THE NOVEL ORAL HSP90 INHIBITOR NVP-HSP990 EXHIBITS POTENT AND BROAD-SPECTRUM ANTI-TUMOR 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 nM IC$_{50}$ values on three of the Hsp90 isoforms (Hsp90$\alpha$, Hsp90$\beta$, and GRP94) and 320 nM IC$_{50}$ 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 pharmacological 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 pharmacological profile and broad spectrum anti-tumor activities, clinical trials have been initiated to evaluate NVP-HSP990 in advanced solid tumors.
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 signalling 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 semi-synthetic benzoquinone ansamycin 17-AAG became the first Hsp90 inhibitor tested in cancer patients. 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, thirteen 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, while seven (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 pharmacological properties. All other Hsp90 inhibitors are fully synthetic small molecules that fall into distinct structural classes including: 1) resorcinol-containing molecules (NVP-AUY922,
KW-2478, STA-9090, and AT13387), 2) purine scaffold (BIIB021, PU-H71 and MPC-3100), 3) imidazopyridine (Debio 0932), 4) 2-aminoterephthalamide (XL888), and 5) 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 re-synthesis, as well as drug pharmacokinetic and pharmacological properties. This complexity makes it difficult to predict anti-tumor 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 (DLTs) of 17-AAG have been demonstrated 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, when compared to intravenous drugs (3).
NVP-HSP990 is an orally available Hsp90 inhibitor and is structurally distinct from other clinical Hsp90 inhibitors. NVP-HSP990 demonstrates potent anti-proliferative 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-amino-7-((R)-4-fluoro-2-(6-methoxypyridin-2-yl)phenyl)-4-methyl-7,8-dihydropyrido[4,3-d]pyrimidin-5(6H)-one, was synthesized at Novartis Institutes for BioMedical Research.

Cell lines

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

Hsp90 binding, ATPase and selectivity profiling assays

The potency of Hsp90 inhibitors for Hsp90α, Hsp90β and Grp94 was determined using AlphaScreen® competition binding assays, and activity against TRAP-1 was assessed
using an ATPase assay (25). Profiling against a panel of human kinases, enzymes and receptors were performed at Cerep (Poitiers, France), Invitrogen (Madison, WI) or internally at Novartis.

**Cell proliferation and apoptosis assays**

Cells were treated with NVP-HSP990 or 17-AAG for 72h and cell viability was determined using Promega’s CellTiter-Glo® Luminescent Cell Viability assay. Soft agar clonogenic assays with primary human tumors were performed at Oncotest GmbH (Freiburg, Germany) (26).

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

Hsp90-p23 co-immunoprecipitation assay and in-cell western assays for client degradation or hsp70 induction were performed as described previously (25).

**Human tumor xenograft studies**

Human tumor xenograft models GTL-16, H1975, BT474 and MV4;11 were implanted subcutaneously (s.c.) with 50% Matrigel™ (Becton Dickinson) in nude (Charles River Laboratories, Wilmington, MA) or SCID mice (Harlan, Livermore, CA). Mice were randomized into cohorts (10 mice/group for efficacy; 3-5 mice/group for PD studies) when tumors reached 200-500 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: ½ (length x [width]²). Relative tumor inhibition was calculated as %T/C = 100 X 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 analysis of variance (ANOVA), followed by a Dunn's or Tukey's post-hoc test (SigmaStat). Differences were statistically significant at p < 0.05.

For PD studies, c-Met (Santa Cruz Biotechnologies, CA), inducible Hsp70 (SPA-810, Stressgen, Ann Arbor, MI), pERK (Cell Signaling, Beverly, MA), EGFR (Cell Signaling), ErbB2 (Zymed, South San Francisco, CA), pAkt\textsuperscript{Ser473} (Cell Signaling), total ERK (Cell Signaling), AKT (Cell Signaling) and actin (Cell Signaling) were evaluated by Western analysis. FLT3 (Santa Cruz Biotechnology) and pFLT3 (Cell Signaling) were detected using an immunoprecipitation/ Western analysis. An ELISA was also performed 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, Tucson, AZ). Antibodies used were Ki-67 (NovoCastra Laboratories, UK), 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 LC-MS, and PK data was analysed using 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 Figure 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 co-crystal structure solved to 1.5Å resolution (manuscript in preparation) demonstrates 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 IC_{50} values of 0.6 nM, 0.8 nM 8.5 nM respectively, while 17-AAG binds to all three Hsp90 isoforms equipotently (Table 1). With NVP-HSP990, >90% inhibition of TRAP1 ATPase activity was observed, with an IC_{50} value of 320 nM. In contrast, 17-AAG had a marginal effect on TRAP1 ATPase activity (10% inhibition at 10 µM).

