin was closed with a suture after the needle was retrieved.

Pharmacokinetic Studies

Methods for conducting pharmacokinetic studies of CUDC-305 in mice have been described in detail previously (28). Briefly, three mice per time point were euthanized; various tissues were collected at different time points following oral administration of CUDC-305. Samples were prepared and analyzed for concentrations of CUDC-305 with PE Sciex API-3000 liquid chromatographic mass spectrometric (LC-MS/MS) system (Applied Biosystems, Inc.).

Pharmacodynamic Studies

Before initiation of efficacy study in each tumor model, a single-dose pharmacodynamic study was carried out to evaluate the compound’s biological effects in vivo at molecular levels. Briefly, mice were dosed orally with CUDC-305 when s.c. implanted tumors reached a volume of 100 to 200 mm³, or orthotopically implanted lung tumors were established about 4 wk after tumor implantation. Tumors were then collected at various time points following treatment. Protein was extracted from tumors using TissueLyser (Qiagen) and T-PER tissue protein extraction reagent (Thermo Fisher Scientific) following the manufacturers’ instructions. Thirty micrograms of protein were routinely used for Western blot analysis as described (28).

Efficacy Studies in Animal Models of Human Cancers

For efficacy studies in subcutaneous tumor models, treatment with compounds started when tumors reached ~80 to 150 mm³. During the course of efficacy studies, tumor sizes and body weights were measured twice weekly. The last tumor measurement was used to calculate the tumor volume change ratio (%ΔT/C = 100 × ΔT/ΔC), a standard metric developed by National Cancer Institute for evaluation of the compound’s antitumor activity in animal models (30).

For efficacy studies using survival as an end point, treatment with compounds started 4 d after orthotopic lung tumor implantation and 5 d after intracranial tumor implantation, respectively. Animals were sacrificed when moribund clinical signs appeared, following Institutional Animal Care and Use Committee protocols.

CUDC-305, formulated in 30% Captisol (Cydex Pharmaceuticals, Inc.), was delivered through oral gavage on an every-other-day (once every two days) dosing schedule at various doses up to 160 mg/kg—its maximum tolerated dose in nude mice. In survival studies in Balb/C nude mice, the highest dose was reduced to 120 mg/kg as a result of the compromised animal condition due to lung and brain tumor implantation surgeries. The dosing regimen of once every two days was chosen based on pharmacokinetic and pharmacodynamic results of CUDC-305 as reported before (28). The control group was dosed with vehicle (30% Captisol) using the same dosing paradigm. Lapatinib formulated in MCT (0.5% methylcellulose plus 0.1% Tween-80 in water) was delivered orally at 75 mg/kg twice daily (twice a day; ref. 31). Erlotinib formulated in MCT was delivered orally at 50 mg/kg daily, a schedule proven to be effective in treating erlotinib-sensitive NSCLC tumors and tolerated by this animal strain. Paclitaxel in Cremophor alcohol formulation was diluted in 0.9% normal saline and injected i.p. at 12.5 mg/kg twice weekly. For all compounds, animals were dosed based on the body weight of each individual animal.
NGCC-305 Displays Potent Affinity to HSP90 Derived from Erlotinib-Resistant NSCLC

The chemical structure and antitumor activity of NGCC-305 have been reported previously (28, 32). To determine whether the compound can interact with HSP90 derived from erlotinib-resistant NSCLC cell lines, we performed a fluorescence polarization assay with geldanamycin competition using cell extracts prepared from cultured H1975 and H1993 NSCLC cell lines, which become resistant to erlotinib due to EGFRT790M mutation and c-MET amplification, respectively. We observed that NGCC-305 strongly bind to cancer-derived HSP90 complex with an IC50 of 61.2 nmol/L in H1975 and 74.2 nmol/L in H1993, respectively (Fig. 1).

