Cancer Therapy: Preclinical

NMS-E973, a Novel Synthetic Inhibitor of Hsp90 with Activity against Multiple Models of Drug Resistance to Targeted Agents, Including Intracranial Metastases

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Abstract

Purpose: Recent developments of second generation Hsp90 inhibitors suggested a potential for development of this class of molecules also in tumors that have become resistant to molecular targeted agents. Disease progression is often due to brain metastases, sometimes related to insufficient drug concentrations within the brain. Our objective was to identify and characterize a novel inhibitor of Hsp90 able to cross the blood–brain barrier (BBB).

Experimental Design: Here is described a detailed biochemical and crystallographic characterization of NMS-E973. Mechanism-based anticancer activity was described in cell models, including models of resistance to kinase inhibitors. Pharmacokinetics properties were followed in plasma, tumor, liver, and brain. In vivo activity and pharmacodynamics, as well as the pharmacokinetic/pharmacodynamic relationships, were evaluated in xenografts, including an intracranially implanted melanoma model.

Results: NMS-E973, representative of a novel isoxazole-derived class of Hsp90 inhibitors, binds Hsp90α with subnanomolar affinity and high selectivity towards kinases, as well as other ATPases. It possesses potent antiproliferative activity against tumor cell lines and a favorable pharmacokinetic profile, with selective retention in tumor tissue and ability to cross the BBB. NMS-E973 induces tumor shrinkage in different human tumor xenografts, and is highly active in models of resistance to kinase inhibitors. Moreover, consistent with its brain penetration, NMS-E973 is active also in an intracranially implanted melanoma model.

Conclusions: Overall, the efficacy profile of NMS-E973 suggests a potential for development in different clinical settings, including tumors that have become resistant to molecular targeted agents, particularly in cases of tumors which reside beyond the BBB. Clin Cancer Res; 19(13); 3520–32. ©2013 AACR.

Introduction

The molecular chaperone Hsp90 is essential for the conformational stability and activity of many key oncogenic proteins, including kinases such as ErbB2, B-Raf, Alk, Flt3, EGFR, RET, KIT, PDGFR, MET. AKT but also transcription factors, telomerase, and other proteins that participate in the activation of the several biologic pathways whose dys-functional activation have been collectively described as constituting the hallmark traits of cancer (1). On the basis of this mechanism of action it is possible, by using a specific inhibitor of Hsp90, to induce the degradation of diverse client proteins and as a consequence to achieve a parallel block of multiple signaling pathways that results in cell death and inhibition of the growth of a broad range of tumors, as already described in several preclinical studies (2).

For these reasons, Hsp90 emerged in the last decade as a major therapeutic target and a great deal of efforts have been dedicated to the discovery of inhibitors, some of them currently under clinical evaluation (3). After the identification of the initial Hsp90 inhibitors, in particular 17-AAG and other semisynthetic ansamycin derivatives such as retaspimycin (4), a new group of fully synthetic second-generation inhibitors were developed with improved preclinical efficacy and “drug-likeness”.

Among the most advanced compounds, retaspimycin and the second-generation representative ganetespib have shown significant clinical benefit, measured as RECIST-
Translational Relevance

The clinical benefit observed with drugs targeting oncogenic kinases is very often transient due to the appearance of resistance linked to secondary mutations in the target molecules, or to the activation of parallel pathways. Also, brain metastases lead to the progression of the disease when drugs do not reach the tumor site due to the presence of the BBB.

This study presents original data on the biochemical and biologic characterization of NMS-E973, a novel second generation inhibitor of Hsp90. This nonansa-mycin compound has antitumor efficacy against a broad range of preclinical tumor models, including models of resistance to some recently approved kinase inhibitors such as vemurafenib and crizotinib. Furthermore, NMS-E973 shows antitumor activity against intracranially implanted tumor xenografts, consistent with its ability to penetrate the BBB. This feature highlights the potential of this new class of molecules with the ability to gain access to the brain, as the most advanced inhibitors of Hsp90 are not brain permeable.

Materials and Methods

Reagents and chemicals

NMS-E973, 5-[2,4-Dihydroxy-6-(4-nitrophenoxy)phenyl]-N-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide was synthesized at Nerviano Medical Sciences S.r.l. laboratories. The synthesis has been previously reported (WO 2010/121963). NMS-E973 and other reference Hsp90 inhibitors were synthesized in house.

Hsp90 binding and selectivity assays

The recombinant proteins have been expressed as Histagged versions of the human Hsp90 (full length for assays and a.a. 9–236 for structure determination), human Grp94 ATPase domain (a.a. 69–337), and human Trap1 (a.a. 26–end), following the published conditions (15–17). Briefly, they have been expressed in Escherichia coli BL21(DE3) at 18°C for 16 hours, then the soluble proteins were purified by affinity chromatography on a Ni-Sepharose column (GE Healthcare). After the overnight cleavage of the hexahistidine tag by the site-specific protease PreScission (GE Healthcare), the final purification was achieved by size exclusion chromatography (Superdex 200, GE Healthcare), and the proteins were stored in PBS buffer containing 10% glycerol and 0.1 mmol/L EDTA.

