The Novel Oral Hsp90 Inhibitor NVP-HSP990 Exhibits Potent and Broad-spectrum Antitumor Activities In Vitro and In Vivo

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Abstract
A novel oral Hsp90 inhibitor, NVP-HSP990, has been developed and characterized in vitro and in vivo. In vitro, NVP-HSP990 exhibits single digit nanomolar IC₅₀ values on three of the Hsp90 isoforms (Hsp90α, Hsp90β, and GRP94) and 320 nanomolar IC₅₀ value on the fourth (TRAP-1), with selectivity against unrelated enzymes, receptors, and kinases. In c-Met amplified GTL-16 gastric tumor cells, NVP-HSP990 dissociated the Hsp90-p23 complex, depleted client protein c-Met, and induced Hsp70. NVP-HSP990 potently inhibited the growth of human cell lines and primary patient samples from a variety of tumor types. In vivo, NVP-HSP990 exhibits drug-like pharmaceutical and pharmacologic properties with high oral bioavailability. In the GTL-16 xenograft model, a single oral administration of 15 mg/kg of NVP-HSP990 induced sustained downregulation of c-Met and upregulation of Hsp70. In repeat dosing studies, NVP-HSP990 treatment resulted in tumor growth inhibition of GTL-16 and other human tumor xenograft models driven by well-defined oncogenic Hsp90 client proteins. On the basis of its pharmacologic profile and broad-spectrum antitumor activities, clinical trials have been initiated to evaluate NVP-HSP990 in advanced solid tumors. Mol Cancer Ther; 11(3); 730–9. ©2012 AACR.

Introduction
Hsp90 is a ubiquitous and abundant molecular chaperone required for protein folding, assembly, and transport (1, 2). Hsp90 ensures the conformational and functional stability of multiple client proteins, including oncoproteins essential for tumor growth and survival (1, 2). The protein folding function of Hsp90 depends on its ATPase activity and inhibition of this intrinsic activity disrupts the Hsp90 client protein interaction. Hsp90 inhibition destabilizes diverse oncoproteins, resulting in simultaneous blockade of multiple tumorigenic signaling pathways, arrest of cell proliferation, and induction of apoptosis (3, 4). Because of the potential therapeutic use in multiple cancer indications, several Hsp90 inhibitors have been identified and are being evaluated as anticancer drugs (3, 5–7).

In 1999, the semisynthetic benzoquinone ansamycin 17-AAG became the first Hsp90 inhibitor tested in patients with cancer. Despite proof-of-concept activity in these trials, 17-AAG suffers from deficiencies such as difficulties in synthesis and formulation, low oral bioavailability, metabolism by polymorphic CYP3A4 and NQO1 enzymes, efflux by P-glycoprotein, and hepatotoxicity (7, 8). Currently, 13 synthetic Hsp90 inhibitors are under assessment in oncology clinical trials. Six inhibitors (IPI-504, NVP-AUY922, KW-2478, STA-9090, AT13387, and BIIB-028) are administered intravenously, whereas 7 (BIIB-021, IPI-493, XL-888, MPC-3100, DS-2248, Debio 0932, and NVP-HSP990) are dosed orally (5–7, 9–17). These inhibitors address some key pharmaceutical limitations of 17-AAG (5–7). IPI-504 (17-AAGH2) and IPI-493 (17-AG) are the reduced form and active metabolite, respectively, of 17-AAG and have improved pharmacologic properties. All other Hsp90 inhibitors are fully synthetic small molecules that fall into distinct structural classes including: (i) resorcinol-containing molecules (NVP-AUY922, KW-2478, STA-9090, and AT13387), (ii) purine scaffold (BIIB021, PU-H71, and MPC-3100), (iii) imidazopyridine (Debio 0932), (iv) 2-aminothephalalimide (XL888), and (v) aminopyrimidine (NVP-HSP990). The chemical structures of BIIB028 and DS-2248 have not yet been disclosed (5–7, 9–17).

Preclinical data in human tumor xenograft models indicate that Hsp90 inhibitors are efficacious in a wide variety of tumor types, consistent with activity against a range of oncoproteins. Antitumor efficacy ranges from minimal effects to tumor growth stasis but rarely tumor regression (9, 14, 15, 18–20). The variance in response between xenograft models may be attributable to differences in client protein dependence on Hsp90, tumor...
dependence on the client protein, kinetics of client protein degradation, and resynthesis, as well as, drug pharmacokinetic and pharmacologic properties. This complexity makes it difficult to predict antitumor response in xenograft models and renders patient stratification in the clinic challenging (2).