NGCC-305 Durably Induces Oncoprotein Degradation in NSCLC Cell Lines with Mutations That Could Confer Resistance to Erlotinib

We then tested whether NGCC-305 can induce degradation of key oncoproteins essential to proliferation and survival of erlotinib-resistant NSCLC cells in vitro. H1975 and H1975 NSCLC cells were incubated with 1 μmol/L NGCC-305 for 7 hours and then cultured in compound-free medium for an additional 0, 17, or 24 hours, respectively, before being analyzed by Western blot (Fig. 2A). As shown in Fig. 2B, NGCC-305 treatment reduced the levels of phospho-MET as well as total MET, the amplification of which is responsible for erlotinib resistance in NSCLC. In addition, NGCC-305 treatment suppressed downstream PI3K/AKT and RAF/ERK signaling as shown by reduced p-AKT/AKT and p-ERK/ERK levels (Fig. 2B). Similarly, brief treatment with NGCC-305 also reduced the levels of phospho and total EGFR and suppressed downstream PI3K/AKT signaling in H1975 NSCLC cells (Fig. 2C). Importantly, oncoprotein degradation in both H1993 and H1975 NSCLC cell lines were even sustained at 24 hours after the compound had been removed (Fig. 2B and C). To correlate this oncoprotein degradation with functional effects, we monitored apoptosis and proliferation after H1975 cells were briefly exposed to NGCC-305 (7 hours) and then cultured in compound-free medium. We found that NGCC-305 induced activation of caspase-3 and caspase-7, increased PARP cleavage (Fig. 2D), and inhibited cell proliferation (Fig. 2E) dose-dependently even after the compound was removed from culture. Thus, NGCC-305 is able to induce degradation of essential oncoproteins, inhibit cancer cell proliferation, and induce apoptosis in erlotinib-resistant NSCLC in vitro.

NGCC-305 Exhibits a Unique Pharmacokinetic Profile

As has been reported, NGCC-305 has a high oral bioavailability in mice (F 96.0%), tends to be selectively retained in tumor tissues longer than in normal ones, and reaches therapeutic levels in brain tissues (28). Here, we continued to characterize its pharmacokinetic property in different organs. NGCC-305 exposure in different organs and tumor is illustrated in Fig. 3A. Following a single oral dosing of NGCC-305 at 30 mg/kg, NGCC-305 exhibited highest exposures in lung tissue (Cmax 91.7 μmol/L, AUC 623.8 μmol/L h) compared with other organs. This unique pharmacologic property of high exposure in the lung may be particularly relevant clinically given the poor prognosis and lack of effective therapies for lung cancer and has encouraged us to further evaluate compound antitumor activity in animal models of NSCLC, especially in those resistant to EGFR inhibitors. Notably, although compound exposure in the lung is very high, its clearance from this organ (T1/2 4.6 hours) is relatively quick, similarly to other normal tissues (T1/2 2–7 hours). At 48 hours after single dosing, exposure in the lung and other normal organs is negligible (Fig. 3A). On the contrary, the clearance of NGCC-305 in tumors is much slower (T1/2 20.4 hours). Even at 48 hours, the therapeutic level of NGCC-305 (1 μmol/L) could still be detected in tumors.

NGCC-305 Induces Degradation of Multiple Oncoproteins In vivo and Exhibits Antitumor Activity in Subcutaneous, Orthotopic, and Brain Metastatic Tumor Models Established from H1975

A pharmacodynamic study in H1975 subcutaneous tumors showed potent inhibition of multiple HSP90 client proteins and induction of apoptosis following a single dose of NGCC-305 at 160 mg/kg (Fig. 3B). Most importantly, the compound induced degradation of mutant EGFR, the gene conferring oncogenicity and erlotinib resistance in the H1975 cell line. The degradation of EGFR was accompanied by inhibition of its downstream signaling molecules of the cell proliferation (RAF, p-ERK) and survival (p-AKT) pathways, with concurrent induction of HSP70, a marker of HSP90 inhibition. Furthermore, NGCC-305 robustly induced apoptosis at both the 6- and 24-hour time points as...
measured by PARP and caspase-3 cleavages (Fig. 3B), in accordance with in vitro observations (Fig. 2D).

