BIIB021, an orally available, fully synthetic small-molecule inhibitor of the heat shock protein Hsp90

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Abstract

Inhibition of heat shock protein 90 (Hsp90) results in the degradation of oncoproteins that drive malignant progression, inducing cell death, making Hsp90 a target of substantial interest for cancer therapy. BIIB021 is a novel, fully synthetic inhibitor of Hsp90 that binds competitively with geldanamycin in the ATP-binding pocket of Hsp90. In tumor cells, BIIB021 induced the degradation of Hsp90 client proteins including HER-2, AKT, and Raf-1 and up-regulated expression of the heat shock proteins Hsp70 and Hsp27. BIIB021 treatment resulted in growth inhibition and cell death in cell lines from a variety of tumor types at nanomolar concentrations. Oral administration of BIIB021 led to the degradation of Hsp90 client proteins measured in tumor tissue and resulted in the inhibition of tumor growth in several human tumor xenograft models. Studies to investigate the antitumor effects of BIIB021 showed activity on both daily and intermittent dosing schedules, providing dose schedule flexibility for clinical studies. Assays measuring the HER-2 protein in tumor tissue and the HER-2 extracellular domain in plasma were used to show interdiction of the Hsp90 pathway and utility as potential biomarkers in clinical trials for BIIB021. Together, these data show that BIIB021 is a promising new oral inhibitor of Hsp90 with antitumor activity in preclinical models. [Mol Cancer Ther 2009;8(4):921–9]

Introduction

Heat shock protein 90 (Hsp90) is a widely expressed molecular chaperone that functions in the maturation and stabilization of cellular proteins (1–3). Hsp90, in complex with other cochaperone proteins, catalyzes the conformational changes of client proteins via its ATPase activity (4). The activity of Hsp90 maintains a variety of client proteins in their active conformation (5). Hsp90 also plays an important role in the regulation of several key oncogenic signaling proteins (6–8) and steroid receptors (9). Mutated proteins are particularly dependent on Hsp90 for the maintenance of the active conformation (2, 3).

Ansamycin drugs such as geldanamycin bind in the ATP-binding site in the NH_2 terminus of Hsp90 (6, 10). This binding inhibits the chaperone activity of Hsp90 and results in proteasomal degradation of the client proteins (5, 11–13). Because tumor cells rely on the activity of client proteins for cell proliferation and survival, drug-induced client protein degradation leads to cytostasis and/or selective cell killing of tumor cell *in vitro* and *in vivo* (14–16).

The semisynthetic Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) is currently in clinical trials for cancer (17–19). However, 17-AAG is expensive to prepare and difficult to formulate. The problematic nature of the formulations may well contribute to the dose-limiting toxicity observed with this compound. 17-AAG is also susceptible to metabolism by NQO1/DT-diaphorase enzymes (20) and to efflux by P-glycoprotein (21). The identification of a synthetic Hsp90 inhibitor would be of great therapeutic interest as it would circumvent the pharmaceutical issues associated with 17-AAG and potentially also avoid the dose-limiting toxicity of the ansamycin class of drugs. Efforts to generate a synthetic inhibitor of Hsp90 have been reported by several groups (22, 29).

BIIB021, formerly CNF2024, is a synthetic, new chemical entity designed to inhibit Hsp90. BIIB021 binds in the ATP-binding pocket of Hsp90, interferes with Hsp90 chaperone function, and results in client protein degradation and tumor growth inhibition. *In vitro* studies showed that treatment of tumor cells with BIIB021 leads to degradation of the Hsp90 client protein, HER-2, and a plethora of other oncogenic signaling proteins. In cell proliferation assays, BIIB021 potently inhibited the growth of cell lines from a variety of tumor types. Furthermore, oral administration of BIIB021 to athymic mice bearing a variety of human tumors resulted in client protein degradation and dose-dependent inhibition of tumor growth. Pharmacokinetic studies show that despite the short serum half-life of BIIB021 in the mouse, the compound can be detected in the tumor tissue at 24 and 48 h after dose; furthermore, the biological effects of Hsp90 inhibition are sustained for >24 h. Thus, it is possible to dose BIIB021 intermittently

two or three times per week in tumor xenograft models and achieve significant antitumor activity. BIIB021, a novel, synthetic inhibitor of Hsp90 that can be administered orally, is a promising new therapeutic for the treatment of cancer that is currently in phase I and II clinical trials in hematopoietic malignancies and solid tumors.

Materials and Methods

Test Compound

Test compounds including 17-AAG and BIIB021, 6-chloro-9-[(4-methoxy-3,5-dimethylpyridin-2-yl-)methyl]-9*H*-purin-2-amine, were synthesized at Biogen Idec (30).

Cell Lines

Tumor cell lines were obtained from the American Type Culture Collection. Cell lines were maintained in DMEM containing 1 mmol/L HEPES and 10% fetal bovine serum (complete DMEM) in a 37°C, 10% CO_2 incubator at 100% humidity or RPMI 1640 containing 1 mmol/L HEPES and 10% fetal bovine serum (complete RPMI 1640) in a 37°C, 5% CO_2 incubator at 100% humidity.