A commercially available FITC-Geldanamycin (InvivoGen) was used as probe in a fluorescence polarization assay, after its reduction as described elsewhere (18).

For competition experiments, a protein concentration of 5 nmol/L for Hsp90 and of 200 nmol/L for Trap1 were used with 0.5 nmol/L probe (final concentrations). After incubation, the dimethyl sulfoxide (DMSO) compound solution was added to the mixture. The plate was incubated for 18 hours at room temperature and then the fluorescence polarization signal was measured. Data were fitted with the program Dynafit version 3.28.039 (BioKin Ltd) or SigmaPlot (SSI) using the mathematical equation for competitive binding of 2 ligands to the receptor (19).

We synthesized a Grp94 binding probe in house by coupling NMS-E973 to the fluorophore Atto-610 (Atto-Tec GmbH). For competition experiments, Grp94 was mixed to the probe at a final concentration of 50 and 20 nmol/L respectively.

Fluorescence polarization displacement assay for Hsc70-FL was set up using a commercially available probe N\(^6\)-amino-Hexyl-EDA-ATTO-590-ATP (JenaBioScience).

Determination of NMS-E973 binding kinetics to Hsp90 was conducted using Biacore T100 with the approach of the kinetic titration as previously described (20). The data were fitted using the 1:1 kinetic titration model of BIASoftware.

NMS-E973 was tested against a panel of 52 diverse kinases as previously described (21). The following in house produced kinases were tested: ABL, ACK1, AKT1, Alk, AUR1, AUR2, BRK, CDC7, CDR2/CYCA, CHK1, CK2alpha/beta, eEF2K, EGFR1, ERK2, FAK, FGER1, Fli3, GSK3beta, Haspin V473-K798, IGFR1, IKK2, IR, Jak1, Jak2, Jak3, Kit, LCK, MELK, MET, MK2, MPS1, MST4, NEK6, NIM, P38alpha, PAK4, PDGFRb, PDK1, PERK, PIM1, PIM2, PKAalpha,
PKCbetaII, PLK1, RET, SULI11, SYK, TRKA, TYK 2, VEGFR2, VEGFR3, ZAP70.

Crystallography

Crystallography studies were conducted using the N-terminal domain of Hsp90α (a.a. 9–236). Crystals of the N-terminal domain of Hsp90 in complex with NMS-E973 were grown using the hanging-drop vapor diffusion method from a solution of 20% MPEG 2000K, 0.2 mol/L magnesium chloride, 0.1 mol/L cacodylate, pH 6.5, at 4°C. The protein was concentrated to 25 mg/mL and compound was added to a final concentration of 2 mmol/L. For data collection, the crystals were transferred to drops containing the equivalent mother liquor with 25% glycerol. Diffraction data were collected at the ESRF (Grenoble, France) on beamline ID23-2. Data were processed using the HKL package (22). The space group is P21. Model building was done using Coot (23) and refinement was done with RefMac (24). The coordinates have been deposited in the Protein Data Bank with code 4b7p together with structure factors and detailed experimental conditions.

Cell culture

Human cancer cell lines were obtained either from the American Type Culture Collection (ATCC) or from the European Collection of Cell Culture. Cells were maintained in the media at serum concentrations recommended by the suppliers in a humidified 37°C incubator with 5% CO2.

Cell line authentication

All cell lines were profiled using DNA fingerprinting technology (AmpFISTR Identifiler Plus PCR Amplification kit, Applied Biosystems) according to the manufacturer’s protocol. The kit amplifies simultaneously 15 tetranucleotide repeated loci and the amelogenin gender marker in a single assay. The short tandem repeat profiles of the analyzed cell lines were compared with DNA fingerprinting databases (e.g., ATCC and DMSZ). All the analyzed profiles showed a similarity more than 80%. In accord with many published studies (25, 26), this is the suggested threshold to declare the correspondence between the examined cell line and the external reference. All cell lines are routinely confirmed at 6-month intervals.

Cell proliferation assay

Exponentially growing cells were seeded in complete medium in a 384-well plate format. Twenty-four hours after seeding, cells were treated with compounds dissolved in 0.1% DMSO, at different concentrations. The cells were incubated at 37°C and 5% CO2, at the end of treatment time, the plates were processed using Cell Titer-Glo assay (Promega) following the manufacturer’s instruction. Cell Titer-Glo is a homogenous method based on the quantification of the ATP present, an indicator of metabolically active cells. Briefly, 25 μL reagent solution is added to each well and after 5 minutes of shaking the microplates, the luminescent signal is read by Envision (PerkinElmer) luminometer. Inhibitory activity was evaluated comparing treated versus control data using sigmoidal equation on Assay Explorer (Symix) program.