Hsp90 also plays key roles in regulating protein function and stability in normal cells (21). Therefore, balancing efficacy and toxicity is essential to achieving a suitable therapeutic index in patients. One approach will be to optimize the route, dose, and schedule of Hsp90 inhibitors. On one hand, the dose-limiting toxicities (DLT) of 17-AAG have been shown to be schedule dependent (22). On the other hand, the dosing schedule might have to be tailored to the duration and extent of the desired suppression of a particular client protein (23). Determining optimal dosing regimens in the clinic is, therefore, integral to fully realizing the therapeutic potential of Hsp90 inhibition. In this regard, oral administration of Hsp90 inhibitors may provide greater dose and schedule flexibility to achieve a maximal therapeutic window than intravenous drugs (3).

NVP-HSP990 is an orally available Hsp90 inhibitor and is structurally distinct from other clinical Hsp90 inhibitors. NVP-HSP990 shows potent antiproliferative activity in multiple tumor cell lines and primary patient samples in vitro and efficacy in various human tumor xenograft models in vivo. These preclinical results, in conjunction with desirable pharmaceutical properties, support further evaluation of NVP-HSP990 in clinical trials.

Material and Methods

**NVP-HSP990 compound**

NVP-HSP990, IUPAC name: \((R)-2\text{-amino}-7-(\text{4-fluoro-}
\text{2-(6-methoxy-pyridin-2-yl phenyl)}-4\text{-methyl}-7,8\text{-dihydropyrido}[4,3\text{-d}]pyrimidin-5(6\text{H})\text{-one}\), was synthesized at Novartis Institutes for BioMedical Research.

**Cell lines**

Cell lines were either obtained from American Type Culture Collection or through material transfer agreements. The GTL-16 human gastric adenocarcinoma tumor cell line was obtained from Dr. S. Giordano, University of Turin, Turin, Italy (24). All cell lines were authenticated by single-nucleotide polymorphism (SNP) analysis at Novartis.

**Hsp90 binding, ATPase, and selectivity profiling assays**

The potency of Hsp90 inhibitors for Hsp90α, Hsp90β, and Grp94 was determined by AlphaScreen competition binding assays, and activity against TRAP-1 was assessed by an ATPase assay (25). Profiling against a panel of human kinases, enzymes, and receptors was carried out at Cerep, Invitrogen, or internally at Novartis.

**Cell proliferation and apoptosis assays**

Cells were treated with NVP-HSP990 or 17-AAG for 72 hours, and cell viability was determined by CellTiter-Glo Luminescent Cell Viability assay from Promega. Soft agar clonogenic assays with primary human tumors were conducted at Oncotest GmbH (26).

**Hsp90-p23 dissociation and in-cell Western assays**

Hsp90-p23 coimmunoprecipitation assay and in-cell western assays for client degradation or Hsp70 induction were conducted as described previously (25).

**Human tumor xenograft studies**

Human tumor xenograft models GTL-16, NCI-H1975, BT474, and MV4;11 were implanted subcutaneously with 50% Matrigel (Becton Dickinson) in nude (Charles River Laboratories) or severe combined immunodeficient mice (SCID; Harlan). Mice were randomized into cohorts (10 mice/group for efficacy; 3–5 mice/group for pharmacodynamic studies) when tumors reached 200 to 500 mm³. NVP-HSP990 was administered orally in a vehicle of 100% polyethylene glycol (PEG400). Tumor caliper measurements were converted into tumor volumes using the formula: \(\frac{1}{2} \times \text{length} \times \text{width}^2\). Relative tumor inhibition was calculated as \(\% T/C = 100 \times \frac{dT}{dC}\), where, \(dT\) or \(dC\) = difference of mean tumor volume of drug treatment (T) or vehicle (C) on the final day of the study and the randomization volume. Statistical comparisons were conducted using a one-way ANOVA, followed by the Dunn or Tukey post hoc test (SigmaStat). Differences were statistically significant at \(P < 0.05\).

