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Antitumor Activity of the Hsp90 Inhibitor IPI-504 in HER2-Positive Trastuzumab-Resistant Breast Cancer

Maurizio Scaltriti[1,4], Violeta Serra[1], Emmanuel Normant[5], Marta Guzman[1], Olga Rodriguez[1], Alice R. Lim[5], Kelly L. Slocum[5], Kip A. West[5], Varenka Rodriguez[5], Ludmila Prudkin[2], José Jimenez[2], Claudia Aura[2], and José Baselga[1,3,4]

Abstract

Hsp90 facilitates the maturation and stability of numerous oncoproteins, including HER2. The aim of this study was to assess the antitumor activity of the Hsp90 inhibitor IPI-504 in trastuzumab-resistant, HER2-overexpressing breast cancer cells. Therapy with trastuzumab, IPI-504, and the combination of trastuzumab and IPI-504 was evaluated in trastuzumab-sensitive and trastuzumab-resistant cells. Inhibition of protein targets, cell proliferation, and tumor growth was assessed *in vitro* and in xenograft models. IPI-504 inhibited proliferation of both trastuzumab-sensitive and trastuzumab-resistant cells. Administration of IPI-504 markedly reduced total levels of HER2 and Akt, as well as phosphorylated Akt and mitogen-activated protein kinase (MAPK), to an equal extent in trastuzumab-sensitive and trastuzumab-resistant cells. IPI-504, used as single agent or in combination with trastuzumab, also inhibited *in vivo* the growth of both trastuzumab-sensitive and -resistant tumor xenografts. As a mechanism for the observed antitumor activity, IPI-504 resulted in a marked decrease in the levels of HER2, Akt, p-Akt, and p-MAPK in trastuzumab-resistant xenografts as early as 12 hours after a single dose of IPI-504. IPI-504–mediated Hsp90 inhibition may represent a novel therapeutic approach in trastuzumab refractory HER2-positive breast cancer. *Mol Cancer Ther; 10(5); 817–24. ©2011 AACR.*

Introduction

Breast cancer is the most common cancer among women of Western countries and is the second leading cause of cancer death in women. Amplification of the *HER2* oncogene has been reported in approximately 20% to 30% of human breast tumors, and HER2 overexpression is associated with poor clinical outcome and recurrent disease (1–4). A milestone in the treatment of breast cancer was the development of trastuzumab, a humanized monoclonal antibody targeting HER2. Single-agent trastuzumab is clinically active and well tolerated among patients with advanced, refractory breast cancer (5–8). Combining trastuzumab with chemotherapy improves survival in metastatic, HER2-positive breast cancer, and this regimen is the current standard of care for this patient population (9, 10).

Despite these advances, intrinsic or acquired resistance to trastuzumab is common and eventually occurs in all patients with metastatic disease. With the exception of some cases in which trastuzumab resistance may occur as a result of loss of HER2 (11), the majority of mechanisms of resistance described to date are the result of continued hyperactivation of HER2 downstream signaling in the presence of trastuzumab-insensitive truncated receptors (12), HER2 dimerization with other receptors (13–15), downstream deletion of tumor suppressor genes such as *PTEN* (16), or activation mutations of phosphoinositide 3-kinase (PI3K; ref. 17). Thus, these tumors still remain heavily dependent on the HER2 pathway for their malignant behavior.

The Hsp90 chaperone complex facilitates the conformational maturation, stability, and activation of numerous wild-type and mutated oncoproteins, including HER2 and mutated EGFR (18). Some of the critical components of the HER2 signaling pathway such as Akt are also client proteins of Hsp90. Accordingly, inhibition of Hsp90 induces proteasomal degradation of HER2 and suppresses growth of breast cancer cells and breast cancer xenograft tumors *in vivo* (18–22). The

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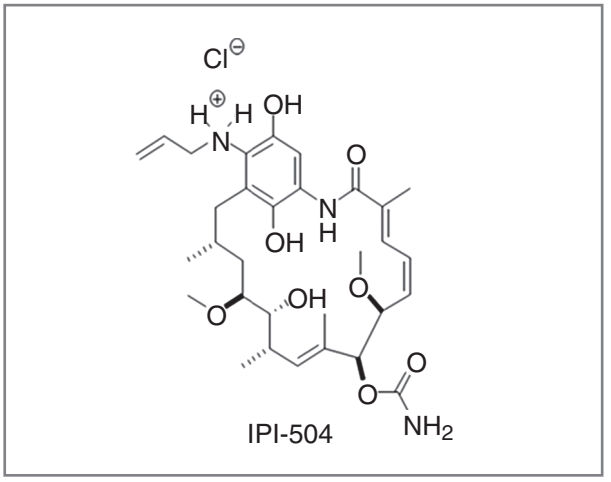

Figure 1. Structure of IPI-504.