Dose-dependent inhibition of tumor growth was determined in the H1975 subcutaneous tumor model following a 3-week dosing regimen (Fig. 3C). The T/C values for the 80, 120, and 160 mg/kg groups were 33.4%, 21.4%, and 10.2%, respectively (P < 0.001). No loss of body weight or other side effects were observed in any of the treatment groups (Fig. 3D).

Because of its preferential exposure in lung tissues, CUDC-305 was further evaluated in H1975 orthotopic lung tumor model. Four days after tumor implantation, treatment with CUDC-305 at 120 mg/kg once every two days for 5 weeks was initiated. The reversible EGFR inhibitor, erlotinib, used as a reference, was dosed orally at 50 mg/kg daily—its maximum tolerated dose in this mouse strain and model. As shown in Fig. 4A, CUDC-305 treatment significantly prolonged animal survival (median survival 50 ± 8 days, P = 0.001) compared with the vehicle control group (median survival 19 ± 11 days), whereas the erlotinib-treated group exhibited no survival advantage (median survival 24 ± 7 days, P > 0.05).

A dose-dependent efficacy study was next carried out in the same orthotopic tumor model. As shown in Fig. 4B, although CUDC-305 dosed at 40 mg/kg minimally extended animal survival (27 ± 3 days, P > 0.05) compared with the vehicle control (22 ± 2 days), CUDC-305 dosed at higher levels (80 or 120 mg/kg) significantly extended animal survival (median survival of 36 ± 6 and 47 ± 2 days, respectively, P < 0.001) following a 5-week dosing regimen.
To further assess biological effects of CUDC-305 in orthotopic lung tumor model, a separate single-dose pharmacodynamic study was carried out in animals bearing H1975 orthotopic lung tumors. Western blot analysis of lung tumors showed that CUDC-305 was able to inhibit HSP90 for as long as 48 hours following a single oral dosing of compound as shown by robust induction of HSP70. In addition, inhibition of p-AKT and induction of apoptosis were observed up to 24 hours following a single oral dosing of the compound (Fig. 4C).

We have reported previously that CUDC-305 was able to cross the brain-blood barrier (Fig. 3A; ref. 28) and prolong animal survival in the intracranial tumor model of U87MG glioblastoma. We next evaluated its efficacy in a brain metastasis model of erlotinib-resistant NSCLC where mice were injected with H1975 intracranially. Lapatinib, a reversible, dual inhibitor of EGFR/HER2 reported to be brain-penetrable (31), was used as a reference. As shown in Fig. 4D, CUDC-305 treatment significantly prolonged survival of animals carrying H1975 intracranial tumors (median survival 32 ± 4 days, \( P = 0.001 \)) following a 4-week dosing regimen, compared with the vehicle control group (median survival 25 ± 3 days). However, lapatinib treatment did not prolong animal survival (median survival 25 ± 3 days, \( P > 0.05 \)).

### CUDC-305 Induces Degradation of Multiple Oncoproteins In vivo and Displays Antitumor Activity in Subcutaneous and Orthotopic Tumor Models Established from A549

The pharmacodynamic study was first conducted in subcutaneous tumor xenografts of A549 NSCLC cell line with \( K\)-ras mutation. As shown in Fig. 5A, potent inhibition of
multiple HSP90 client proteins was observed following a single oral dosing of CUDC-305 at 160 mg/kg. The downregulated signaling molecules include RTK HER2 and its downstream key regulators of cell proliferation (RAF, p-ERK) and survival (p-AKT) pathways, with concurrent induction of HSP70 at both the 6- and 24-hour time points (Fig. 5A).

The antitumor activity of CUDC-305 was assessed in the A549 subcutaneous tumor xenograft model. Tumor stasis (T/C 9.5%, P < 0.001) was observed following a 3-week dosing regimen (Fig. 5B). No loss of body weight or other side effects were observed in the treatment group (data not shown).