For combination assays, NMS-E973 and drug solutions, prepared immediately before use, were added to the cells in scalar doses, in 7 point dose–response curves at constant ratios, using a separate pipetting step for each drug. When sequential, rather than simultaneous combinations were studied, the first drug was added to culture 24 hours after cell seeding, and the second drug 48 hours after seeding (i.e. at time = 24 hours). At time = 72 hours, cells were lysed using 50 μL/well of Cell Titer-Glo reagent solution (Promega).

The data were expressed as percentage of viability versus DMSO controls and analyzed using a proprietary software (Beremiz) according to the Chou Talalay equation (27). Combination indices (CI) were calculated for mutually nonexclusive drugs where a CI <0.3 indicates strong synergism; 0.3–0.8 synergism, 0.8–1.2 additivity; 1.3–3 antagonism; >3 strong antagonism.

Cell-based assays

The mechanism of action of the compound was investigated using cell lines treated for 24 hours with compounds at the indicated concentration of DMSO as control. Client protein degradation was determined by Western blot analysis, using DMSO as a control. Immunoblotting was done according to standard procedures (28) and using the following antibodies: anti-Hsp70, anti-Hsp90 (Enzo Life Sciences), anti-Fl3, anti-B-Raf, anti-PARP1, anti-EGFR (Santa Cruz Biotechnology), anti-ERK, anti-pThr199 NPM, anti-EGFR, anti-STAT5, anti-pY694 STAT5, anti-pThr201/202 ERK, anti-pSer217/221 Mek1/2, anti-pSer21/221 Mek1/2, anti-S6, anti-pSer240/244 S6, anti-cleaved Caspase3, anti-pThr199 NPM (Cell Signaling); anti-Histone H3 (Millipore). The SuperSignal Chemiluminescence Kit (Pierce) was used for detection.

Human xenograft models and antitumor efficacy studies

All procedures adopted for housing and handling of animals were in strict compliance with European and Italian Guidelines for Laboratory Animal Welfare. A total of 3 × 106 A375 or A2780 cells were transplanted subcutaneously in athymic nu/nu mice (Harlan). Mice bearing a minimal tumor mass (130–180 mm3) were randomized into vehicle and treated groups made of 7 animals each unless differently stated. Intravenous treatments started the day after randomization, with different schedules as described. Tumor dimensions were measured regularly using Vernier calipers and tumor volume was calculated according to the following formula: length (mm) × width2 (mm)/2.

The percentage of tumor growth inhibition (%TGI) was calculated as follows:

$$\%\ TGI = \frac{Mean\ tumor\ volume\ of\ treated\ group}{Mean\ tumor\ volume\ of\ control\ group} \times 100$$
Toxicity was evaluated on the basis of body weight reduction. At the end of the experiment, mice were sacrificed and gross autopsy findings were reported. Tumor-free animals at 90 days after tumor implant were considered cured. For the leukemia studies, female severe combined immunodeficient (SCID) mice (Harlan) were used. Animals were exposed to γ-irradiation, 200 Rads of whole body gamma-irradiation, within 24 hours of injection of cells. To obtain growth as a solid tumor, 5 x 10^6 MOLM-13 cells were injected subcutaneously and treatment initiated when tumor size reached 130 to 200 mm^3. Tumor dimensions were monitored during the experiment and TGI was estimated of the steady-state drug concentration in plasma to be maintained for observing tumor stabilization.

A pharmacokinetic and pharmacodynamic approach, based on a previously published model (29, 30) was applied to the efficacy data of NMS-E973. This model links the dosing regimen to the tumor growth dynamics independently from the schedule and levels of dose. A quantitative estimate of the drug potency is assessed through the parameters k_2, the proportionality factor linking the plasma concentration to the effect, and C_50, which provides an estimate of the steady-state drug concentration in plasma (C_50) to be maintained for observing tumor stabilization.

Pharmacokinetics

The pharmacokinetics of NMS-E973 was investigated in an ancillary group of 3 tumor-bearing mice. Blood samples for the pharmacokinetic assessment were collected, and the drug was assayed in plasma using liquid chromatography/tandem mass spectrometry techniques (29). The pharmacokinetics of NMS-E973 was evaluated using a 2 compartment model after single intravenous administration of 10 mg/Kg.

Data analysis

Winnonlin Program (Pharsight) was used both for compartmental pharmacokinetic and pharmacokinetic/pharmacodynamic analysis. Parameters were estimated by using weighted nonlinear least squares (with weight 1/\(y^2\) observed).

In vivo imaging of A375-LUC intracranially implanted

Intracranial model. Balb/c male nude mice, aged 6 to 8 weeks, were anesthetized and stereotactically implanted in the caudate nuclei region with 1 x 10^6 melanoma A375-LUC cells in 2 μL. With a microdrill, a small hole was done at the chosen coordinates and cells were injected at the speed of 1 μL every minute. Hole was closed using bone wax and the wound with sterile autoclips. After the end of surgery, mice were monitored for recovery until complete wakening.