Hsp90 Binding Assay

For fluorescence polarization competition measurements, the FITC-geldanamycin probe (20 nmol/L) was reduced with 2 mmol/L TCEP at room temperature for 3 h, after which the solution was aliquoted and stored at -80°C until used. Recombinant human Hsp90α (0.8 nmol/L; Stressgen) and reduced FITC-geldanamycin (2 nmol/L) were incubated in a 96-well microplate at room temperature for 3 h in the presence of assay buffer containing 20 mmol/L HEPES (pH 7.4), 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 20 mmol/L Na_2MoO_4, 2 mmol/L DTT, 0.1 mg/mL BGG, and 0.1% (v/v) CHAPS. Following this preincubation, competitor in 100% DMSO was then added to final concentrations of 0.2 nmol/L to 10 μmol/L (final volume 100 μL, 2% DMSO). The reaction was incubated for 16 h at room temperature and fluorescence was then measured in an Analyst plate reader, excitation = 485 nm, emission = 535 nm. High and low controls contained no compound or no Hsp90, respectively. The data were fit to a four-parameter curve using GraphPad Prism and IC_{50} values were generated. The IC_{50} values were converted into inhibition constants (K_i) using the modified Cheng-Prusoff equation as described previously (31).

HER-2 Degradation Assay

MCF-7 cells (5×10^5) in complete DMEM were plated per well in 24-well plates. The cells were propagated for 24 h before compound addition. Solutions (1 mmol/L) of the compounds to be tested were prepared in DMSO and serially diluted in complete DMEM. Cells were incubated in the presence of serially diluted compound for 16 h. Following incubation, cells were rinsed with PBS and then trypsinized. After stopping the trypsin reaction with fetal bovine serum, the cell suspensions were washed in PBS containing 0.2% bovine serum albumin and 0.2% sodium azide (BA buffer) and then resuspended in 100 μL phycoerythrin-conjugated anti-HER-2 IgG antibody (Becton Dickinson Immunocytometry Systems). Untreated cells were resuspended in anti-KLH antibody as background controls. Cells were

incubated 15 min at room temperature, washed twice in 200 μL BA buffer, resuspended in 200 μL BA buffer, and transferred to 5 mL round polystyrene tubes. An additional 250 μL BA buffer was then added to each tube. Samples were analyzed using a FACSCalibur flow cytometer equipped with argon-ion laser that emits 15 mW of 488 nm light for excitation of the phycoerythrin fluorochrome.

Cell Proliferation Assay

A modified tetrazolium salt assay was used to measure the inhibition of tumor cell growth (32). Cells were added to 96-well plates and propagated for 24 h before compound addition. The compound was serially diluted and added at a concentration range of 3 to 1,000 nmol/L to the plated cells. DMSO (0.03-0.003%) was included as a vehicle control. Cells were incubated in the presence of compound for 5 days. After incubation phenazine methosulfate (stock concentration 1 mg/mL) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (stock concentration 2 mg/mL; Promega) were mixed at a ratio of 1:20 and added to each well of a 96-well plate. Reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt gave rise to a soluble formazan product that was secreted into the culture medium. After 4 h incubation, the formazan product was quantitated spectrophotometrically at a wavelength of 490 nm. Data were acquired using SOFTmaxPRO software, and 100% viability was defined as the A_{490} of DMSO-treated cells stained with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (the mean A_{490} of cells treated with DMSO at a range of 0.03-0.003%). Percent viability of each sample was calculated from the A_{490} values as follows: % viability = ($A_{490 nm}$ sample / $A_{490 nm}$ DMSO-treated cells $\times$ 100). The IC_{50} was defined as the concentration that gave rise to 50% inhibition of cell viability.

Western Blotting

Cell pellets were prepared in lysis buffer [10 mmol/L HEPES, 42 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1% Triton X-100, freshly supplemented with 1× protease inhibitor cocktail (Pierce)]. The lysate was quantified by BCA assay (Pierce) and normalized. Equal amounts of protein was loaded onto 4% to 12% Tris-glycine gels and subsequently transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5% TBS-Tween 20 and primary antibodies were added and incubated at room temperature for 1 h with shaking. The blots were washed extensively in TBS-Tween 20 before secondary antibodies were added for overnight incubation at 4°C with gentle shaking. The blots were again washed extensively and developed with SuperSignal West Femto substrate (Pierce).

Antibody sources were rabbit antibodies to HER-2/*neu*, phospho-HER-2 (Y1248), phosphatidylinositol 3-kinase p85 (Upstate Biotechnology), rabbit antibodies to insulin-like growth factor (IGF)-I receptor, AKT, phospho-AKT (S473), Raf-1, Cdk4, Cdk6, cyclin D, and progesterone receptor (Santa Cruz Biotechnology), rabbit anti-phospho-Raf (S259; Cell Signaling Technology), and rat anti-Hsp90α and mouse

Table 1. Structure and *in vitro* effects of BIIB021 compared with 17-AAG

BIIB021
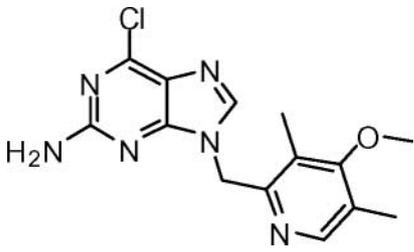
6-chloro-9-((4-methoxy-3,5-dimethylpyridin-2-yl)methyl–9*H*-purin-2-amine

	BIIB021	17-AAG
	K_i (nmol/L) ± SD	K_i (nmol/L) ± SD
Hsp90 binding assay	1.7 ± 0.4	4.6 ± 0.5
	EC$_{50}$ (nmol/L) ± SD	EC$_{50}$ (nmol/L) ± SD
HER-2 degradation assay (MCF-7 cells)	38 ± 10	12 ± 5

anti-Hsp70 monoclonal antibody (Stressgen Biotechnologies). The secondary antibodies were purchased from Jackson ImmunoResearch Lab.