The antitumor activity of CUDC-305 was further evaluated in A549 orthotopic lung tumor model. Erlotinib was dosed orally at 50 mg/kg daily as a reference. As shown in Fig. 5C, CUDC-305 treatment significantly prolonged survival of animals bearing A549 orthotopic lung tumors (median survival 50 ± 8 days, P = 0.001) following a 4-week dosing regimen, compared with the vehicle control group (median survival 38 ± 6 days), whereas erlotinib treatment failed to prolong animal survival (median survival 43 ± 11 days, P > 0.05).

**CUDC-305 Enhances the Antitumor Activity of a Standard-of-Care Agent in the H1975 Tumor Model**

CUDC-305 was evaluated in combination with paclitaxel in the erlotinib-resistant H1975 subcutaneous tumor model. As shown in Fig. 5D, CUDC-305, when delivered at 160 mg/kg as a single agent, significantly inhibited tumor growth (T/C 15.4%, P < 0.001). Paclitaxel also inhibited tumor growth significantly (T/C 35.9%, P < 0.001). However, an enhanced antitumor effect was observed when the two agents were combined (3.9% regression, P < 0.05).

**Figure 4.** A, efficacy study in the H1975 orthotopic lung tumor model compared with erlotinib (n = 10). H1975 cells (2 × 10⁶) were implanted orthotopically to the left lung of nude mice. Starting 4 d after tumor implantation, mice were treated with CUDC-305 (120 mg/kg, orally, once every two days), erlotinib (50 mg/kg, orally, once a day), or vehicle control. CUDC-305 treatment for 5 wk significantly prolonged animal survival (P = 0.001), whereas erlotinib displayed no therapeutic benefit (P > 0.05). B, dose-dependent efficacy study in H1975 orthotopic lung tumor model (n = 11). Treatment with CUDC-305 (20, 40, 80, and 120 mg/kg) started 4 d after tumor implantation. CUDC-305 dose-dependently extended animal survival following a 5-wk dosing regimen. C, pharmacodynamic study in H1975 orthotopic lung tumors. Animals bearing H1975 orthotopic lung tumors were treated with CUDC-305 at 160 mg/kg once. Tumors were collected at various time points (t = 2–3) and subjected to Western blot analysis. Sustained induction of HSP70 and apoptosis and inhibition of oncoprotein phospho-AKT were observed. D, efficacy study in H1975 intracranial metastasis model (n = 10). H1975 cells (5 × 10⁶) were implanted intracranially into nude mice. Starting 5 d after tumor implantation, mice were treated with CUDC-305 (120 mg/kg, orally, once every two days), lapatinib (75 mg/kg, orally, twice a day), or vehicle control. CUDC-305 treatment for 4 wk significantly prolongs animal survival (P = 0.001) in contrast to the lack of lapatinib efficacy (P > 0.05).
Discussion

CUDC-305 has been characterized as a potent HSP90 inhibitor with unique pharmacologic properties, including high oral bioavailability, sustained tumor retention, and therapeutic exposure in brain tissue (28). Its high exposure in lung tissue is of tremendous clinical importance in NSCLC therapy, especially in those cases with erlotinib resistance. Based on the observation that CUDC-305 tends to be more retained in tumors (subcutaneous, $T_{1/2}$ 20.4 hours) relative to normal tissues ($T_{1/2}$ 3–7 hours) as reported previously (28), we expect that an even higher exposure of CUDC-305 in lung tumor tissue relative to normal lung tissue can be achieved.

In the current pharmacodynamic studies, we observed more robust inhibition of pAKT in orthotopic lung tumor (Fig. 4C) than in subcutaneous tumor (Fig. 3B), both established from the same cell line. This result may be explained by more extended exposure of CUDC-305 in lung tumor, which warrants further investigation. Although CUDC-305 exposure in normal lung is high, its clearance is quick ($T_{1/2}$ 4.6 hours), similarly to that observed in other normal organs, thus avoiding
compound accumulation and toxicity in normal lung tissues following a dosing regimen of once every two days.