Bioluminescence imaging. Twelve animals were imaged with an IVIS Lumina device (Xenogen, CaliperLS). After intraperitoneal injection of β-luciferin (150 mg/Kg), animals were anesthetized in 2% to 3% isoflurane atmosphere and 10 minutes after injection of luciferin, mice were imaged. Images were analyzed and scaled after all acquisitions using appropriate computer software (Living Image version 3.00; Xenogen Corporation).

Magnetic resonance imaging. MRI was conducted before and 6 days after last treatment on 3 control and 3 treated animals. The treatment started 5 days after the injection of cells. A 7.0 T Bruker Pharmascan instrument was used; anesthetized animals (isoflurane gas anesthesia) were positioned prone in the animal bed and inserted into the radiofrequency coil (diameter 38 mm) inside the magnet. T2-weighted coronal scans (RARE: TR = 5,000 ms; TEeff = 57 ms; rare factor = 8; 4 averages; FOV = 2.5”2.5 cm²; Sl. Thickn. = 1 mm; matrix = 256”128; acq. time = 5 minutes and 20 seconds) were acquired to better visualize tumors. A macro was used to calculate total tumor volume, starting from the bidimensional area of all contiguous slices and slice thickness.

Results

NMS-E973 is a potent and selective inhibitor of HSP90

A fragment-based approach coupled to an intensive medicinal chemistry effort led to the identification of the isoxazole derivative NMS-E973 (WO 2010/121963), a novel potst Hsp90 inhibitor structurally unrelated to ansamycins (Fig. 1A and Supplementary Fig. S1).

NMS-E973 binds to the ATP binding site of Hsp90α with a D_C50 of <10 nmol/L in a fluorescence polarization displacement assay. As this assay has a sensitivity limited close to the nanomolar concentration of the Hsp90 protein used in the assay, we used a direct binding method, Surface Plasmon Resonance analysis, to define a more accurate K_D of 0.346 nmol/L. We then studied the selectivity of the compound towards Grp94 and Trap1, the structurally related endoplasmic reticulum and mitochondrial isoforms of Hsp90, which are also reported to be overexpressed in tumors and possibly involved in cancer development (31–33). We calculated a K_D of 4.5 nmol/L (>10 fold selectivity) for Grp94, whereas a K_D of 670 nmol/L (~200 fold selectivity) was obtained for Trap1. No activity was observed when the compound was tested against a panel of 52 diverse protein kinases, as well as against the biologically related ATPase Hsc70 (IC_50 >10 μmol/L).

As expected, on the basis of its high biochemical potency, the crystal structure of NMS-E973 bound to the N-terminal domain of Hsp90 in the ATP binding site (Fig. 1A) shows a broad network of hydrogen bonding interactions, in addition to those between Aasp93 and the phenolic hydroxyl already described for other inhibitors. The resorcinol moiety forms a network of water-mediated interactions with the neighborly residues Gly97, Ser52, and Leu48. The 4-nitro-phenoxy group linked to the resorcinol residue is located near a flexible region formed by residues 103–111 and causes its partial rearrangement without, however, inducing the opening of the lipophilic pocket described for purine-based inhibitors. Additional interactions are provided by the isoxazole-3-carboxamide...
group making hydrogen bonds with the carbonyl backbone of Gly97 and with the nitrogen side chain atom of Lys58, and a supplementary binding contribution is given by a salt interaction of the piperidine nitrogen with the carboxyl of Asp102.

**NMS-E973 inhibits cancer cell proliferation and induces client protein degradation**

When profiled against a panel of 140 human tumor cell lines of various tissue origins and molecular background, NMS-E973 shows a widespread antiproliferative activity,
with an average IC$_{50}$ of 1.6 μmol/L and 15 cell lines with an IC$_{50}$ < 100 nmol/L (Fig. 1B and Supplementary Table S1).

As expected, because of the broad mechanism of action of Hsp90 inhibitors, it was not possible to unambiguously associate the pattern of sensitivity observed across the cell panel with mutations in a single gene. However, we observed that all of the most sensitive cell lines are driven by the disregulated activation of known oncogenic pathways (Table 1), including Alk mutations and ErbB2 overexpression, alterations well known to induce clinical sensitivity to Hsp90 inhibitors, as well as Flt3, B-Raf, PI3K, and RAS mutations.

We characterized the in vitro and in vivo effects and mechanism of action of NMS-E973 in some of these sensitive molecular backgrounds.

The acute myeloid leukemia (AML) cell line MOLM-13 is known to be driven by the presence of an internal tandem duplication (ITD) in the Flt3 kinase, leading to constitutive activation of its downstream signaling pathway and to STAT5 phosphorylation. Incubation of the MOLM-13 cells for 24 hours with NMS-E973 induced degradation of Flt3 and inhibition of its signaling pathway, as shown by the decrease of STAT5 phosphorylation, as well as of the AKT and MAPK pathways, as shown by the reduction of S6 and MAPK phosphorylation, with the typical induction of Hsp70 that follows Hsp90 inhibition (Fig. 1C).