Animal Studies

All animal studies were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (ILAR, 1996) and under the guidance of the Biogen Idec Institutional Animal Care and Use Committee. BALB/c and athymic mice were obtained from Harlan Sprague-Dawley at age 6 to 8 weeks. The mice were maintained in sterilized cages in a ventilated caging system (Lab Products) with a 12 h light/12 h dark photoperiod at temperature of 21°C to 23°C and a relative humidity of 50 ± 5%. Irradiated pelleted food (Harlan Teklad) and autoclaved deionized water were provided *ad libitum*. Animals were identified by the use of individually numbered ear tags. N87 tumor fragments (~2 mm^3) were implanted s.c. in the right flank of the animal. BT474 tumor cells (1×10^7) were injected together with Matrigel basement membrane matrix (BD Biosciences) s.c. in the right flank of the animal. Before BT474 inoculation, 1.7 mg/90 day release 17β-estradiol pellets (Innovative Research of America) were placed s.c. Tumor dimensions were measured using calipers and tumor volumes were calculated using the equation for an ellipsoid sphere $(l \times w^2) / 2 = mm^3$, where l and w refer to the larger and smaller dimensions collected at each measurement, respectively. Tumor volumes were measured and animals were weighed and monitored for toxicity at least twice weekly. *P* values were calculated using the two-tailed Student's *t* test to assess the difference in tumor volumes between control and treated groups. *P* < 0.05 was considered significant.

Pharmacokinetic Analysis

The high-performance liquid chromatography system consisted of an Agilent 1100 series binary pump, Agilent 1100 series autosampler, Agilent 1100 series photodiode array detector, and a Zorbax 300SB-C18, 3.5 μm particle size column (4.6 × 150 mm). A gradient method was used with mobile phase A consisting of water with 0.1% TFA. Mobile phase B was composed of acetonitrile with 0.05% TFA. After equilibration with 95% A/5% B, the mobile phase mixture was changed to 5% A/95% B over 9 min with a total run time of 15 min. The flow rate was 1.0 mL/min. Absorbance was monitored at 313 nm. A five-point standard curve ranging from 100 to 10,000 ng/mL was prepared by spiking BIIB021 (from a 1 mg/mL BIIB021 stock solution) into pooled mouse serum (BioChemMed BALB/c female mouse serum). Standards and analytic samples were prepared for analysis by adding 485 μL acetonitrile to 15 μL serum sample followed by mixing on a vortex and centrifuging at 20,000 × *g* for 10 min. The organic layer was transferred to a 96-well Deep-well Microplate (Fisher Scientific) and evaporated under nitrogen (TurboVap 96, Zymark). The pellet was reconstituted with 150 μL mobile phase (95% A/5% B). The high-performance liquid chromatography injection volume was 100 μL. The limit of quantitation for this method was 100 ng/mL.

Biomarker Assays

Both HER-2 and IGF-binding protein-2 (IGFBP-2) were measured as described (33). Briefly, an IGFBP-2 ELISA kit from R&D Systems was used to determine IGFBP-2 level. Diluted capture antibody (100 μL) was coated into 96-well plates at 4°C overnight. The plate was washed and blocked, and the samples and standards were added, incubated for 2 h, and detected with detection antibody followed by streptavidin-horseradish peroxidase and its substrates. The absorbance was determined using a microplate reader set at 450 and 540 nm. For HER-2 level in mice serum samples, an ELISA kit from Calbiochem was employed. The assay was done according to the manufacturer's protocol. The absorbance was detected using the same spectrophotometric plate reader at dual wavelengths of 450/540 nm. For both studies, statistical analyses employed the one-tailed Student's *t* test.

Tumor Processing

Snap-frozen tumors were thawed in 37°C water bath and kept on ice. The thawed tissue was later transferred onto the lid of Petri dish and added with 50 μL ice-cold Western Lysis Buffer [10 mmol/L HEPES, 42 mmol/L KCl, 5 mmol/L MgCl$_2$, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1% Triton X-100, 1× protease inhibitor cocktail (Pierce)]. The tissue was then cut into small pieces. Residual skin was removed and the remaining tissue clumps were further chopped down and transferred into 250 to 500 μL ice-cold Western Lysis Buffer. Sonication at setting 3 on a Fisher Scientific Sonic Dismemberator 550 was done until no residual solid material was visible. The lysate was then centrifuged at 12,000 rpm (14,000 × *g*), 4°C for 5 min. Supernatant was collected into a fresh tube on ice. Total protein concentration was quantified by BCA kit (Pierce). Western blot was done as described above.

Table 2.