NVP-HSP990 did not show significant binding or functional activities against a panel of 83 diverse receptors/enzymes involved in major physiological functions (Supplementary Table 1). The selectivity of NVP-HSP990 was also evaluated in a panel of 51 different kinases with IC_{50} values > 5 µM in all kinases tested. Additionally, NVP-HSP990 (10 µM) did not affect 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 co-chaperone 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
(Figure 1B, Supplementary Figure 1). Levels of the Hsp90 client c-Met, and of Hsp70
were measured using a TRF (time-resolved fluorescence) in-cell western assay following
24h treatment with NVP-HSP990 or 17-AAG. NVP-HSP990 treatment resulted in a
dose-proportional decrease in c-Met (EC$_{50}$= 37 nM) and induction of Hsp70 (EC$_{50}$= 20
nM) in GTL-16 cells (Table 1).

In addition, the effect of Hsp90 inhibitors on signalling 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 demonstrated by the decreased level of phosphorylated
AKT and ERK using in-cell western analysis. The potency (EC$_{50}$) of these compounds to
inhibit ERK and AKT phosphorylation, respectively, was 11 nM and 6 nM for NVP-
HSP990, and 21 nM and 20 nM for 17-AAG (Table 1).

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

The potential for elimination of NVP-HSP990 by drug efflux pumps was assessed in two pairs of isogenic cell lines differing in expression of drug efflux pump P-glycoprotein (Pgp): HCT116 (parental) vs. HCT116 (Pgp+++) and KB3.1 vs. KB8.5 (Pgp+++) (Figure 1C). NVP-HSP990 showed equal potency in these pairs of cell lines. NVP-HSP990 also inhibited growth of NCI-H69 (GI_{50}=40 \text{ nM}), 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 over-expressing HCT116 (Pg+++) and KB8.5 (Pgp+++) cells, and in NQO1 negative NCI-H69 cells.

**PK-PD and tolerability screening of Hsp90 inhibitors**

NVP-HSP990 was selected from a series of closely related compounds based on its pharmacokinetic (PK) and pharmacodynamic (PD) properties, and improved tolerability. In these studies, GTL-16 tumored mice were used to evaluate plasma PK and Hsp90 inhibition following a single dose of candidate Hsp90 inhibitors. To assess Hsp90 inhibition \textit{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 PD markers. In an effort to characterize diverse compounds in the series, compounds were sorted according to basic PK parameters. Clearance (CL) and oral half life (t_{1/2}) 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_{1/2}) and dose-normalized exposure, measured as area under the curve (AUC p.o. 0-last). Using these three variables, compounds were distributed into two classes (A and B; Figure
2A). These classes were associated with differences in PD. Given that the volume of distribution (Vss) were generally high (1.3 – 24.3 L/kg), it was not used to stratify compounds. As shown in Figure 2A-B and Supplementary Figure 2A, compounds with relatively short \( t_{1/2} \) were generally associated with lower extent and shorter duration of Hsp70 induction/c-Met inhibition than compounds exhibiting a longer \( t_{1/2} \). Both \( t_{1/2} \) and AUC (not shown) correlated with the duration of PD response.

To connect PK and PD with tolerability, Hsp90 inhibitors were administered for five days on three 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 (dose eliciting < 15% weight loss in absence of adverse clinical symptoms). In general, the shorter \( t_{1/2} \) compounds were tolerated with more frequent daily dosing, whereas the longer \( t_{1/2} \) compounds were not tolerated with daily dosing at dose levels predicted to confer significant PD modulation.

To tailor dose regimens to different tumor indications with oncogenic client proteins having different kinetics of degradation and re-synthesis, an Hsp90 inhibitor which supported dosing over a range of dose schedules is desirable. NVP-HSP990 was selected based on its intermediate profile which supported dosing with several tolerated regimens in mice at dose levels that conferred Hsp90 inhibition following a single dose (Figure 2). The MTD for NVP-HSP990 in nude mice was 0.5 mg/kg qd, 5 mg/kg 2qw and 15 mg/kg qw, respectively.