In A375 melanoma cells NMS-E973 induced degradation of B and C-Raf proteins, associated with a dual inhibition of the MAPK and AKT pathways and with induction of apoptosis as shown by PARP cleavage (Fig. 1C) and by time-lapse microscopy, which shows that the compound induces an initial accumulation in mitosis (Supplementary Fig. S2, green arrowheads), followed by extensive pyknosis and membrane blebbing damage (Supplementary Fig. S2, red arrowheads).

The AKT and MAPK pathways were also simultaneously inhibited in the A2780 ovarian cancer cell line upon treatment. Again, these modulations were associated with the feedback induction of Hsp70 expression (Fig. 1C).

**Pharmacokinetics and tissue distribution of NMS-E973**

To explore the possibility to use NMS-E973 for in vivo experiments, its pharmacokinetic properties were investigated in mice. When administered intravenously to 3 mice at 10 mg/Kg, the compound resulted suitable for in vivo administration with high exposure levels, compatible with biologic activity, a half life of more than 5 hours and a high volume of distribution, and associated to a moderate systemic clearance (Fig. 2A). The kinetics of tissue distribution of the compound in plasma, brain, liver, and tumor over time were determined in mice xenografted with the A375 tumors after treatment with the active dose of 60 mg/Kg (Fig. 2B). Significant levels of NMS-E973 were detected in tumors for 3 days, with a more rapid elimination from liver and plasma. Interestingly, significant concentrations of NMS-E973, exceeding those observed in plasma, were observed in brain, indicating the capacity of the compound to penetrate the BBB.

**NMS-E973 inhibits the growth of A375 tumors subcutaneously or intracranially implanted in mice**

NMS-E973 was administered at 60 mg/Kg twice daily i.v. in the A375 xenograft model according to 2 schedules: (i) every other day for 12 days and (ii) 3 days on/1 day off/3 days on (3-1-3, one cycle). Both schedules resulted in tumor shrinkage and TGI of 74% and 89%, respectively (Fig. 2C).

### Table 1. Tissue of origin and the key oncogenic alterations present in the most sensitive cell lines (IC$_{50}$ < 100 nmol/L), with their antiproliferative activity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue</th>
<th>NMS-E973 IC$_{50}$ (μmol/L)</th>
<th>Altered oncogenic pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU-4475</td>
<td>Carcinoma breast</td>
<td>0.013</td>
<td>B-Raf</td>
</tr>
<tr>
<td>EVSA-T</td>
<td>Carcinoma breast</td>
<td>0.016</td>
<td>HER2</td>
</tr>
<tr>
<td>MV-4-11</td>
<td>Leukemia acute monocytic</td>
<td>0.029</td>
<td>FLT3</td>
</tr>
<tr>
<td>HT-1080</td>
<td>Fibrosarcoma</td>
<td>0.034</td>
<td>NRAS</td>
</tr>
<tr>
<td>MOLM-13</td>
<td>Leukemia acute myeloid</td>
<td>0.035</td>
<td>FLT3</td>
</tr>
<tr>
<td>L-363</td>
<td>Myeloma multiple</td>
<td>0.049</td>
<td>NRAS</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Neuroblastoma</td>
<td>0.051</td>
<td>ALK</td>
</tr>
<tr>
<td>CAL-51</td>
<td>Carcinoma breast</td>
<td>0.056</td>
<td>PI3KCA</td>
</tr>
<tr>
<td>HCC1954</td>
<td>Carcinoma breast ductal</td>
<td>0.061</td>
<td>HER2</td>
</tr>
<tr>
<td>A2780</td>
<td>Adenocarcinoma ovary</td>
<td>0.069</td>
<td>PTEN</td>
</tr>
<tr>
<td>BT-474</td>
<td>Carcinoma breast ductal</td>
<td>0.073</td>
<td>HER2</td>
</tr>
<tr>
<td>HCC1419</td>
<td>Carcinoma breast ductal</td>
<td>0.076</td>
<td>HER2</td>
</tr>
<tr>
<td>RKO</td>
<td>Adenocarcinoma colon</td>
<td>0.084</td>
<td>B-Raf</td>
</tr>
<tr>
<td>HDQ-P1</td>
<td>Carcinoma breast</td>
<td>0.089</td>
<td>HER2</td>
</tr>
<tr>
<td>A-375</td>
<td>Melanoma</td>
<td>0.133</td>
<td>B-Raf</td>
</tr>
</tbody>
</table>

NOTE: The complete list of antiproliferative data is shown in Supplementary Table S1.
associated to a maximum body weight loss of 9% and 13%, which rapidly recovered at the end of the treatment. This activity was superior to that of the inhibitor AUY922 (34), one of the second generation Hsp90 inhibitors currently in clinical phase II, administered at 50 mg/Kg every other day for 12 days (Fig. 2C). Interestingly, administration of a second cycle of NMS-E973 with the intermittent schedule 3-1-3 to large regrown tumors (0.5 g), was again efficacious, with tumor regression and 1 of 7 mice tumor free at day 92 (Fig. 2C).