Among secondary erlotinib resistance in NSCLC, c-MET amplification occurs in about 20% of cases. MET amplification can transactivate ERBB3 (HER3), resulting in PI3K activation independent of EGFR kinase activity (19, 20). Thus, irreversible EGFR inhibitors currently under clinical development may not be able to overcome acquired erlotinib resistance due to MET amplification. Here, we show that CUDC-305 is able to interact with H1993-derived HSP90 with a low IC₅₀ (74.2 nmol/L) and strongly and durably induce degradation of both c-MET and the downstream AKT and ERK signaling proteins even after the compound was removed (Fig. 2B). Due to difficulty in establishing the tumor model from H1993, CUDC-305 efficacy in MET-mutated NSCLC could not be shown in vitro. Nonetheless, based on these in vitro results, we believe that targeting HSP90 may be a better approach to overcome MET amplification-mediated resistance in NSCLC than independently targeting either EGFR or MET.

A major mechanism of secondary resistance to erlotinib in NSCLC is the gatekeeper EGFR mutation in exon 20 (EGFR<sup>T790M</sup>), which is responsible for half of the NSCLC cases with acquired resistance (8–10). To overcome EGFR<sup>T790M</sup>-mediated erlotinib resistance, irreversible EGFR inhibitors have been tested at various stages (12–16). However, initial results in early clinical trials are disappointing. It has been reported that only limited antitumor activity has been achieved for EGFR irreversible inhibitors in EGFR<sup>T790M</sup>-driven transgenic animal models, possibly due to incomplete blockade of the PI3K/AKT/mTOR signaling, a cancer cell survival pathway that could be blocked by HSP90 inhibitors (17, 18). In addition, toxicity concerns have been raised over irreversible EGFR inhibitors because of their cross-reactivity with a key cysteine residue (equivalent to Cys-797 in EGFR) that is present in several other vital kinases (11).

We first showed that CUDC-305 exhibits high affinity for HSP90 complex from H1975 (IC₅₀ 61.2 nmol/L; Fig. 1) harboring both an erlotinib-sensitizing mutation in exon 21 (L858R) and a secondary erlotinib-resistant mutation in exon 20 (T790M). We further showed that CUDC-305 is able to induce degradation of mutant EGFR (Fig. 2C), the oncoprotein that enhances receptor catalytic activity and confers resistance to reversible tyrosine kinase inhibitors. The inhibition of mutant EGFR is robust because this inhibition was sustained even at 24 hours after the compound was removed (Fig. 2C). We also observed that CUDC-305 robustly depleted mutant EGFR at a much lower concentration (0.3 μmol/L) in cultured H1975 cells (data not shown). These results support the hypotheses that mutant EGFR protein may possess a greater dependence on HSP90 function for its stability and, therefore, is more sensitive to HSP90 inhibitor-induced degradation than wild-type EGFR (33, 34). In addition to EGFR degradation, CUDC-305 is able to durably suppress AKT and ERK signaling pathways. These biological effects correlate with potent inhibition of cell proliferation (IC₅₀ 140 nmol/L; ref. 28; Fig. 2E) and induction of apoptosis in treated H1975 cells by CUDC-305 (Fig. 2D).

Pharmacodynamic study in H1975 tumors further showed that CUDC-305 was able to induce degradation of mutant EGFR and downstream signaling molecules of the cell proliferation and survival pathways in H1975 tumors (Fig. 3B). Inhibition of both EGFR and downstream AKT signaling may be an advantage for CUDC-305 to overcome resistance to EGFR inhibitors in NSCLC (18), in contrast with irreversible EGFR inhibitors. It is noticeable that each HSP90 client protein has a different dynamic of degradation although HSP90 is consistently inhibited as indicated by robust HSP70 induction. For example, EGFR degradation is more obvious at the 24-hour than at the 6-hour time point, whereas p-AKT and p-ERK degradation peaks at 6 hours, suggesting differential timing of dependency of these oncoproteins on HSP90 chaperone function. Nonetheless, robust induction of apoptosis as measured by caspase-3 cleavage has been observed at both the 6- and 24-hour time points.