For pharmacodynamic studies, c-Met (Santa Cruz Biotechnologies), inducible Hsp70 (SPA-810; Stressgen), p-ERK (Cell Signaling), epidermal growth factor receptor (EGFR; Cell Signaling), ErbB2 (Zymed), p-Akt (Cell Signaling), total extracellular signal-regulated kinase (ERK; Cell Signaling), AKT (Cell Signaling), and actin (Cell Signaling) were evaluated by Western blot analysis. FLT3 (Santa Cruz Biotechnology) and p-FLT3 (Cell Signaling) were detected using an immunoprecipitation/Western blot analysis. An ELISA was also conducted for c-Met (Invitrogen) and inducible Hsp70 (StressXpress EKS-700; Stressgen).

For immunohistochemistry, paraffin-embedded tumor slices were stained using an automated slide stainer (Discovery XT; Ventana Medical Systems). Antibodies used were Ki-67 (NovoCastra Laboratories), c-Met (Cell Signaling), and inducible Hsp70 (Stressgen).

**Pharmacokinetics**

The plasma pharmacokinetics of Hsp90 inhibitors were evaluated in CD-1 mice after a 5 mg/kg intravenous (in 15% or 20% Captisol) dose or 10 mg/kg oral (in PEG400) dose. Bioanalysis was conducted by quantitative liquid chromatography/mass spectrometry, and pharmacokinetic data were analyzed with standard noncompartmental methods (WinNonLin).
Results

NVP-HSP990 is a potent and selective Hsp90 inhibitor

High throughput screening in conjunction with structure-based lead optimization led to the identification of the novel potent Hsp90 inhibitor NVP-HSP990. As shown in Fig. 1A, NVP-HSP990 is based on a 2-amino-4-methyl-7,8-dihydropyrido[4,3-d]pyrimidin-5(6H)-one scaffold, which is structurally distinct from other known Hsp90 inhibitors. A cocrystal structure solved to 1.5Å resolution (manuscript in preparation) shows that NVP-HSP990 binds to the N-terminal ATP-binding domain of Hsp90. Potency against Hsp90 isoforms were determined using biotinylated geldanamycin-binding inhibition (Hsp90α, Hsp90β, and Grp94) and ATPase activity (TRAP1) assays (Table 1). NVP-HSP990 potently inhibits Hsp90α, Hsp90β, and Grp94 with IC50 values of 0.6, 0.8, and 8.5 nmol/L respectively, whereas 17-AAG binds to all 3 Hsp90 isoforms equipotently (Table 1). With NVP-HSP990, more than 90% inhibition of TRAP1 ATPase activity was observed, with an IC50 value of 320 nmol/L. In contrast, 17-AAG had a marginal effect on TRAP1 ATPase activity (10% inhibition at 10 μmol/L).

NVP-HSP990 did not show significant binding or functional activities against a panel of 83 diverse receptors/enzymes involved in major physiologic functions (Supplementary Table S1). The selectivity of NVP-HSP990 was also evaluated in a panel of 51 different kinases with IC50 values more than 5 μmol/L in all kinases tested. In addition, NVP-HSP990 (10 μmol/L) did not affect the ATPase activity of topoisomerase II, a GHKL (Gyrase, Hsp90, Histidine Kinase, MutL) family ATPase, closely related to Hsp90.

NVP-HSP990 induces a signature response of Hsp90 inhibition and inhibits growth of a wide range of tumor cells

The GTL-16 cell line was selected as the primary model for characterization of NVP-HSP990 because of overexpression/amplification of the receptor tyrosine kinase c-Met, a client protein of Hsp90, and its dependency on c-Met for growth and survival (24). Three readouts were selected as a unique signature of Hsp90 inhibition; rapid dissociation of the cochaperone p23 from Hsp90, client protein depletion, and compensatory induction of Hsp70 (24). In GTL-16 cells, NVP-HSP990 rapidly destabilized the Hsp90-p23 complex in a time- and concentration-dependent manner (Fig. 1B, Supplementary Fig. S1). Levels of the Hsp90 client c-Met and of Hsp70 were measured using a time-resolved fluorescence-in-cell Western assay following 24-hour treatment with NVP-HSP990 or 17-AAG. NVP-HSP990 treatment resulted in a dose proportional decrease in c-Met (EC50 value = 37 nmol/L) and induction of Hsp70 (EC50 value = 20 nmol/L) in GTL-16 cells (Table 1).