Hsp90 inhibition–mediated degradation of HER2 is enhanced with the addition of trastuzumab (23), and an early clinical trial that combined the Hsp90 inhibitor 17-AAG with trastuzumab has shown signs of efficacy among patients with trastuzumab refractory breast cancer (23, 24). 17-AAG is a potent inhibitor of Hsp90, but its clinical development has been hampered by pharmacologic liabilities, including poor aqueous solubility, which could limit patient safety and the therapeutic index.

IPI-504 (retaspimycin hydrochloride) is a water-soluble, selective Hsp90 inhibitor capable of interconvertion to 17-AAG *in vivo* (see molecule structure in Fig. 1). Both 17-AAG and IPI-504 have shown activity in multiple models of solid (e.g., lung, breast, pancreatic, and melanoma) and hematologic (e.g., chronic myelogenous leukemia and multiple myeloma) cancers (25–28). In HER2-positive human breast cancer cell lines, IPI-504 potently downregulates HER2 and inhibits cell growth (27). IPI-504 is currently under clinical investigation in a phase I/II trial in combination with docetaxel that is focused on patients with non–small cell lung cancer.

An unsolved question that is pertinent to the development of Hsp90 inhibitors is the level of activity of this class of agents in trastuzumab-resistant cells. The results described here show that IPI-504 treatment induces HER2 and Akt degradation with consequent cell growth inhibition in several models of trastuzumab-resistant cells, both *in vitro* and *in vivo*. The addition of trastuzumab does not seem to provide significant benefits to the antitumor activity of IPI-504. These data suggest that treatment with of IPI-504 may represent an effective therapeutic strategy in trastuzumab refractory breast cancer.

Materials and Methods

Cell lines and treatments

BT474, SKBR-3, and HCC1569 cells were obtained from the American Type Culture Collection (ATCC). BT474 and SKBR-3 cell (purchased in 2004) were maintained in Dulbecco's Modified Eagle Medium/Ham F12 1:1 (DMEM/F12) supplemented with 10% FBS and 2 mmol/L L-glutamine (Life Technologies, Inc. Ltd.) at 37°C in 5% CO_2. HCC1569 cells (purchased in 2006) were maintained in RPMI-1640 medium containing 2 nmol/L L-glutamine (Invitrogen) supplemented with 10% FBS (Hyclone) at 37°C in 5% CO_2. BT474, SKBR-3, and HCC1569 cells were authenticated by ATCC (DNA fingerprinting, karyotyping, and morphology study), and cells were not passaged/maintained for more than 6 months after resuscitation. BT474 and SKBR-3 HER2-positive breast cancer cell lines, with acquired resistance to trastuzumab (BT474R and SKBR-3R), were obtained by culturing parental cells with increasing concentrations of trastuzumab for longer than 18 months. Several independent subclones refractory to the antiproliferative effects of trastuzumab were isolated from the pool of resistant cells and used for further analyses. BT474R and SKBR-3R clones were authenticated in-house by single-nucleotide polymorphism array analyses in 2008. Levels of HER2 and phosphorylated HER2 were not significantly different between parental and resistant cells (data not shown). Furthermore, there were no observed changes in trastuzumab binding affinity to HER2 in resistant cells relative to parental cells (data not shown).

BT474 cells transfected with empty pBabe vector (BT474 empty) and stably overexpressing the p110α subunit of PI3K bearing the H1047R mutation (BT474H1047R) were obtained as previously described (29) and not passaged/maintained for more than 6 months.

Trastuzumab (kindly provided by F. Hoffmann-La Roche, Basel, Switzerland) was dissolved in sterile apyrogen water (stock solution of 21.4 mg/mL) and stored at 4°C. IPI-504 (Infinity Pharmaceuticals) was dissolved in citrate 50 mmol/L buffer, pH 3.3, and EDTA as a stock solution at 20 mmol/L and stored at −20°C.

In vitro proliferation assays and cell-cycle analysis

Cell proliferation was studied using the cell proliferation reagent WST-1 (Roche) according to the manufacturer's protocol. Briefly, 8×10^3 cells were seeded in triplicate in 96-well plates and treated for 5 days, with either trastuzumab or IPI-504 as indicated. Viable cells were estimated on the basis of their ability to metabolize tetrazolium salt WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate, to formazan by mitochondrial dehydrogenases. Quantification of the formazan dye directly correlates with the number of metabolically active cells and was analyzed by a scanning microplate reader (ELISA reader). Results are shown as means ± SE.