(A) Antiproliferation activity of BIIB021 in tumor cell lines in culture

Tumor cell line	Origin	IC_{50} (μmol/L)
BT474	Breast carcinoma	0.14
MCF-7	Breast carcinoma	0.31
N87	Gastric carcinoma	0.06
HT29	Colon carcinoma	0.19
H1650	Non-small cell lung cancer	0.15
H1299	Non-small cell lung cancer	0.16
H69	Small cell lung cancer	0.25
H82	Small cell lung cancer	0.25

(B) Summary of tumor growth inhibition with BIIB021

Tumor model	Dose (mg/kg)	Weekly schedule	Tumor type	% Tumor growth inhibition	P
N87	125	qdx5	Gastric	87	0.0001
BT474	120	qdx5	Breast	94	0.00005
CWR22	120	qdx5	Prostate	85	0.02
U87	75	bidx5	Glioblastoma	70	0.02
SKOV3	124	qdx5	Ovarian	67	0.003
Panc-1	124	qdx5	Pancreatic	51	0.002

Histology

Tumors were collected and fixed in 10% buffered formalin at various time points after administration of control vehicle or compound. The tissues were embedded in paraffin and slices of tissue (5 μm thick) were placed on charged slides (Comparative Biosciences). HER-2 was visualized using the Hercept-Test kit (DAKO) according to the product instructions.

Results

BIIB021 Binds to Hsp90 and Induces Client Protein Degradation

BIIB021 is a novel, synthetic inhibitor of Hsp90 optimized for its potency, selectivity, inhibition of cell proliferation, and favorable pharmacokinetic profile. The structure of BIIB021 is shown in Table 1. BIIB021 exhibits competitive binding with geldanamycin and binds the ATP pocket of Hsp90. A fluorescent polarization assay was developed for Hsp90α using FITC-labeled geldanamycin, a well-characterized ATP competitive inhibitor (34–36). The binding affinities (K_i values) for BIIB021 and 17-AAG to Hsp90α were found to be 1.7 ± 0.4 and 4.6 ± 0.5 nmol/L, respectively (Table 1).

BIIB021 was tested for selectivity against another target class, the protein kinases, which also bind ATP. The protein kinases tested were Aurora-A, CHK2, c-RAF, cSrc, IKKα, MEK1, MAPK1, MAPK2, PDK1, Plk3, PKCα, Cdk2/cyclin A, p38α, PKB/AKT, EGFR, HER-2, Flt-3, IGF-I receptor, JNK1, and PDGFRα. In addition, BIIB021 was tested for inhibitory activity against another ATPase, Na$^+$K$^+$ ATPase. No significant inhibition (<10% inhibitor activity) was
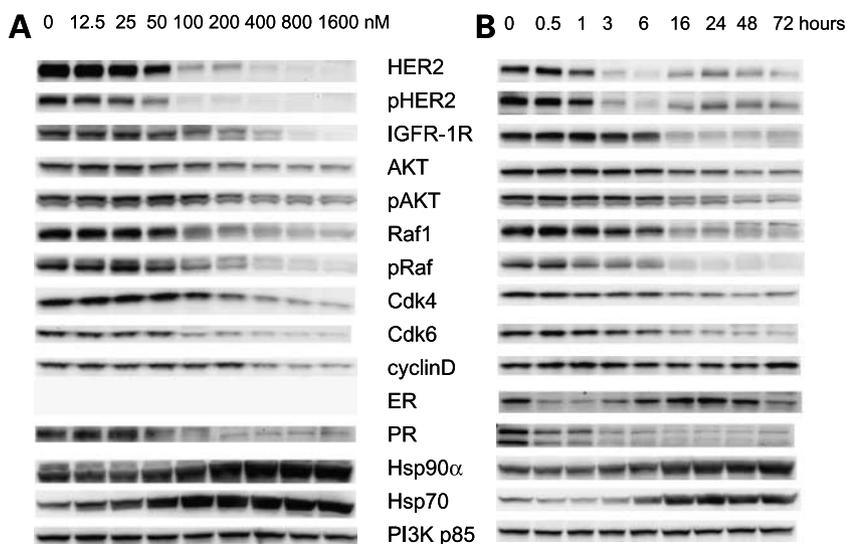

Figure 1. Western blots of Hsp90 client proteins and heat shock proteins after treatment of MCF-7 human tumor cells with BIIB021 *in vitro*. **A,** MCF-7 cells were treated with the indicated concentrations of BIIB021 for 24 h and collected and processed for Western blotting. **B,** MCF-7 cells were treated with 400 nmol/L BIIB021 for the indicated periods after which the cells were collected and prepared for Western blot analysis (ER changes were detected at 100 nmol/L BIIB021).