Antitumor activity of CUDC-305 was shown in the subcutaneous as well as orthotopic lung tumor models (Fig. 4A and B). In addition to CUDC-305 sensitivity in H1975 cells, high exposures of the compound in lung tissue may also contribute to its potent efficacy observed in the orthotopic lung cancer model. Consistently, we observed robust biological effects of CUDC-305 in the orthotopically implanted lung tumors (Fig. 4C). Based on these results, we speculate that inhibition of HSP90 may be a better approach in overcoming T790M-mediated erlotinib resistance in NSCLC therapy.

In addition to its effectiveness in lung-originated tumor models, the efficacy of CUDC-305 in the H1975 brain metastasis model is also encouraging. About 10% of NSCLC patients have brain metastases upon diagnosis and another 25% to 40% will ultimately develop them. Indeed, the majority (30–70%) of brain metastases originate from lung tumors (35). Due to the lack of therapeutic agents able to cross the blood-brain barrier, brain metastases invariably carry a poor prognosis, with a 1-year survival rate of less than 10%. Hence, new anticancer agents are urgently needed. We previously reported that CUDC-305 is able to penetrate the blood-brain barrier, reach a therapeutically meaningful level in brain, and prolongs animal survival in an intracranial glioblastoma model (28). Here, we showed that CUDC-305 effectively prolongs animal survival in an intracranial metastasis tumor model implanted with an erlotinib-resistant NSCLC (Fig. 4D).

In a combination study, CUDC-305 was able to enhance paclitaxel in the H1975 subcutaneous tumor model (Fig. 5D). It has been reported that T790M mutation in H1975 selectively activates antiapoptotic signals through AKT and signal transduction and activator of transcription 5, making H1975 resistant to chemotherapeutic drugs (25). In addition to its direct induction of apoptosis (Fig. 3C), CUDC-305 may sensitize tumor cells to paclitaxel by suppressing AKT, or other antiapoptosis pathways as a result of HSP90 inhibition (36), which requires further investigation.

Among NSCLC with primary resistance to EGFR inhibitors, K-ras mutations account for 15% to 30% of cases. K-ras mutations constitutively activate downstream RAF/ERK
signaling and increase the activity of multiple survival pathways, independent of upstream EGFR activity (22, 23). In cell-based assays reported previously (28), we noticed that CUDC-305 is able to potently inhibit proliferation of erlotinib-resistant, K-ras–mutated NSCLC cell lines (A549, H460, Calu-6, and H2122) with a potency similar to that observed in erlotinib-sensitive, wild-type K-ras cell line.

A pharmacodynamic study in A549-derived tumors showed that CUDC-305 is able to downregulate RTK HER2 and key components of the PI3K/akt and RAF/ERK signaling cascades downstream of RAS and HER2 (Fig. 5A). This result suggests that, in addition to K-ras mutations, preexisting or reactivated HER2 may contribute to erlotinib resistance in A549 cells. HER2 probably heterodimerizes with EGFR, transphosphorylates HER3, and controls PI3K survival signals in ErbB-driven cancers (37). Consistent with its pharmacodynamic effects, CUDC-305 displays potent antitumor activity in the A549 subcutaneous tumor model (Fig. 5B). Most importantly, CUDC-305 is able to significantly prolong animal survival in the A549 orthotopic lung tumor model (Fig. 5C), further supporting its pharmacologic activity in NSCLC with primary resistance to EGFR inhibitors due to K-ras mutations.

In summary, CUDC-305 is a novel, oral, small-molecule inhibitor of HSP90 that exhibits potent antitumor activity against NSCLC cell lines resistant to erlotinib due to various genetic deregulations. In addition, CUDC-305 displays a favorable safety profile (data not shown). Therefore, CUDC-305 is a promising agent for the treatment of erlotinib-resistant NSCLC, which presents a serious unmet medical need.

Disclosure of Potential Conflicts of Interest
All authors: employees and shareholders, Curis, Inc.

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