In addition, the effect of Hsp90 inhibitors on signaling cascades downstream of c-Met was evaluated. Constitutively active c-Met in GTL-16 causes activation of both ERK and AKT pathways. Hsp90 inhibition by NVP-HSP990 or 17-AAG in GTL-16 cells inhibited ERK and AKT activation, as showed by the decreased level of phosphorylated AKT and ERK using in-cell Western blot analysis. The potency (EC50) of these compounds to...
inhibit ERK and AKT phosphorylation, respectively, was 11 and 6 nmol/L for NVP-HSP990 and 21 and 20 nmol/L for 17-AAG (Table 1).

The effects of NVP-HSP990 on cell proliferation were tested in a panel of human tumor cell lines (Fig. 1C). NVP-HSP990 inhibited growth of all tumor cell lines evaluated irrespective of cancer types or genetics, with nanomolar potency (GI<sub>50</sub> value of 4–40 nmol/L). Five cell lines were selected as tumor xenograft models to represent a diversity of tumor types and oncogenic drivers, and the antiproliferative activity of NVP-HSP990 against these cell lines in vitro is shown in Table 1. To expand these analyses, we also assessed the antiproliferative activity of NVP-HSP990 on a panel of 45 primary patient derived tumor models in soft agar assays (Fig. 1D). NVP-HSP990 was active against most patient derived tumors from a variety of cancer types ex vivo.

The potential for elimination of NVP-HSP990 by drug efflux pumps was assessed in 2 pairs of isogenic cell lines differing in expression of drug efflux pump P-glycoprotein (Pgp; HCT116 parental) versus HCT116 (Pgp<sup>+++</sup>) and KB3.1 versus KB8.5 (Pgp<sup>+++</sup>; Fig. 1C). NVP-HSP990 showed equal potency in these pairs of cell lines. NVP-HSP990 also inhibited growth of NCI-H69 (GI<sub>50</sub> = 40 nmol/L), a small cell lung cancer cell line that does not express NQO1. In contrast, the cellular sensitivity to 17-AAG was dramatically decreased in Pgp overexpressing HCT116 (Pgp<sup>+++</sup>) and KB8.5 (Pgp<sup>+++</sup>) cells and in NQO1 negative NCI-H69 cells.

Pharmacokinetics–pharmacodynamics and tolerability screening of Hsp90 inhibitors

NVP-HSP990 was selected from a series of closely related compounds based on its pharmacokinetic and pharmacodynamic properties and improved tolerability. In these studies, GTL-16-tumored mice were used to evaluate plasma pharmacokinetic and Hsp90 inhibition following a single dose of candidate Hsp90 inhibitors. To assess Hsp90 inhibition in vivo, it would be ideal to directly measure the Hsp90 ATPase activity. However, there is no practical approach readily available. Instead, c-Met degradation and Hsp70 induction were used as surrogate pharmacodynamic markers. In an effort to characterize diverse compounds in the series, compounds were sorted according to basic pharmacokinetic parameters. Clearance and oral half-life (t<sub>½</sub>) varied significantly among the compounds. In considering the oral pharmacokinetics, all the compounds had acceptable bioavailability (range from 30%–100%), with a range in oral half-life (t<sub>½</sub>) and dose-normalized exposure, measured as area under the curve (AUC 0-last). Using these 3 variables, compounds were distributed into 2 classes (A and B; Fig. 2A). These classes were associated with differences in pharmacodynamics. Given that the volume of distribution (V<sub>d</sub>) were generally high (1.3–24.3 L/kg), it was not used to stratify compounds. As shown in Fig. 2A and B, Supplementary Fig. S2A, compounds with relatively short t<sub>½</sub> were generally associated with lower extent and shorter duration of Hsp70 induction/c-Met inhibition than compounds exhibiting a longer t<sub>½</sub>. Both t<sub>½</sub> and AUC (not shown) correlated with the duration of pharmacodynamic response.

To connect pharmacokinetics and pharmacodynamics with tolerability, Hsp90 inhibitors were administered for 5 days on 3 dose regimens (single dose, daily, or every 3 days), and body weights were measured daily. A maximum-tolerated dose (MTD) was determined for each regimen (defined as the dose eliciting <15% weight loss in absence of adverse clinical symptoms). In general, the shorter t<sub>½</sub> compounds were tolerated with more frequent daily dosing, whereas the longer t<sub>½</sub> compounds were not tolerated with daily dosing at dose levels predicted to confer significant pharmacodynamic modulation.