Western blot

Cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer (50 mmol/L HEPES, pH 7.0, 10%

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glycerol, 1% Triton X-100, 5 mmol/L EDTA, 1 mmol/L $MgCl_2$, 25 mmol/L NaF, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 0.5 mmol/L orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride). Tumor xenografts were minced into ice-cold lysis buffer, using a Labgen 125 tissue homogenizer (Cole-Palmer).

Lysates were centrifuged at 15,000 × *g* for 20 minutes at 4°C, and supernatants were removed and assayed for protein concentration using the DC Protein assay (Bio-Rad). Protein extracts were resolved by SDS-PAGE acrylamide and electrophoretically transferred to nitrocellulose membranes. Membranes were hybridized with the following primary antibodies: mouse monoclonal anti-total HER2 (CB11; Biogenex); rabbit polyclonal phospho-Akt (Ser 473), rabbit polyclonal phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr202/Tyr204), rabbit polyclonal total Akt, and rabbit polyclonal total MAPKs (Cell Signaling Technology). HER2 antibody were incubated in TBS-Tween buffer (T-TBS, 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20)/5% nonfat dry milk. Anti–p-Akt, p-MAPKs, total Akt, and total MAPKs were incubated in T-TBS/5% bovine serum albumin. Mouse and rabbit HRP-conjugated secondary antibodies (Amersham Biosciences) were used at 1:3,000 in T-TBS/5% nonfat dry milk. Protein–antibody complexes were detected by chemiluminescence with Immobilon Western HRP substrate (Millipore, catalogue no. WBKLS0500).

In vivo xenograft studies

Mice (Harlan Laboratories) were maintained as previously described (12). A 17β-estradiol pellet (Innovative Research of America) was inserted subcutaneously to each mouse 1 day before cell injection.

For all the experiments, 2×10^7 cells were injected into the right flanks of 10 mice for each experimental condition. Established tumors were treated with trastuzumab, IPI-504, or the combination as following: trastuzumab (10 mg/kg in sterile PBS) or sterile PBS (control) was given intraperitoneally twice weekly. IPI-504 (100 mg/kg) was administered intraperitoneally thrice weekly. IPI-504, trastuzumab, and the combination treatments were tolerable. No significant toxicity was noticed among the treatment arms.

Tumor growth was measured with digital calipers as indicated and tumor volume was determined using the formula: (length × width^2) × (π/6). At the end of the experiments, the animals were anesthetized with 1.5% isofluorane–air mixture and killed by cervical dislocation. Results are depicted as means of tumor volume ± SE.

Statistical analysis

For *in vitro* assays and nude mice experiments, comparisons between groups were made using a 2-tailed Student's *t* test. When more than 2 groups were compared, we used the 1-way ANOVA test. Differences for which $P \geq 0.05$ were considered statistically significant.

Results

Antiproliferative activity of IPI-504 in trastuzumab-resistant cells

The isolation and characterization of the trastuzumab-resistant cells BT474R, SKBR-3R, and BT474H1047R is described in Materials and Methods.

BT474R, SKBR-3R, BT474H1047R, and HCC1569 cells were confirmed to be refractory *in vitro* to the antiproliferative effects of trastuzumab compared with parental cells or cells transfected with empty vector that served as trastuzumab-sensitive controls (Fig. 2).

To test the sensitivity of trastuzumab-resistant cells to the inhibition of Hsp90 in terms of cell growth, we treated both control and trastuzumab-resistant cells with increasing concentration of IPI-504 for 5 days. IPI-504 inhibited in a dose-dependent fashion the growth of both trastuzumab-sensitive and -resistant cells (Fig. 2). At low concentration of IPI-504, BT474H1047R and SKBR-3R cells were slightly less sensitive than controls (Fig. 2B and C) but, nevertheless, a robust inhibition of growth was observed at clinically achievable concentrations of the inhibitor. Data are representative of experiments repeated in triplicate.

IPI-504 decreases HER2 protein expression and inhibits both Akt and MAPKs pathways

Both control and trastuzumab-resistant cells were exposed to increasing concentrations of IPI-504 for 24 hours, and HER2 protein levels were subsequently assessed. Treatment with IPI-504 resulted in a marked dose-dependent HER2 downregulation (Fig. 3).