Figure 2. Inhibition of tumor growth by BIIB021 on a daily dose schedule. Athymic mice bearing established N87 gastric (**A**) or BT474 breast (**B**) tumors were treated with BIIB021 orally at the indicated doses qdx5 for 5 and 4 wk, respectively. Tumors were measured using Vernier calipers on the indicated days and tumor volumes were reported as mean ± SE tumor volumes for groups of 8 to 10 mice. *, $P < 0.05$, significant differences between treated and control groups (Student's t test). Plasma biomarkers for Hsp90 inhibitors. HER-2 extracellular domain (**C**) and IGFBP-2 levels (**D**) in plasma from mice bearing established BT474 tumors 24 h after receiving a single dose of BIIB021.
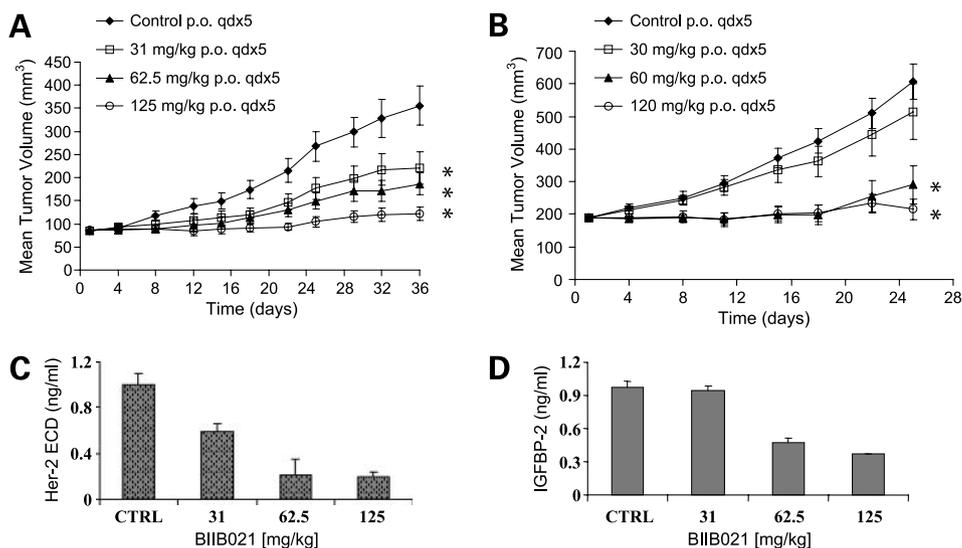
observed at 10 μmol/L with BIIB021. These studies showed that BIIB021 is selective for Hsp90 over kinases and another ATPase.

Degradation of HER-2 is an on-target effect of Hsp90 Inhibitors (12). Consequently, the primary cell-based assay used to select potent Hsp90 inhibitors was a HER-2 degradation assay developed in MCF-7 cells. MCF-7 cells were treated with compound for 16 h followed by flow cytometric measurement of HER-2 levels. The EC_{50} was defined as the concentration that gave rise to 50% degradation of the HER-2 protein. In this assay, the EC_{50} for HER-2 degradation of BIIB021 in MCF-7 cells was 38 ± 10 nmol/L and the EC_{50} for 17-AAG was 12 ± 5 nmol/L (Table 1). The EC_{50} values for two synthetic Hsp90 inhibitors that were recently reported to have entered clinical trials (37) were also obtained using this assay. The EC_{50} value for SNX-2112 (28, 37) was 22 ± 3 nmol/L and the EC_{50} value for NVP-AUY922 (29) was 7.2 ± 1 nmol/L in the HER-2 degradation assay.

BIIB021 Inhibits Tumor Cell Proliferation

The effects of BIIB021 on cell proliferation in a variety of tumor cell lines are shown in Table 2. The cell growth-inhibitory activity of BIIB021 was determined in cell lines with a range of concentrations of BIIB021 for 5 days followed by colorimetric measurement of cell viability [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay]. The IC_{50} was defined as the concentration of drug that resulted in 50% inhibition of cell growth. The IC_{50} for BIIB021 ranged from 0.06 μmol/L in N87 cells to 0.31 μmol/L in MCF-7 cells and was 0.14 μmol/L in BT474 cells.

Effects of BIIB021 on Client Proteins *In vitro*

The effect of BIIB021 on Hsp90 client and heat shock proteins was tested over a range of concentrations in the MCF-7 tumor cell line. The cells were exposed to concentrations of 12.5 to 1,600 nmol/L BIIB021 for 24 h. Expression and/or phosphorylation of various client and heat shock proteins were determined by Western blotting (Fig. 1A). The phos-

phorylation and protein levels of HER-2 were significantly reduced at a concentration of 50 nmol/L and barely detectable at 100 nmol/L BIIB021. Degradation on several other client proteins, including IGF-I receptor, AKT, Raf-1, Cdk4, Cdk6, and progesterone receptor were observed after treatment with 100 to 200 nmol/L BIIB021. Induction of the heat shock proteins, Hsp90α and Hsp70, were observed in a concentration-dependent manner. As expected, there was no change in expression level of the nonclient protein phosphatidylinositol 3-kinase p85 subunit.

In a time-course study, MCF-7 cells were treated with 400 nmol/L BIIB021 for various lengths of time, up to 72 h. The same group of client proteins and heat shock proteins was analyzed again by Western blotting. As can be seen in Fig. 1B, MCF-7 cells treated with BIIB021 exhibited decreased levels the Hsp90 client proteins HER-2, estrogen receptor, and progesterone receptor as early as 1 to 3 h after treatment. Decreased levels of total and phospho-AKT and Raf-1 occurred in the 6 to 16 h timeframe. Degradation of the Cdk4 and Cdk6 was also observed 16 h after treatment. Again, no change in the phosphatidylinositol 3-kinase p85 levels was observed. An increase in Hsp90α, Hsp70, and Hsp27 levels was detected from ~6 h and persisted for up to 72 h after treatment.

In vivo Antitumor Effects of BIIB021 Administered Orally on a Daily Schedule

BIIB021 was tested for antitumor efficacy in human tumor xenografts grown in nude mice. BIIB021 was administered to animals bearing N87 stomach carcinoma tumors at doses of 31, 62.5, and 125 mg/kg, once daily, from Monday to Friday, for 5 weeks. As can be seen from the results in Fig. 2A, BIIB021 showed significant activity in this study. On the final study day, the observed tumor growth inhibition was 46% ($P = 0.02$) with 31 mg/kg, 65% ($P = 0.002$) with 62.5 mg/kg, and 87% ($P = 0.0001$) with 125 mg/kg. The mean body weights of the control and treated groups did not differ significantly over the course of dosing. There was one death in the 125 mg/kg group on day 21.