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

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

NVP-HSP990 plasma drug concentrations were also analysed (Figure 2C). Following a 15 mg/kg dose, peak concentrations of NVP-HSP990 in plasma was 1467 ng/ml (1h, ~4 µM). Applying a correction factor for 74% mouse plasma protein binding, the fraction of unbound drug was estimated to be 1 µM, confirming that drug concentrations were attained above the cellular EC<sub>50</sub> (Table 1). Subsequently, at 48 hours post-dose, plasma concentrations were at the lower limit of quantification (approximately 6.7 ng/ml), coinciding with PD rebound.
NVP-HSP990 activity in human tumor xenograft models

Given the broad antitumor activity of NVP-HSP990 in vitro, its efficacy of NVP-HSP990 was tested 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 PD 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) model (expressing FLT3- ITD); two human non-small cell lung cancer (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 (Figure 3). GTL-16 tumored mice were treated with either vehicle or NVP-HSP990 at 0.5 mg/kg daily, 2.5- 5 mg/kg twice weekly or 5-15 mg/kg weekly (Figure 3A, Supplementary Figure 3). To connect efficacy with PD response, a single dose PD assessment was conducted in parallel (Figure 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 Ki67 staining (Figure 3C, Supplementary Figure 3B).

Minimal antitumor activity was observed with 0.5 mg/kg qd, the highest tolerated dose on a daily regimen, and this was associated with minimal c-Met inhibition, albeit inducing Hsp70 (Figure 3A, 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 72h (Figure
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. Since 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 (Figure 4A, 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 30-35% AML patients (28). Given this frequent occurrence of FLT3 ITD mutations in AML patients, 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-ITD by its sensitivity to selective FLT3 kinase inhibitors (29). Both treatment regimens resulted in significant anti-tumor efficacy compared to the group receiving the dosing vehicle alone (%T/C of 3% with twice
weekly and 5% regressions weekly; p < 0.05) (Figure 4C). No statistical difference was observed between the two NVP-HSP990 treatment cohorts (p>0.05). Efficacy was accompanied by FLT3 degradation, Hsp70 induction and pronounced cleaved PARP induction at the 24 hour time point, indicative of tumor cell death (Figure 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 NSCLC patients 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 anti-cancer 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 EGFR inhibitor gefitinib.

NVP-HSP990 was administered orally to NCI-H1975 tumor-bearing mice at 0.5 mg/kg qd x 14, 5 mg/kg twice weekly or 15 mg/kg weekly (Figure 5A, B). All three dosing regimens were tolerated and resulted in marked tumor growth inhibition (p<0.05 vs. vehicle) (Figure 5A, B). The anti-tumor efficacy induced by 15 mg/kg of NVP-HSP990 treatment correlated with reduced EGFR protein levels at the 24h 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 (Figure 5 C,D), where treatment regimens of 0.5 mg/kg qd and 5,
20, 15 mg/kg weekly all resulted in significant tumor growth inhibition compared to the group treated with the dosing vehicle alone (p < 0.05).

**DISCUSSION**

To date, there are thirteen new Hsp90 inhibitors at various stages of clinical development (5-7, 9-17). The earlier geldanamycin analogs (i.e. 17-AAG or 17-DMAG), despite potent *in vitro* and *in vivo* preclinical activity, have not demonstrated 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, pharmacological 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 PK-PD screen pointed to key PK parameters that predicted the extent and duration of Hsp90 inhibitor response. Compounds with acceptable oral bioavailability were binned into two 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 PK-PD relationship established a
framework for dose selection in efficacy studies. Compounds with transient PD 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, longer duration of PD response observed with group B inhibitors restricted dosing frequency to once or twice weekly in order to achieve tolerated and efficacious doses. From the assessment of compounds at both ends of the spectrum, NVP-HSP990 was selected based on intermediate PK 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 demonstrated anti-tumor 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 72h. In contrast, in EGFR (H1975) or FLT3 (MV4;11) dependent models, the primary oncogenic clients were down-regulated for a shorter duration, and largely recovered to pre-treatment 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 signalling 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 signalling 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 anti-tumor actions of Hsp90 inhibitors, thus additional studies are required to refine the PK-PD-efficacy relationship to extrapolate drug exposure with optimal tumor inhibition.