Because continued activation of HER2 downstream signaling pathways is a common feature of trastuzumab resistance (see Introduction), we decided to explore the effects of IPI-504 on critical elements of these pathways. Akt and MAPKs signaling pathways are activated in breast cancer cells by HER2 (30). Akt is dependent on Hsp90 for protein maturation and stability, whereas MAPKs are not (31). Incubation of IPI-504 potently suppressed both Akt and MAPKs phosphorylation in both sensitive and trastuzumab-resistant cells (Fig. 3). Total levels of Akt decreased in all 4 cell lines in a dose-dependent manner. However, levels of total MAPKs were not significantly altered with IPI-504 treatment. Similar results were obtained also with SKBR-3 and SKBR-3R cells (Supplementary Fig. S1). Data are representative of experiments repeated in triplicate.

In vivo antitumor activity of IPI-504 in trastuzumab refractory tumors

To expand our results to the *in vivo* setting, we evaluated tumor growth inhibition in xenografts derived from BT474 and BT474R in response to IPI-504 and trastuzumab dosing. Treatments were started 22 days after cell injection when tumors were well established. IPI-504 and trastuzumab independently induced tumor regression of trastuzumab-sensitive BT474 cell-derived

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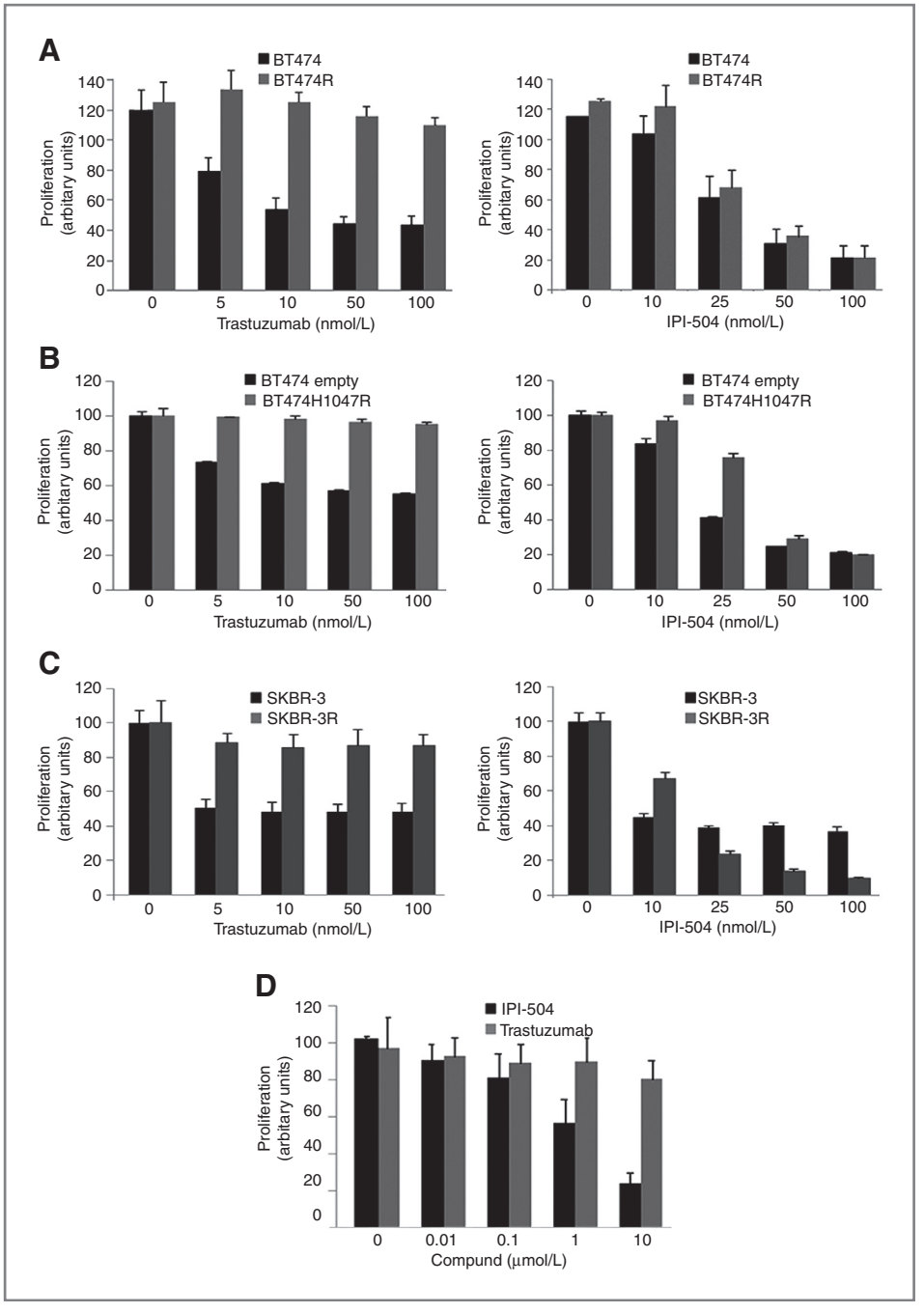