To tailor dose regimens to different tumor indications with oncogenic client proteins having different kinetics of degradation and resynthesis, an Hsp90 inhibitor that

### Table 1. Biochemical and cellular potency of NVP-HSP990

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hsp90&lt;sub&gt;x&lt;/sub&gt;</th>
<th>Hsp90&lt;sub&gt;β&lt;/sub&gt;</th>
<th>Grp94</th>
<th>TRAP1 ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP-HSP990</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>8.5 ± 0.4</td>
<td>320 ± 97</td>
</tr>
<tr>
<td>17-AAG</td>
<td>14.9 ± 0.7</td>
<td>12.3 ± 1.7</td>
<td>16.7 ± 1.3</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cellular potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTL-16 (EC&lt;sub&gt;50&lt;/sub&gt; ± SE, nmol/L)</td>
</tr>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>NVP-HSP990</td>
</tr>
<tr>
<td>17-AAG</td>
</tr>
</tbody>
</table>
supports dosing over a range of dose schedules is desirable. NVP-HSP990 was selected on the basis of its intermediate profile that supported dosing with several tolerated regimens in mice at dose levels that conferred Hsp90 inhibition following a single dose (Fig. 2). The MTD for NVP-HSP990 in nude mice was 0.5 mg/kg every day, 5 mg/kg twice weekly, and 15 mg/kg every week.

The summary of the single-dose pharmacokinetics of NVP-HSP990 studied in CD1 mice is presented in Table 2. Following intravenous administration, NVP-HSP990 displayed moderately low clearance (19 mL/min/kg) with a plasma $t_{1/2}$ of 2.5 hours. Following oral dosing, NVP-HSP990 was absorbed rapidly with peak plasma concentrations observed at 1 hour ($t_{\text{max}}$). The volume of distribution was high (2 L/kg), indicating extensive tissue distribution. NVP-HSP990 exhibited 76% oral bioavailability in mice. Similar pharmacokinetic parameters were obtained in GTL16-tumor-bearing mice (Table 2). In an equilibrium dialysis mouse plasma protein binding assay, 74% of NVP-HSP990 was protein bound.

To further characterize the pharmacokinetic–pharmacodynamic relationship of NVP-HSP990, a single dose of 15 mg/kg NVP-HSP990 was administered to GTL16-tumor-bearing mice. Twenty-four hours after treatment, c-Met protein levels in tumors decreased by 80% compared with levels in tumors from mice treated with vehicle (Fig. 2C, Supplementary Fig. S2B). A sustained (>50% inhibition) decrease in c-Met protein levels was observed for 72 to 120 hours, with recovery to baseline levels detected by 172 hours (Fig. 2C). The induction of Hsp70 protein levels tracked reciprocally with c-Met degradation, with peak Hsp70 levels observed by 24 hours and returning to baseline by 172 hours. Significant inhibition of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways, as measured by reduced phosphorylation levels of ERK and AKT, was observed for up to 24 hours postdose, appearing more transient than the reduction in c-Met levels in the GTL-16 model (Fig. 2D).

NVP-HSP990 plasma drug concentrations were also analyzed (Fig. 2C). Following a 15 mg/kg dose, peak concentrations of NVP-HSP990 in plasma was 1,467 ng/mL (1 hour, $C_{\text{max}}$). Applying a correction factor for 74% mouse plasma protein binding, the fraction of

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Table 2. Pharmacokinetics of NVP-HSP990 in CD1 mice or GTL16-tumor-bearing mice

<table>
<thead>
<tr>
<th>Model</th>
<th>Route</th>
<th>$t_{\text{max}}$ h</th>
<th>$C_{\text{max}}$, ng/mL</th>
<th>AUC (0, last), ng h/mL</th>
<th>$V_{\text{ss}}$, L/kg</th>
<th>Clearance, mL/min/kg</th>
<th>%F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1 mice</td>
<td>Intravenous</td>
<td>2.5</td>
<td>6,648</td>
<td>4,333</td>
<td>2</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD1 mice</td>
<td>Oral, 10 mg/kg</td>
<td>3.4</td>
<td>1.280</td>
<td>6,566</td>
<td>-</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>GTL16-tumor-bearing nude mice</td>
<td>Oral, 15 mg/kg</td>
<td>3.0</td>
<td>1,467</td>
<td>12,200</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
unbound drug was estimated to be 1 μmol/L, confirming that drug concentrations were attained above the cellular EC50 value (Table 1). Subsequently, at 48 hours postdose, plasma concentrations were at the lower limit of quantification (~6.7 ng/mL), coinciding with pharmacodynamic marker rebound.