An advantage of Hsp90 inhibitors is their ability to affect multiple onco-proteins simultaneously, including targets considered “undruggable”. This is relevant given emerging data demonstrating resistant phenotypes arising from mutation, activation of alternative signalling 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 utility 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 therapies. 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 cancer patients who would benefit from this treatment. Clinical data from 17-AAG trials have established no clear patient stratification approach. To address this, we are performing a comprehensive screen with HSP90 inhibitors in over 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 demonstrated in a variety of cancers with well defined oncogenic clients, supporting clinical evaluation in a range of solid tumor and hematological malignancies.
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REFERENCES


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

Biochemical potency (IC$_{50}$ ± SD, nM) of NVP-HSP990 and 17-AAG for Hsp90α, Hsp90β, Grp94, and TRAP1.

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<th>Compound</th>
<th>Hsp90α</th>
<th>Hsp90β</th>
<th>Grp94</th>
<th>TRAP1 ATPase</th>
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<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>
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<td>17-AAG</td>
<td>14.9±0.7</td>
<td>12.3±1.7</td>
<td>16.7±1.3</td>
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Cellular potency of NVP-HSP990 and 17-AAG.

<table>
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<th>Compound</th>
<th>GTL-16 (EC$_{50}$ ± SE, nM)</th>
<th>Proliferation (GI$_{50}$ ± SE, nM)</th>
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Table 2. Pharmacokinetics of NVP-HSP990 in CD1 mice or GTL16 tumored mice.

<table>
<thead>
<tr>
<th>Model</th>
<th>Route</th>
<th>t\textsubscript{1/2} (h)</th>
<th>t\textsubscript{max} (h)</th>
<th>(C_0) or C\textsubscript{max} (ng/mL)</th>
<th>AUC(0, last) (ng*hr)/mL</th>
<th>V\textsubscript{ss} (L/kg)</th>
<th>CL (mL/min/kg)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1 mice I.v</td>
<td></td>
<td>2.5</td>
<td>-</td>
<td>6648</td>
<td>4333</td>
<td>2</td>
<td>19</td>
<td>-</td>
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<tr>
<td>CD1 mice Oral 10 mg/kg</td>
<td></td>
<td>3.4</td>
<td>1.1</td>
<td>1280</td>
<td>6566</td>
<td>-</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>GTL16 tumored nude mice Oral 15 mg/kg</td>
<td></td>
<td>3.0</td>
<td>1.0</td>
<td>1467</td>
<td>12200</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Chemical structure (A) and cellular activity (B-D) of NVP-HSP990. (B) In vitro effects of NVP-HSP990 on p23-Hsp90 complex. GTL-16 cells were treated with DMSO, NVP-HSP990 (250 nM) or 17-AAG (500 nM) at indicated times (5, 30, 120 min). The p23 bound to hsp90 was determined by immunoprecipitation/Western blot analysis. (C,D) In vitro antiproliferative activity on human tumor cell lines (C) and primary tumors ex vivo (D). #, cell line not expressing NQ01; +, P-gp overexpressing cell line.

Figure 2. PK-PD relationship of various Hsp90 inhibitors in vivo. (A) Single dose mouse PK parameters (AUC, CL, and t½) for various compounds. B) Extent and duration of Hsp70 response in GTL-16 tumors with examples from group A and B compounds (15 mg/kg), that are structural analogs of NVP-HSP990, * p<0.05 vs vehicle; (C) PK-PD of single oral dose of 15 mg/kg NVP-HSP990 on c-Met and Hsp70 levels in GTL-16 tumors in nude mice (n=2 studies). (D) PD responses on AKT and ERK pathways with NVP-HSP990 (15 mg/kg).

Figure 3. Efficacy and PD of NVP-HSP990 in GTL-16 tumor model. (A) GTL-16 tumored mice were administered either vehicle or NVP-HSP990 at indicated dose/schedules, * p<0.05 vs vehicle. (B) Western blot analysis for c-Met and Hsp70 for NVP-HSP990 at select dose levels. (C) Immunohistochemical staining for Ki67. Magnification of 200x.
Figure 4. Efficacy and PD of NVP-HSP990 in BT-474 and MV4;11 tumor models. 
BT-474 (A, B) or MV4;11 (C, D) tumored mice were treated vehicle or NVP-HSP990 at 
indicated doses/schedules, * p<0.05 vs vehicle. PD readouts (B, D) were evaluated 
using Western blot analysis.