Figure 2. Antiproliferative activity of IPI-504 in trastuzumab-resistant cells. Five days proliferation of BT474R (A), BT474H1047R (B), and SKBR-3R (C) cells in response to increasing concentrations of trastuzumab or IPI-504 was compared with controls. D, five days proliferation of HCC1569 cells in response to increasing concentrations of trastuzumab or IPI-504. The experiments were done 3 times.

xenografts (Fig. 4A). Xenografts derived from BT474R cells continued to grow in the presence of trastuzumab but were still sensitive to IPI-504 (Fig. 4B). When used in combination, IPI-504 and trastuzumab added only marginal benefits to IPI-504 monotherapy (Fig. 4B).

Low expression of PTEN (16) or the presence of PI3K-activating mutations (such as the E545K and the H1047R; ref. 17) have been shown to correlate with decreased sensitivity to trastuzumab-based therapy in HER2-positive breast cancer patients. Aiming to evaluate whether inhibition of Hsp90 could be a valid therapeutic strategy to target trastuzumab resistance driven by deregulation of the PI3K pathway, we tested the antitumoral activity of IPI-504 in xenografts bearing the aforementioned aberrations. We treated both BT474 empty and BT474H1047R-derived xenografts with IPI-504 and trastuzumab and found that both controls and cells bearing the PI3K mutation respond similarly to Hsp90 inhibition (Fig. 5A and B). Interestingly, the H1047R mutation of the p110α subunit of PI3K was not found sufficient to

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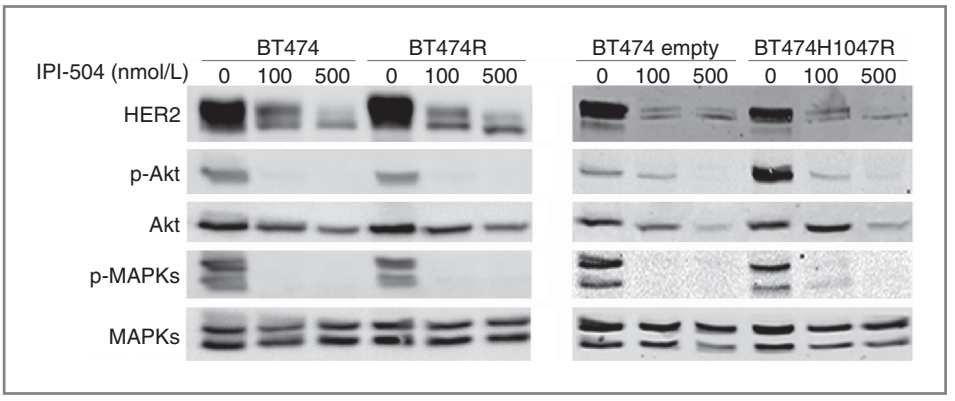

Figure 3. Inhibitory effects of IPI-504 on Akt and MAPKs pathways. Western blots showing the effects of increasing concentrations of IPI-504 for 24 hours on the expression of total levels of both HER2 and Akt, and the phosphorylation of both Akt and MAPKs in BT474, BT474R, BT474 empty, and BT474H1047R cells. Total MAPKs serve as loading controls. The experiments were done 3 times.

decrease *in vivo* trastuzumab sensitivity of BT474 cells (data not shown). This phenomenon has been observed previously in the HER2-amplified KPL-4 breast cancer cell line (32) and independently verified by our group as well (data not shown).

We then assessed the efficacy of IPI-504 in tumors derived from HCC1569 cells, a recognized preclinical model with activated PI3K pathway due to low levels of PTEN (33). Tumors derived from this cell line were treated with trastuzumab, IPI-504, or the combination (Fig. 5C). As expected, IPI-504 as a single agent was more efficacious than trastuzumab in inhibiting tumor growth in HCC1569 xenografts. The combination was not significantly superior to IPI-504 used as a single agent. The inhibitory effects of IPI-504 on both Akt and MAPKs pathways was confirmed also in this cell line (Supplementary Fig. S2).