**NVP-HSP990 activity in human tumor xenograft models**

Given the broad antitumor activity of NVP-HSP990 in vitro, its efficacy was evaluated in multiple xenograft models dependent on established Hsp90 client proteins. As different clients could exhibit varying kinetics of synthesis and degradation in vivo, these studies were conducted to understand the mechanistic relationship between extent and duration of pharmacodynamic response and efficacy. The tumor models included GTL-16 gastric carcinoma (amplified c-Met); BT-474 breast cancer (ErbB2 overexpressing/ER+); MV4;11 acute myelogenous leukemia (AML; expressing FLT3-ITD); 2 human non–small cell lung carcinoma (NSCLC) models; A549 expressing wild-type (WT) EGFR; and NCI-H1975 expressing mutant EGFR (L858R and T790M).

**Efficacy in the GTL-16 gastric cancer model.** NVP-HSP990 efficacy was evaluated in the GTL-16 tumor model at a range of dose levels up to the MTD for each dose regimen (Fig. 3). GTL-16 tumor-xenograft-bearing mice were treated with either vehicle or NVP-HSP990 at 0.5 mg/kg daily, 2.5 to 5 mg/kg twice weekly, or 5 to 15 mg/kg weekly (Fig. 3A, Supplementary Fig. S3). To connect efficacy with pharmacodynamic response, a single-dose pharmacodynamic assessment was conducted in parallel (Fig. 3B). NVP-HSP990 treatment resulted in dose proportional antitumor efficacy with twice weekly or weekly regimens. No significant weight loss or overt signs of toxicity were observed in treated mice (not shown). Tumor growth inhibition correlated with a reduced proliferative index evaluated by Ki-67 staining (Fig. 3C, Supplementary Fig. S3B).

Minimal antitumor activity was observed with 0.5 mg/kg every day, the highest tolerated dose on a daily regimen, and this was associated with minimal c-Met inhibition, albeit inducing Hsp70 (Fig. 3A and B). In contrast, significant antitumor inhibition at 5 mg/kg twice weekly or 15 mg/kg weekly was associated with a marked decrease in c-Met for 72 hours (Fig. 3B). At the MTD, efficacy of the twice weekly schedule appeared slightly more pronounced but not statistically different from weekly treatment. Collectively, these data suggest that prolonged suppression of c-Met for the dosing interval was associated with maximal activity.

**Efficacy in the BT-474 breast cancer model.** ErbB2 and steroid receptors are oncogenic drivers involved in breast cancer progression and have been shown to be Hsp90 client proteins (27). In this context, the activity of NVP-HSP990 was evaluated in the ErbB2 overexpressing/ER-BT-474 breast cancer xenograft model. Mice implanted with BT-474 were given supplemental estradiol to support tumor growth. Because the estradiol supplementation alone caused some body weight loss in mice, only doses up to 10 mg/kg NVP-HSP990 were tolerated on a weekly schedule in this model. NVP-HSP990 administered at 5 or 10 mg/kg weekly produced significant inhibition of tumor growth (% T/C of 12% and 6%, respectively) and a single dose of NVP-HSP990 caused a reduction of ErbB2 protein level (Fig. 4A and B).

**Efficacy in the MV4;11 AML model.** The tyrosine kinase receptor FLT3 is an Hsp90 client protein. Activating internal tandem duplications (ITD) in the juxtamembrane domain of FLT3 have been identified in 30% to 35% patients with AML (28). Given this frequent
occurrence of FLT3-ITD mutations in patients with AML, we investigated the activity of NVP-HSP990 given twice weekly at 5 mg/kg and weekly at 15 mg/kg in the MV4;11 xenograft model, which expresses FLT3-ITD. MV4;11 has been shown to be dependent on FLT3 kinase inhibitors (29). Both treatment regimens resulted in significant antitumor efficacy compared with the group receiving the dosing vehicle alone (%T/C of 3% with twice weekly and 5% regressions weekly; \( P < 0.05 \); Fig. 4C). No statistical difference was observed between the 2 NVP-HSP990 treatment cohorts (\( P > 0.05 \)). Efficacy was accompanied by FLT3 degradation, Hsp70 induction, and pronounced PARP cleavage at the 24-hour time point, indicative of tumor cell death (Fig. 4D).