Antitumor activity was also observed in mice bearing established xenografts of the human breast cancer line BT474 that expresses high levels of the Hsp90 client protein, HER-2 (Fig. 2B). In this study, 30, 60, and 120 mg/kg BIIB021 was administered orally, once daily, from Monday through Friday, for 4 weeks. The mean tumor volume results from the study showed that BIIB021 had significant activity. On day 24, 22% (not significant), 76% ($P = 0.001$), and 94% ($P = 0.0001$) tumor growth inhibition were observed at doses 30, 60, and 120 mg/kg, respectively. There was one death in the high-dose group on day 16. A summary of tumor growth inhibition, including additional tumor xenograft models, is shown in Table 2. As can be seen, significant antitumor activity was also observed in the CWR22 prostate, U87 glioblastoma, SKOV3 ovarian, and Panc-1 pancreatic tumor xenograft models.

Evaluation of Hsp90 Inhibition in Plasma

Plasma from mice bearing BT474 tumors was collected 24 h after a single oral dose of BIIB021 to identify circulating biomarkers that would be useful for measuring the

activity of Hsp90 inhibitors. These biomarkers are being evaluated for potential clinical application. ELISA-based assays were done to compare plasma from control and BIIB021-treated mice for circulating HER-2 extracellular domain (HER-2 ECD) and IGFBP-2 (33). A dose-dependent decrease in plasma levels of both HER-2 ECD (Fig. 2C) and IGFBP-2 (Fig. 2D) was observed.

The pharmacodynamic effects of a single oral dose of BIIB021 were evaluated in the BT474 xenograft model (Fig. 3). Control vehicle or 30, 60, or 120 mg/kg BIIB021 was administered to mice bearing BT474 tumors. Six hours after the dose, tumors, spleens, and plasma were collected and tumor and spleen lysates were prepared. Western blot analysis showed dose-dependent degradation of key client proteins including HER-2, Raf-1, and cyclin D (Fig. 3A) in tumor tissue. A decrease in the phosphorylation state of HER-2, AKT, and Raf-1 was also observed. Oral treatment with BIIB021 also resulted in the induction of Hsp70 in both tumor and spleen tissue (Fig. 3A and B). Protein levels of a non-Hsp90 client protein, phosphatidylinositol 3-kinase p85, remained constant. In a further experiment, formalin-fixed, paraffin-embedded BT474 tumor sections were prepared for immunohistochemical staining for the HER-2 protein using an antibody directed against HER-2 (HercepTest kit; DAKO). Staining of control tissue detected HER-2 on the cell membranes of BT474 tumor cells. Eight hours after an oral dose of 150 mg/kg BIIB021, HER-2 levels on the surface of the cells had markedly decreased (Fig. 3C).

BIIB021 Mouse Pharmacokinetic and Dose Schedule

The pharmacokinetic profile of BIIB021 was evaluated in mice. A single dose of 75 or 150 mg/kg BIIB021 was administered orally to BALB/c mice and serum samples were collected over 4 h to evaluate the level of BIIB021 using high-performance liquid chromatography (Fig. 4A). The maximum serum concentration was observed by 5 min, and the C_{max} was 10.2 and 19.5 μg/mL for the 75 and 150 mg/kg doses, respectively. The half-life of the compound was 0.5 to 1 h in mouse serum and the exposure (AUC) through the last time point collected was 2.3 and 9.1 μg h/mL, respectively.

Despite the short half-life of BIIB021 in serum, it was measurable in tumors at 24 and 48 h (Fig. 4B). Athymic mice with BT474 tumors were administered 75 and 150 mg/kg BIIB021 orally and the tumors and spleens were collected at 8, 24, and 48 h. A portion of the tumor tissue was prepared for pharmacokinetic evaluation as before, and the spleens and the remaining tumor tissue were prepared for Western blot analysis of protein levels. As can be seen in Fig. 4B, BIIB021 is readily detectable in the tumor at 8 h and remains detectable for up to 2 days. Western blot analysis showed a decrease in HER-2 protein in the tumor at 6 h (Fig. 4C). By 24 h, HER-2 levels increased; by 48 h, the levels were similar to control HER-2 levels. In addition, phospho-AKT and cyclin D protein levels were decreased at 6 h and returned to control levels by 24 h. Raf-1 protein levels decreased slightly. Western blot analysis also showed an increase in Hsp70 protein levels at 6 and 24 h. These levels were close to control levels by 48 h.
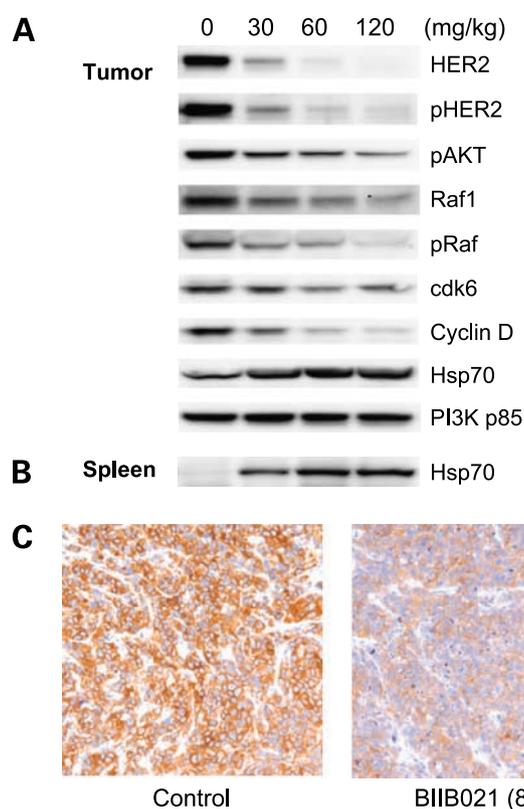