Effects of IPI-504 on HER2 and downstream signaling

Expression of HER2, p-Akt, p-MAPKs, Akt, and MAPKs was evaluated in both BT474R and BT474H1047R xenografts at the end of the experiments depicted in Figs. 4 and 5. In Fig. 6A, we show decreased levels of total HER2, p-Akt, and p-MAPKs in xenografts treated with IPI-504. Total Akt levels were unaffected in these tumors. To further analyze the effects of IPI-504 on HER2 and downstream signaling *in vivo*, we conducted a pharmacodynamic study treating BT474R tumors with a single dose of IPI-504 (100 mg/kg) and follow the expression of these markers overtime. The levels of HER2, p-Akt, and p-MAPKs were significantly decreased 12 hours after a single dose of 100 mg/kg IPI-504 (Fig. 6B). Total levels of Akt were decreased only after 24 hours. Maximum downregulation of HER2 was achieved at 24 hours after treatment initiation, whereas maximal decreases of activated Akt and MAPKs were reached between 12 and 24 hours. At 48 hours, p-Akt, p-MAPKs, and, less evidently, HER2 expression started to recover whereas Akt levels remained suppressed. These results were also reproduced in cultured BT474 and BT474R cells, showing good correlation between *in vivo* and *in vitro* data (Supplementary Fig. S3).

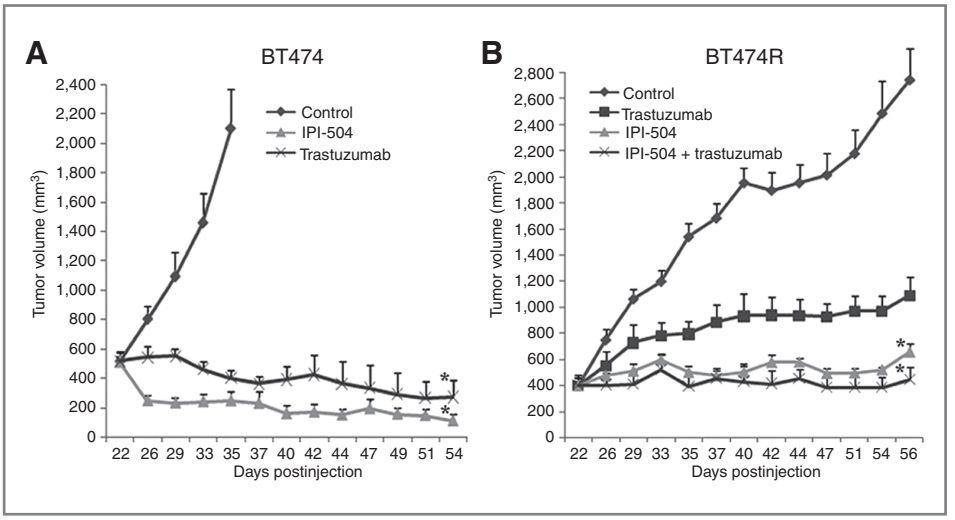

Figure 4. Antitumor activity of IPI-504 in BT474R xenografts. A, tumor growth inhibition of BT474 xenografts in response to IPI-504 or trastuzumab. Data are expressed as mean ± SE. *, $P < 0.01$ between treatment groups versus untreated control. B, tumor growth inhibition of BT474R xenografts in response to IPI-504, trastuzumab, or the combination of the 2 agents. Data are expressed as mean ± SE. *, $P < 0.01$ of IPI-504 and combination groups versus control.