To study the ex vivo mechanism of action, the compound was administered at 60 mg/Kg twice daily, and at different time points after the second administration, biomarker modulation was investigated in lysates from A375 xenograft tumors. Inhibition of Hsp90 resulted in decreased MAPK pathway activation at early time-points (up to 6–12 hours), followed at later time points (12–24 hours) by induction of apoptosis, measured as caspase 3 activation and upregulation of Hsp70 levels. Most biomarkers returned to control levels after 24 to 48 hours, although significant recovery of AKT and BRAF was observed by 12 hours (Fig. 2D). To achieve sustained modulation of these biomarkers, we therefore conducted efficacy experiments in which we administered the compound every 12 hours (twice daily). On the basis of the observed presence of compound in the brain, the efficacy of NMS-E973 was tested in an intracranial model in which luciferized A375 tumor cell lines were injected in the caudate nuclei region of the right brain hemisphere, and 5 days later, treated with 60 mg/Kg twice daily with one cycle of the intermittent schedule 3-3-3 (3 days on/3 days off/3 days on). Bioluminescence imaging of the luciferized tumor cells showed a clear signal decrease after treatment, with an inhibition of 92% of the luminescence signal in the treated group (Supplementary Fig. S3) compared with the control group. Magnetic resonance (MRI) was also used to monitor tumor localization and growth 6 days after the end of the treatment, showing 77% decrease in tumor volume in treated groups with respect to controls, associated with extensive necrosis visible as areas of lower signal intensity (Fig. 3).

**Treatment with NMS-E973 is active in A2780 xenograft tumors**

As the ovarian carcinoma A2780 cell line is one of the most sensitive to treatment with NMS-E973 in vitro, the efficacy of the compound was tested in vivo in 2 experiments...
using different schedules and doses, and a pharmacokinet-
ic/pharmacodynamic approach was applied on the basis of
a previously published model that links the dosing regimen
to the tumor growth dynamics, generating an algorithm
able to estimate and predict the efficacy in different condi-
tions (29). As shown in Fig. 4A, treatment with either 30 or
60 mg/Kg of NMS-E973 for 10 consecutive days resulted in
significant tumor growth delay (TGI 53% and 74%, respec-
tively). On the basis of these results, an algorithm was
generated that was able to accurately fit the data (Fig. 4A,
lines). It can be used to calculate the CT parameter, which
provides an estimate of the steady-state plasma drug con-
centration needed to observe tumor stabilization. As pre-
picted by the algorithm, treatment with either 30 or 60 mg/
Kg twice daily with one cycle of the intermittent 3-1-3
schedule resulted in higher efficacy (TGI 68% and 91%,
respectively; Fig. 4B) associated to a moderate body weight
loss (4.5 and 7.7%, respectively).

Fitting the data on the A375 and A2780 xenograft tumor
models, we obtained similar CT, around 2 to 2.3
mol/L (Fig. 4C). This parameter is helpful to guide preclinical
inhibitor development and can help to estimate the active
dose in humans.

**NMS-E973 induces tumor eradication of a Flt3ITD
dependent leukemia model**

The in vivo antitumor efficacy of NMS-E973 against the
MOLM-13 AML cell line, driven by the Flt3ITD mutation,
was then analyzed. Intravenous administration of NMS-
E973 at 60 mg/Kg twice with one cycle of the intermittent
schedule 3-1-3 to SCID mice subcutaneously inoculated
with the MOLM-13 cells induced cure in all treated mice,
with 9 of 10 animals alive at day 120 at the end of treatment (Fig. 5B). This
activity was superior to that observed for sunitinib, highly active against Flt3, when administered at 40 mg/Kg daily for 20 consecutive days (death of all animals by day 41; Fig. 5B). Although we did not conduct ex vivo biomarker modulation studies in this experiment, the ability of NMS-E973 to induce degradation of Flt3ITD was confirmed in MOLM-13 cells in vitro (Fig. 1C).

**NMS-E973 is active in resistance models**

Emergence of resistance is a common feature of targeted therapy with kinase inhibitors, often resulting from mutations of critical residues in the ATP pocket, such as those observed after treatment with imatinib in AML (Abl T315I) and in GIST (Kit D816V, T670I, and PDGFRα D842V), or crizotinib in lung adenocarcinoma (Alk F1174L, L1196M, C1156Y, G1269A; ref. 35).