Figure 3. Dose-dependent effects on Hsp90 client proteins and heat shock proteins in the BT474 breast tumor xenograft model. Athymic mice bearing established BT474 breast tumors were treated with a single oral dose of BIIB021. Tumors were collected at 6 h post-dose and flash-frozen for Western blot analysis or 8 h and fixed in 10% buffered formalin and embedded in paraffin. **A,** Western blot analysis of tumor lysates using specific antibodies as described in Materials and Methods. **B,** Western blot analysis of spleen lysates using anti-Hsp70 antibody. **C,** immunohistochemical evaluation using the HercepTest (DAKO) to assess HER-2 levels in BT474 tumor tissue 8 h after treatment with BIIB021.
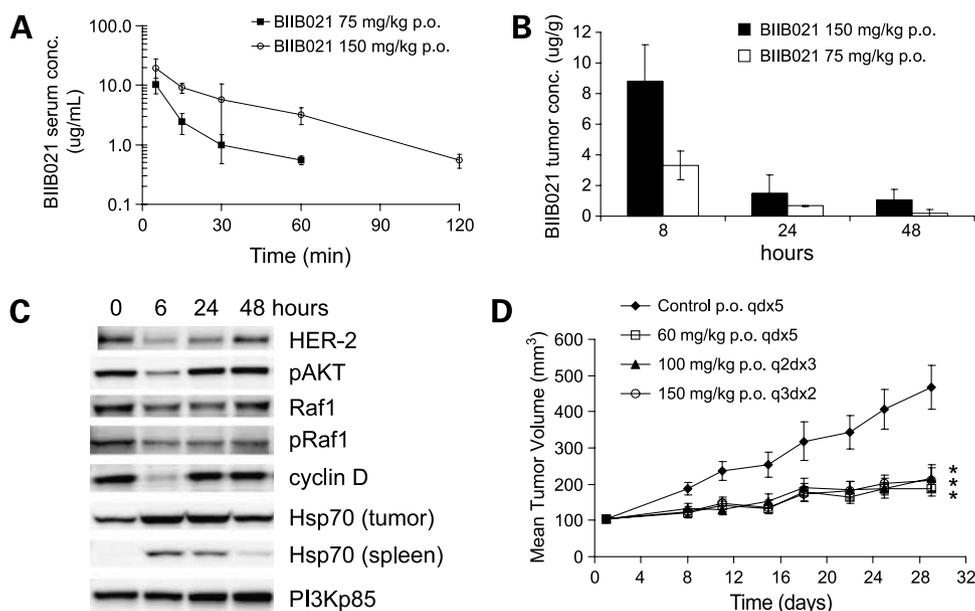
Figure 4. BIIB021 pharmacokinetic profile in mice and dose schedule evaluation in the N87 xenograft model. Serum (**A**) and tumor (**B**) levels of BIIB021 after a single oral dose of BIIB021 at 75 and 150 mg/kg. **C,** athymic mice bearing established BT474 breast tumors were treated with a single oral dose of BIIB021 at 150 mg/kg. Tumors were collected at 6, 24, and 48 h post-dose and flash-frozen for Western blot analysis. **D,** athymic mice bearing established BT474 breast tumors were treated with BIIB021 orally with a total weekly of 300 mg/kg for 4 wk on the indicated weekly dose schedules, 60 mg/kg qdx5 (from Monday to Friday), 100 mg/kg q2dx3 (Monday, Wednesday, and Friday), or 150 mg/kg q3dx2 (Monday, Friday). Tumors were measured using Vernier calipers on the indicated days and tumor volumes were reported as mean ± SE tumor volumes for groups of 8 to 10 mice. *, $P < 0.05$, significant differences between treated and control groups (Student's *t* test).

The schedule dependency of BIIB021 was evaluated in the N87 xenograft model (Fig. 4D) and BT474 model (data not shown). The dosing regimen was based on administration of 300 mg/kg BIIB021 per week. Mice received 60 mg/kg on a daily schedule for 5 days per week, 100 mg/kg three times per week, or 150 mg/kg twice per week. As can be seen from the results in Fig. 4D, each dosing regimen showed a significant ($P < 0.05$) inhibition of tumor growth by up to 70% to 80% by 28 days post-treatment. Furthermore, there was no significant difference between the dosing regimens. The results for the BT474 study were similar, with a 75% to 98% tumor growth inhibition ($P < 0.05$) and no significant differences between the groups (data not shown).