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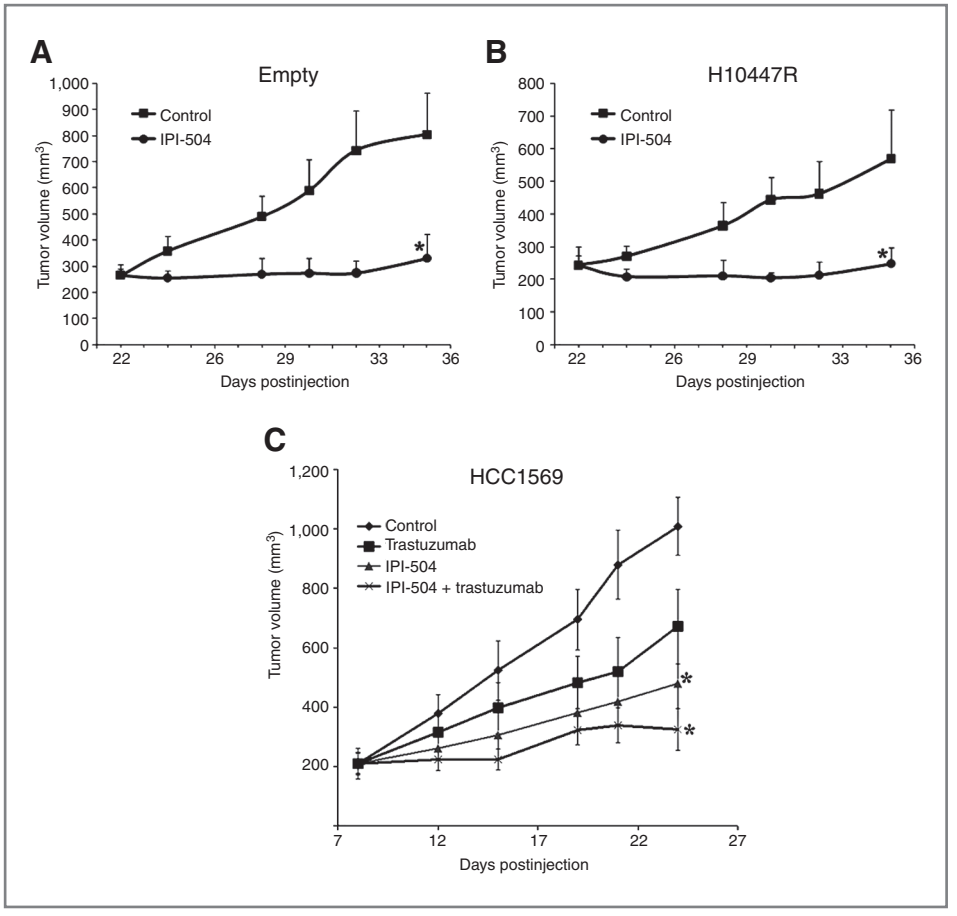

Figure 5. Antitumor activity of IPI-504 in BT474H1047R and HCC1569 xenografts. A, tumor growth inhibition of BT474 empty xenografts in response to IPI-504. Data are expressed as mean ± SE. *, $P < 0.01$ versus control xenografts. B, tumor growth inhibition of BT474H1047R xenografts in response to 100 mg/kg IPI-504. Data are expressed as mean ± SE. *, $P < 0.05$ versus control xenografts. C, tumor growth inhibition of HCC1569 xenografts in response to 50 mg/kg IPI-504, trastuzumab, or the combination of the 2 agents. Data are expressed as mean ± SE. *, $P < 0.05$ of IPI-504 and combination groups versus control.

Discussion

In the last decade, the treatment of HER2-positive breast cancer has significantly improved by the introduction of anti-HER2 agents such as trastuzumab and, more recently, tyrosine kinase inhibitors (34, 35). Despite these advances, resistance to anti-HER2 agents is frequent and new anti-HER2 therapies are being explored (36). Among these novel approaches, Hsp90 inhibitors have been considered as potential agents because they result in decreased HER2 expression, and there is already some evidence of antitumor activity with these agents (23, 37).

Hsp90 is described to interact with more than 200 different client proteins (http://www.picard.ch/downloads/Hsp90interactors.pdf), including numerous oncogenes (18, 38). Hsp90 may regulate the maturation, stability, and/or trafficking of these proteins. For instance, maturing and fully mature forms of HER2 depend on Hsp90 association for stability and, in fact, HER2 is considered one of the most sensitive client proteins to Hsp90 inhibition (39).

In this study, we investigated whether Hsp90 inhibitors would also be active in HER2-positive cell lines with either primary (intrinsic) or secondary (acquired) resistance to trastuzumab. We show that cells/tumors that developed resistance to trastuzumab following chronic exposure to the antibody (BT474R and SKBR-3R) were highly sensitive to IPI-504. Similar results were observed in tumor models bearing the H1047R PI3K activating mutation or low levels of PTEN, known to be clinically confirmed mechanisms of intrinsic trastuzumab resistance (16, 17). Furthermore, it has also been recently shown that IPI-504 reduces tumor growth of JIMT-1 xenografts, which are believed to be intrinsically resistant to trastuzumab because of upregulation of the membrane-associated mucin 4 (27). Taken together, these data suggest that IPI-504 treatment can counteract multiple trastuzumab-resistant mechanisms, which may offer a potential clinical path forward for intervention in this setting. As a potential mechanistic explanation for the observed activity on resistant cells, we show that IPI-504 not only downregulates HER2 but also prevents phosphorylation of both Akt and MAPKs in trastuzumab-resistant cells. Because continued signaling via HER2-dependent pathways is still observed in the majority of trastuzumab-resistant cells, the silencing of these pathways by IPI-504 could partially explain its activity in this setting.