**Efficacy in the NSCLC models.** Many NSCLCs express wild-type EGFR. However, somatic mutations in the kinase domain of EGFR occur in approximately 10% of patients with NSCLC and alter tumor response to EGFR kinase inhibitors, with the most common mutation occurring at L858, conferring sensitivity and at T790 conferring resistance (30). To assess the anticancer activity of Hsp90 inhibition against NSCLC models in mice, the efficacy of NVP-HSP990 was examined in tumor xenografts derived from the A549 (WT EGFR) and NCI-H1975 (L858R and T790M mutations in EGFR) cell lines. The NCI-H1975 line was previously described to be resistant to the EGFR kinase inhibitors, with partial recovery at 72 hours. Reduced phosphorylation levels of AKT and ERK tracked with the decrease in EGFR protein, confirming pathway inhibition. Antitumor activity was also observed in the WT EGFR A549 model (Fig. 5C and D), where treatment regimens of 0.5 mg/kg every day and 5, 20, and 15 mg/kg weekly all resulted in significant tumor growth inhibition (\( P < 0.05 \) vs. vehicle; Fig. 5A and B). The antitumor efficacy induced by 15 mg/kg of NVP-HSP990 treatment correlated with reduced EGFR protein levels at the 24-hour time point in treated tumors relative to mice treated with the dosing vehicle control, with partial recovery at 72 hours. Reduced phosphorylation levels of AKT and ERK tracked with the decrease in EGFR protein, confirming pathway inhibition. Antitumor activity was also observed in the WT EGFR A549 model (Fig. 5C and D), where treatment regimens of 0.5 mg/kg every day and 5, 20, and 15 mg/kg weekly all resulted in significant tumor growth inhibition compared with the group treated with the dosing vehicle alone (\( P < 0.05 \)).

**Discussion**

To date, there are 13 new Hsp90 inhibitors at various stages of clinical development (5–7, 9–17). The earlier geldanamycin analogues (i.e., 17-AAG or 17-DMAG), despite potent in vitro and in vivo preclinical activity, have not shown clear clinical benefit (5, 31). It is believed that the disappointing clinical activity is due to their poor pharmaceutical properties, selectivity, and toxicity profiles in patients (22, 23, 31). Given this precedent, we set out to identify novel Hsp90 inhibitors with a superior potency, pharmacologic, and tolerability profile.

NVP-HSP990 binds to the ATP catalytic pocket of Hsp90 and interferes with its chaperone function. NVP-HSP990 is highly potent and selective for Hsp90 and represents one of the most potent oral Hsp90 inhibitors reported (5–7, 9–17).
The GTL-16 model was selected as a screening model due to its growth and survival dependency on the Hsp90 client protein c-Met (24). Our pharmacokinetic-pharmacodynamic screen pointed to key pharmacokinetic parameters that predicted the extent and duration of Hsp90 inhibitor response. Compounds with acceptable oral bioavailability were binned into 2 categories, group A and B based on plasma AUC, $t_{1/2}$, and clearance. Generally, group A compounds had short $t_{1/2}$, eliciting only partial and transient inhibition, whereas group B compounds had longer $t_{1/2}$, resulting in sustained c-Met inhibition. This pharmacokinetic–pharmacodynamic relationship established a framework for dose selection in efficacy studies. Compounds with transient pharmacodynamic response required more frequent administrations to achieve efficacy. In general, group A compounds were efficacious on a daily schedule. Extension of the dosing interval to once or twice weekly compromised efficacy, which suggests that prolonged c-Met suppression is required to achieve maximal tumor growth inhibition. In contrast, the longer duration of pharmacodynamic response observed with group B inhibitors restricted dosing frequency to once or twice weekly to achieve tolerated and efficacious doses. From the assessment of compounds at both ends of the spectrum, NVP-HSP990 was selected on the basis of intermediate pharmacokinetic characteristics relative to groups A and B, which resulted in greater schedule flexibility and therapeutic index.