Discussion

The development of novel, small-molecule inhibitors that are effective against a specific molecular target is an important strategy in the search for new treatments for cancer. Hsp90 is a widely expressed molecular chaperone that plays an important role in the regulation of key oncogenic signaling proteins (15, 38, 39). Inhibition of Hsp90 has been shown to result in the destabilization and degradation of proteins involved in tumor cell signaling (5, 40). These Hsp90 client proteins are part of several different pathways (HER-2, AKT, cell cycle, transcription factors, and hormone receptors; refs. 2, 3). Thus, Hsp90 inhibition provides an attractive approach for targeting multiple signaling pathways important in tumor growth.

BIIB021, a fully synthetic compound (30), is a new chemical entity designed to inhibit Hsp90 that offers the potential for a novel therapeutic treatment for cancer. In the current studies, BIIB021 was shown to bind competitively with geldanamycin in the ATP-binding site of Hsp90. In this binding affinity assay, BIIB021 showed higher binding affinity for Hsp90. The K_i for BIIB021 was 1.7 nmol/L and the IC_{50} for 17-AAG was 4.6 nmol/L. Furthermore, BIIB021 is not susceptible to metabolism NQO1/DT-diaphorase enzymes or to efflux by P-glycoprotein, thus avoiding some of the liabilities of 17-AAG.[3]

One of the consequences of inhibiting Hsp90 in cells is that proteins that are dependent on Hsp90 for proper activity and folding, so-called client proteins, are degraded via the proteasome pathway (5). HER-2 is one of the most sensitive client proteins identified to date (7, 12, 14). A cell-based HER-2 degradation assay was therefore implemented as a primary screen for Hsp90 inhibitors. The assay, run in MCF-7 tumor cells, was used as a screen to identify novel synthetic compounds that were potent inhibitors of Hsp90. BIIB021 induced the degradation of HER-2 in this assay with an EC_{50} value of 38 nmol/L. In other cell-based studies, treatment of MCF-7 cells with BIIB021 resulted in the degradation of many of the key client proteins that are known to be dependent on Hsp90 in a time- and dose-dependent manner. BIIB021 also inhibited the growth of a variety of human tumor cell lines *in vitro*.

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[3] H. Zhang, submitted for publication.

The antitumor activity of oral BIIB021 was shown when the compound was administered daily, 5 days per week for 4 or 5 weeks, in two high HER-2 xenograft models, the BT474 breast carcinoma model and the N87 gastric carcinoma model. Antitumor activity was also shown in models of other client proteins including the CWR22 prostate model, which expresses the androgen receptor in addition to HER-2 (15). In the BT474 tumor xenograft model, a single oral dose of BIIB021 led to the degradation of key client proteins as measured by Western blot analysis of tumor cell lysates and by immunohistochemistry with an anti-HER-2 antibody. Decreased levels of circulating HER-2 extracellular domain and IGFBP-2 were observed in plasma. Induction of a heat shock response as measured by increased levels of Hsp70 protein was also observed in both tumor tissue and spleen, indicating that this synthetic compound has the same mechanism of action as the natural product geldanamycin and its derivative 17-AAG as well as other Hsp90 inhibitors (41, 42). For novel compounds undergoing clinical trials, it is useful to have biomarkers that will aid in determining clinically active doses. Assays that measure the HER-2 protein, HER-2 extracellular domain, and Hsp70 protein levels in patients can be evaluated for this purpose.

Pharmacokinetic studies show that BIIB021 has a relatively short half-life in serum. However, the compound can be measured in tumor tissue as far as 48 h post-treatment. Maximal HER-2 degradation is seen at 6 to 8 h and is still below normal levels at 24 h. In addition, Hsp70 induction is observed at 6 and 24 h, and relative to control, levels remain elevated at 48 h. Intermittent dosing schedules also indicate that the effects of BIIB021 last for >24 h because the compound can be administered daily, three times weekly, or twice weekly at the same total weekly dose with the achievement of similar tumor growth inhibition.

The flexibility of dosing afforded by BIIB021 could be valuable clinically. Early clinical trials with 17-AAG revealed that this drug is unsuitable for administration on schedules more frequent than twice weekly. Daily treatment resulted in dose-limiting hepatotoxicity at subtherapeutic doses (19) and subsequent trials have employed exclusively biweekly, weekly, or even more intermittent schedules (17). A recent report describing a novel proteasome inhibitor (drugs whose mode of action overlaps with that of Hsp90 inhibitors) showed that the ability to give the drug on consecutive days conferred significantly enhanced antitumor activity relative to bortezomib, which can only be given twice per week (43). In this study, we observed that some client proteins (e.g., HER-2) disappear rapidly after application of Hsp90 inhibitors, whereas others (e.g., AKT) persist for days in the presence of drug, confirming previous findings with ansamycin and non-ansamycin drugs (23, 39). Thus, tumors driven by different client proteins may respond optimally to different schedules of treatment with Hsp90 inhibitors.

In conclusion, BIIB021 is a synthetic, orally bioavailable Hsp90 inhibitor that binds selectively to Hsp90. BIIB021 causes HER-2 degradation, an increase in the induction of heat shock proteins, and growth inhibition in human tumor cell lines. Oral administration of BIIB021 also resulted in similar pharmacodynamic effects *in vivo*. Tumor growth inhibition was observed in several human tumor xenografts both on a daily times five schedule and on intermittent schedules. BIIB021 is a promising new chemical entity being tested in clinical trials.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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