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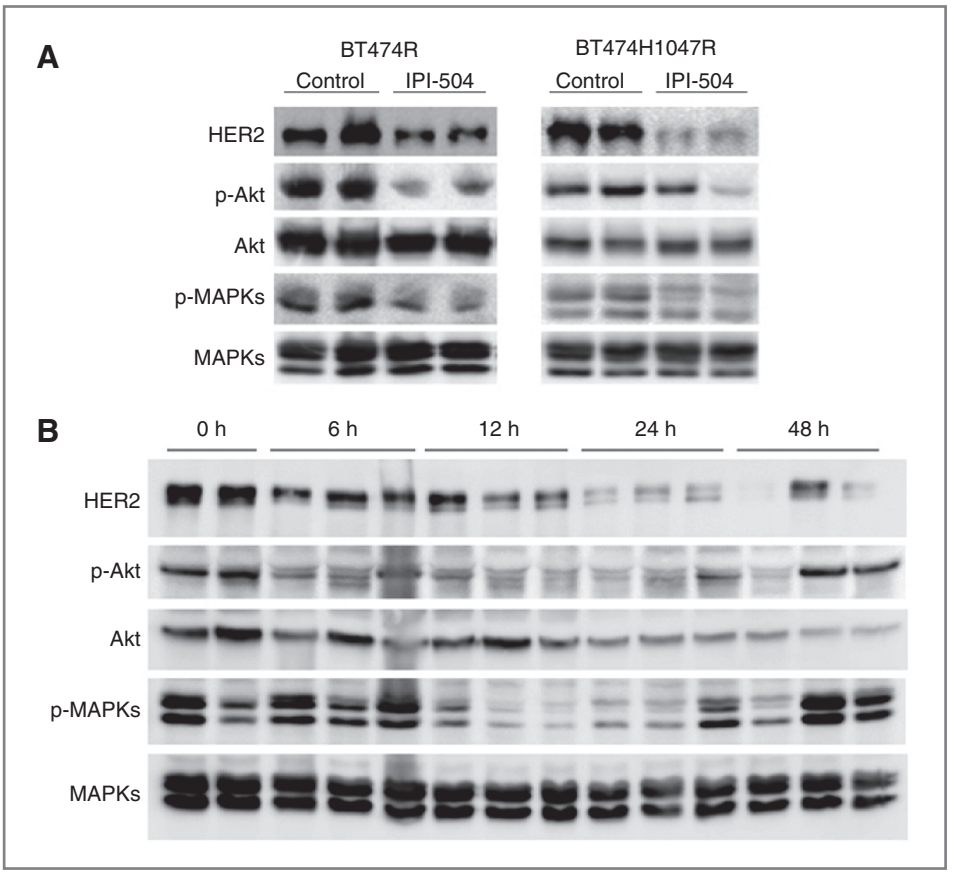

Figure 6. Western blots showing the effects of IPI-504 on HER2 and downstream signaling pathways in BT474R and BT474H1047R tumors. A, expression levels of HER2, p-Akt, Akt, and p-MAPK in both BT474R and BT474H1047R tumors at the end of the tumor growth experiments. Total MAPKs serve as loading controls. B, time course experiment showing the effects of a single administration of IPI-504 (100 mg/kg) on HER2, Akt, p-Akt, and p-MAPKs levels in BT474R tumors. Total MAPKs serve as loading controls.

Interestingly, both BT474R and HCC1569 tumors were not entirely refractory to trastuzumab treatment. This phenomenon is most likely because of antibody-dependent cell-mediated cytotoxicity, proved to be one of the mechanisms of action of trastuzumab *in vivo* (40).

In conclusion, our results suggest that IPI-504–mediated inhibition of Hsp90 may be a valid therapeutic approach to target HER2-positive breast cancer patients who have become refractory to trastuzumab therapy. Our data confirm that Hsp90 inhibition is a promising strategy for trastuzumab-resistant HER2-amplified breast cancer.

Disclosure of Potential Conflicts of Interest

E. Normant, A.R. Lim, K.L. Slocum, K.A. West, and V. Rodriguez are Infinity Pharmaceuticals employees.

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