As expected for Hsp90 inhibitors, NMS-E973 was equally active in the inhibition of Ba/F3 cells dependent on Alk or mutants insensitive to crizotinib (F1174L, L1196M, C1156Y and F1174C, Supplementary Fig. S4).

In melanoma, resistance to treatment with vemurafenib and the short duration of responses have not been associated to B-Raf kinase mutations so far, but they are linked to the activation of alternative signaling pathways (10, 11). Also, in colon, an escape mechanism from B-Raf inhibition through the activation of EGFR signaling has been suggested. In fact, B-Raf–mutant colorectal cancer cell lines have been described to harbor higher basal levels of EGFR/pEGFR compared with melanoma cells (12), with an increase in pEGFR after vemurafenib treatment (13). As all the activated pathways identified so far are reported to be sensitive to Hsp90 inhibition in vitro (36), treatment with Hsp90 inhibitors as single agent or in combination with B-Raf inhibitors could potentially eradicate cancer cells that have acquired resistance.

Combined in vitro treatment of A375 melanoma cells with NMS-E973 and PLX-4720 shows a synergic antiproliferative activity (Fig. 6A), further supporting the rationale to test in vivo the combination of the 2 drugs. Simultaneous administration of NMS-E973 at 60 mg/Kg i.v. twice daily with one cycle of the 3-1-3 schedule, together with B-Raf inhibitor PLX-4720 at 100 mg/Kg per os for 10 days, showed an efficacy superior to the treatments as single agents, as judged by the delay observed in tumor regrowth after initial regression (Fig. 6B), supporting the rationale to combine Hsp90 inhibitors with vemurafenib in melanoma.
About 8% to 10% of colon carcinomas harbor the B-RafV600E oncogenic lesion and these patients show a very limited response to vemurafenib (37, 38). Given the poor prognosis of these patients, there is a need of new effective treatments for this patient population and it is suggested that combining B-Raf inhibitors with drugs with different mechanism of action could overcome treatment resistance in colon cancer.

We found that RKO cells that are resistant to B-Raf inhibitors (IC_{50} = 8.5 μmol/L for vemurafenib, >10 μmol/L for PLX-4720) are sensitive to Hsp90 inhibitors, with an IC_{50} of 84 nmol/L for NMS-E973. We analyzed the molecular basis for this sensitivity in RKO cells treated with PLX-4720 and NMS-E973, as single agents or in combination. We observed that PLX-4720, even at the highest dose of 10 μmol/L, only partially inhibits B-Raf activity in these cells, with a modest decrease of MEK and ERK phosphorylation and lack of caspase activation, possibly due to the observed concomitant increase in phospho-EGFR, which has been described as a potential mechanism of resistance to B-Raf inhibition in colorectal carcinoma (12, 13). Significantly, treatment with NMS-E973 induces downregulation of both total and phospho-EGFR, reduction of MEK, ERK, and S6 phosphorylation, coupled with caspase activation as a single agent and is able to markedly enhance suppression of BRAF signaling by PLX-4720 when combined with this agent.

Moreover, treatment with NMS-E973 induced a degradation of C-Raf and B-Raf proteins, which was partially abrogated by combined treatment with PLX-4720. These results suggest that treatment with NMS-E973 represents a potential approach to bypass escape mechanisms from B-Raf inhibition in this tumor context.

Accordingly, we treated nude mice bearing RKO xenograft tumors with NMS-E973 (60 mg/Kg twice daily) with the

<table>
<thead>
<tr>
<th>Ratio of combination</th>
<th>C.I @ 70%</th>
<th>C.I @ 90%</th>
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<tr>
<td>1 : 0.5</td>
<td>0.56</td>
<td>0.36</td>
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<tr>
<td>1 : 1</td>
<td>0.65</td>
<td>0.43</td>
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<tr>
<td>1 : 2</td>
<td>0.75</td>
<td>0.54</td>
</tr>
<tr>
<td>1 : 4</td>
<td>0.83</td>
<td>0.66</td>
</tr>
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Figure 6. A, combination experiment of NMS-E973 with PLX-4720. Twenty-four hours after the seeding of exponentially growing A375 cells, the experiment was started by addition of PLX-4720, followed 24 hours later by the treatment with scalar doses of NMS-E973. C.I. calculated for mutually nonexclusive drugs are indicative of synergism. B, antitumor efficacy in A375 model (7 mice mice/group). NMS-E973 was administered with the schedule 60 mg/Kg twice daily, 3-1-3, on-off-on, whereas PLX-4720 was given orally for 10 consecutive days. The combination resulted in an efficacy superior to the single treatments, as judged by the delay observed in tumor regrowth after initial regression, and also by the increase in median survival time for the combination of the drugs (59 days), as compared to the MST of 32, 39, and 42 days registered for controls, PLX-4720 and NMS-E973, respectively. C, modulation of Hsp90-dependent markers after 24 hour incubation of RKO cells with the indicated doses of NMS-E973 and PLX-4720 as single agents or in combination. D, in vivo antitumor efficacy in the RKO model resistant to vemurafenib. RKO cells were inoculated subcutaneously in 7 nude mice/group, and NMS-E973 was administered with the schedule 60 mg/Kg bid, 3-1-3, on-off-on.
Discussion