NVP-HSP990 was evaluated in tumor xenograft models driven by different oncogenic client proteins. In each model, NVP-HSP990 showed antitumor activity accompanied by client degradation and Hsp70 induction. However, these models show intriguing differences in the kinetics of client degradation and pathway inhibition. In the GTL-16 model, NVP-HSP990 treatment suppressed c-Met and simultaneously induced Hsp70 for 72 hours. In contrast, in EGFR- (NCI-H1975) or FLT3 (MV4;11)-dependent models, the primary oncogenic clients were downregulated for a shorter duration and largely recovered to pretreatment expression levels by 72 hours. This may result from the differences in client degradation and resynthesis rates and/or client dependence on Hsp90 in different aspects of client protein function. Interestingly, downstream signaling pathways (e.g., AKT and MAPK) were inhibited in concordance with client protein reduction and fully recovered at 72 hours in both the GTL-16 and H1975 models. Recovery of AKT and MAPK activation in the presence of sustained c-Met suppression in GTL-16 is unclear. In the FLT3-ITD model MV4;11, no inhibition of p-AKT or p-ERK was observed at 24 hours, although cleaved PARP induction was marked. It is possible that inhibition of Akt or MAPK pathways might occur earlier. Despite the differences in the duration of client protein suppression and downstream signaling inhibition, twice weekly or weekly NVP-HSP990 dosing is equally effective and sufficient to block tumor growth in vivo for various xenograft models. These data point to the mechanistic complexity of antitumor actions of Hsp90 inhibitors, thus additional studies are required to refine the pharmacokinetic–pharmacodynamic–efficacy relationship to extrapolate drug exposure with optimal tumor inhibition.

**Figure 5.** Efficacy and pharmacodynamics of NVP-HSP990 in H1975 and A549 tumor models. NCI-H1975 (A and B) or A549 (C and D) tumor-bearing mice were administered either vehicle or NVP-HSP990 at indicated doses/schedules. *, $P < 0.05$ versus vehicle. Pharmacodynamics (B and D) were evaluated using Western blot analysis qd, daily; qw, weekly; 2qw, twice weekly.
An advantage of Hsp90 inhibitors is their ability to affect multiple oncoproteins simultaneously, including targets considered “undruggable.” This is relevant given emerging data showing resistant phenotypes arising from mutation, activation of alternative signaling pathways, or feedback loops seen with therapeutics targeting a single oncogene or pathway (32). NVP-HSP990 was equally efficacious in EGFR WT and mutant (gefitinib resistant) NSCLC models, suggesting clinical use in cancers resistant to targeted therapies (30). It remains to be seen whether resistance emerges with prolonged Hsp90 inhibition (33).

NVP-HSP990 addresses most issues that have hampered 17-AAG clinical development. Preclinically, NVP-HSP990 is well tolerated and is not hepatotoxic, unlike 17-AAG. Likewise, NVP-HSP990 is not a Pgp substrate or a major CYP450 inhibitor (data not shown), and hence, has low drug–drug interaction potential, allowing for combination with other agents. In addition, cellular sensitivity to NVP-HSP990 is not affected by NQO1, an enzyme that converts 17-AAG to the more potent hydroquinone (17-AAGH2). The lack of NQO1 expression could be one mechanism of metabolism-based resistance for 17-AAG.

One challenge for Hsp90 inhibitor development is the selection of patients with cancer who would benefit from this treatment. Clinical data from 17-AAG trials have established no clear patient stratification approach. To address this, we are carrying out a comprehensive screen with Hsp90 inhibitors in more than 400 genomically profiled tumors with diverse lineages and genotypes. Results of this study are anticipated to help define cancers that would be most susceptible to Hsp90 inhibition.

In summary, the preclinical activity of NVP-HSP990 was showed in a variety of cancers with well-defined oncogenic clients, supporting clinical evaluation in a range of solid tumor and hematologic malignancies.

Disclosure of Potential Conflicts of Interest


Acknowledgments

The authors thank Susan Fong, Julie Lin, Dachuan Lei, Fernando Salangang, Flora Tang, Yan Tang, Wei Zhang, Ursula Jeffry, Kay Huh, Song Lin, and Keshi Wang for their excellent technical assistance. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 29, 2011; revised November 28, 2011; accepted December 22, 2011; published OnlineFirst January 12, 2012.

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The Novel Oral Hsp90 Inhibitor NVP-HSP990 Exhibits Potent and Broad-spectrum Antitumor Activities In Vitro and In Vivo


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