Figure 5. Efficacy and PD of NVP-HSP990 in H1975 and A549 tumor models. NCI-
H1975 (A, B) or A549 (C, D) tumored mice were administered either vehicle or NVP-
HSP990 at indicated doses/schedules, * p<0.05 vs vehicle. PD readouts (B, D) were 
evaluated using Western blot analysis.
**Figure 1**

**A**

NVP-HSP990

Molecular weight = 379.4

**B**

<table>
<thead>
<tr>
<th></th>
<th>17AAG (500 nM)</th>
<th>NVP-HSP990 (250 nM)</th>
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</thead>
<tbody>
<tr>
<td>DMSO</td>
<td></td>
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</tr>
<tr>
<td>5'</td>
<td></td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>120'</td>
<td></td>
<td></td>
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</tbody>
</table>

**C**

![Graph showing GI50 values for 17-AAG and NVP-HSP990](image)

**D**

![Bar chart showing Hsp90 and p23 protein expression](image)
Figure 2

A

CL, iv

Type A

Cpd A

NVP-HSP990

Type B

Cpd B

AUC, po

$\tau_{1/2}$, po

B

Hsp70 (fold vs vehicle)

Cpd A

Cpd B

* * *

Time

Vehicle 24h 72h 120h

C

NVP-HSP990 15 mg/kg

Plasma cMet Hsp70

Time (hours)

Hsp70 (fold induction vs Vehicle)

D

Vehicle 8h 24h 72h 120h

pAkt$^{Ser473}$

Total Akt

pERK1/2

ERK1/2
**Figure 3**

A. GTL-16 (cMet)

- **Mean tumor volume (mm³) ± SE**
  - **Days post-randomization**

```
0 2 4 6 8 10 12 14 16 18
```

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 2 4 6 8 10 12 14 16 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP-HSP990 0.5 mg/kg, qd</td>
<td>●</td>
</tr>
<tr>
<td>NVP-HSP990 5 mg/kg, 2qw</td>
<td>▲</td>
</tr>
<tr>
<td>NVP-HSP990 15 mg/kg, qw</td>
<td>▼</td>
</tr>
</tbody>
</table>

B. NVP-HSP990

- **(a) 0.5 mg/kg**
- **(b) 5 mg/kg**
- **(c) 15 mg/kg**

```
Vehicle (a) 0 5 mg/kg
NVP-HSP990 0.5 mg/kg, qd
NVP-HSP990 5 mg/kg, 2qw
NVP-HSP990 15 mg/kg, qw
```

C. Vehicle  

- **NVP-HSP990 5 mg/kg, 2qw**
Figure 4

A

Breast BT474 (ErbB2/ ER+)

- Vehicle
- NVP-HSP990, 5 mg/kg qw
- NVP-HSP990, 10 mg/kg qw

Mean tumor volume (mm³) ± SE

Days post-randomization

B

- 15 mg/kg

0 8hr 24hr 72hr

ErbB2
Hsp70
Actin

C

AML MV4-11 (FLT3 ITD)

- Vehicle
- NVP-HSP990 5 mg/kg, 2qw
- NVP-HSP990 15 mg/kg qw

Mean tumor volume (mm³) ± SE

Days post-randomization

D

- FLT-3
- Hsp70

Vehicle 24 hr 72 hr 120 hr

5 mg/kg

- pAKT (S473)
- pERK1/2
- cl. PARP

15 mg/kg
Figure 5

A. H1975 (EGFR L858R/T790M)

B. 15 mg/kg

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<tr>
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<th>120 hr</th>
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<td>p85 (PI3K)</td>
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<td>pERK 1/2</td>
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<td>ERK 1/2</td>
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C. A549 (EGFR WT)

D. 15 mg/kg

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<thead>
<tr>
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<th>Vehicle</th>
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<th>8 hr</th>
<th>24 hr</th>
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Molecular Cancer Therapeutics

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