Despite the significant number of molecules that have entered clinical studies (39–41), the development of Hsp90 inhibitors has been delayed so far by the weak potency of the first-generation drugs that resulted in modest clinical activity, by the difficulty to optimize the treatment schedule towards a suitable therapeutic window, and by the challenge to identify the most sensitive patient population. However, there is currently a new wave of interest in this field linked to the promising clinical activity observed recently with retasipymcin and ganetesib in Alk-driven NSCLCs (5, 6) and in ErbB2-positive metastatic breast cancers (7, 42, 43), that allowed to identify clinical settings in which schedule optimization of agents with higher potency resulted in permissive conditions for selected Hsp90 inhibitors.

Here, we showed the characterization of NMS-E973, a lead compound belonging to a novel isoxazole series structurally unrelated to ansamycins. NMS-E973 binds with high potency and selectivity to the ATP binding pocket of Hsp90aa, shows broad antiproliferative activity in vitro associated to the degradation of Hsp90 client proteins such as Flt3, B-Raf, AKT, with the resultant block of key pathways for tumor cell progression, such as the Raf/MAPK, PI3K/AKT, and JAK/STAT signal transduction pathways.

In vivo, NMS-E973 showed high antitumor efficacy in all the models tested, including A375 and A2780 xenografts, achieving tumor shrinkage also after rechallenge of large tumor masses. Schedule optimization supported by an efficient pharmacokinetic/pharmacodynamic model allowed identification of the 3-1-3 treatment as an efficient way to maximize the efficacy of the compound at tolerated doses.

NMS-E973 showed efficacy in models representative of clinical settings with high medical need. The best approach to the treatment of Flt3ITD AML is currently undefined, and multiple clinical trials are investigating inhibitors of the Flt3 kinase. These agents showed, so far, clinical activity in patients with Flt3-mutant AML that correlates with effective in vivo suppression of the Flt3 target, although it is very often transient, possibly due to inadequate dosing or insufficient selectivity of these drugs that may reduce their tolerability (44). NMS-E973 treatment of the MOLM-13 Flt3ITD tumor cell line caused efficient target degradation and silencing of the pathway and resulted in complete eradication of the tumors after both subcutaneous and intravenous tumor cell implantation. This activity was superior to that of the multi-kinase inhibitor sunitinib in the same experiment and compared favorably with the activity of AC220 (45), the most potent and selective Flt3 inhibitor described so far, tested in the same model (data not shown). Resistance mutations to Flt3 inhibitors in clinics are beginning to emerge, such as the D835Y mutation in the tyrosine kinase domain (45), raising the opportunity for future studies with NMS-E973 in this setting.

Metastases to vital organs of difficult surgical access are the ultimate cause of death when not controlled, and brain metastases are the most life-threatening among the secondary localizations of cancers such as melanoma, NSCLC, and breast cancers for their unresponsiveness to chemotherapeutic treatments. Brain cancers represent another crucial unmet medical need, and although the presence and function of the BBB in brain tumors is still poorly understood, it is likely that small tumors could be protected from therapeutics by the barrier itself, creating a "sanctuary" site (46, 47). Among Hsp90 inhibitors, SNX-5422 does not reach the brain (48), the efficacy of AUY-922 in brain cancers has been restricted so far to subcutaneously implanted glioblastomas due to low BBB permeability (49), and only CUDC-305 (50) is reported to have the capacity to penetrate the brain barrier. We showed that NMS-E973 enters the brain and slows the progression of an intracranially implanted melanoma model and would suggest the opportunity of investigating clinical efficacy on brain tumors while potential CNS effects should clearly be closely monitored.

Vemurafenib and crizotinib are recently approved kinase inhibitors that have shown unprecedented activity in melanoma and Alk-positive NSCLC, respectively. However, the efficacy of these drugs is limited by target mutations (for Alk), resistance mechanisms based on activation of additional pathways and progression due to brain metastases. Also, vemurafenib has failed to show activity in B-Raf-mutated colon cancers. The preclinical features of NMS-E973 have the potential to overcome these limitations, based on its high activity towards the known Alk resistance mutations and the V600E vemurafenib resistant tumors, the broad spectrum of Hsp90 target proteins, the ability to penetrate the blood brain barrier and its proven activity in intracranial tumor models. The observed synergy with the B-Raf inhibitor PLX-4720 in melanoma may also suggest additional therapeutic combinations in some tumors.

The results obtained for NMS-E973 show the high potential of this class in the Hsp90 arena, including the ability to pass the BBB which is an uncommon feature among the compounds described so far. We are currently completing the lead optimization of the NMS-E973 class to allow the final selection of the candidate for clinical studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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