Inhibition of HSP90 with AUY922 induces synergy in HER2 amplified trastuzumab resistant breast and gastric cancer

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Abstract

Heat Shock Protein 90 (HSP90) enables the activation of many client proteins of which the most clinically validated is HER2. NVP-AUY922, a potent HSP90 inhibitor, is currently in Phase II clinical trials. To explore its potential clinical utility in HER2-amplified breast and gastric cancers, we evaluated the effect of AUY922 alone and in combination with trastuzumab in both trastuzumab-sensitive and resistant models. A panel of 16 human gastric and 45 breast cancer cell lines, including 16 HER2-amplified (3 and 13 respectively) were treated with AUY922 over various concentrations. In both breast and gastric cancer, we used cell lines and xenograft models with conditioned trastuzumab-resistance to investigate the efficacy of AUY922 alongside trastuzumab. Effects of this combination on downstream markers were analyzed via western blot. AUY922 exhibited potent anti-proliferative activity in the low nanomolar range (< 40nM) for 59 of the 61 cell lines. In both histologies, HER2-amplified cells expressed greater sensitivity to AUY than HER2-negative cells. In conditioned trastuzumab-resistant models, AUY922 showed a synergistic effect with trastuzumab. In vitro, the combination induced greater decreases in HER2, a G2 cell cycle arrest, and increased apoptosis. In a trastuzumab-resistant gastric cancer xenograft model, the combination of AUY922 and trastuzumab showed greater antitumor efficacy than either drug alone. These data suggest that AUY922 in combination with trastuzumab has unique efficacy in trastuzumab-resistant models. The combination of HSP90 inhibition and direct HER2 blockade represents a novel approach to the treatment of HER2-amplified cancers and clinical trials based on the above data are ongoing.
INTRODUCTION

In human breast and gastric cancer, HER2 overexpression and/or amplification have been detected in approximately 20-30% of patients and correlates with poorer clinical outcomes (1-3). In both breast and gastric cancer, HER2 gene amplification is accompanied by increased expression of the gene product in the cell membrane resulting in growth and transformation (4). Since the approval of trastuzumab and lapatinib, there have been considerable efforts to identify ideal drug-drug partnerships to optimize anti-HER2 activity (5). Furthermore, while many patients initially respond to anti-HER2 therapies, many will ultimately develop resistance to these agents (6). Heat shock protein 90 (HSP90) is an ATP-dependent, evolutionarily conserved molecular chaperone that assists in the structural folding and stabilization of a wide range of cellular proteins. HSP90 accounts for 1-2% of all proteins and it consists of 3 domains; a 24-28 kDa NH2-terminal region, a 33-44 kDa middle region and an 11-15 kDa COOH domain, all of which are involved in ATP binding (7). Most recently, it has become clear that cancer cells in particular express increased levels of active HSP90 and that mutated oncogenic proteins are more reliant on the function of HSP90, and therefore more susceptible to its inhibition (8). In fact, gastric adenocarcinomas have been shown to express higher levels of HSP90 especially in those tumors with lymph node metastasis (9). Additionally, a strong inverse correlation has been shown between HSP90 expression in breast tumor samples with overall patient survival (10). Among the many described client proteins of HSP90 and certainly the most validated clinical target is the HER2 oncoprotein (11, 12).

NVP-AUY922 is part of the isoxazole HSP90-inhibitor family and inhibits ATPase activity with an IC₅₀ of 30 nM (Figure1). This molecule is a non-geldanamycin analog which have the promise of more prolonged target inhibition and which so far have not been associated with the same degree of hepatotoxicity as their geldanamycin counterparts. AUY922 exerts its effects by binding to the ATPase domain of the HSP90 N-terminal preventing HSP90 from its chaperone functions. This leads to the proteosomal degradation of many relevant client proteins (13). Blocking HSP90 leads to significant decrease in ErbB2 protein levels in HER2 positive breast cancer and gastric cancer cell lines, causing growth inhibition by decreasing ErB2 expression and downstream signaling via ErBB3 and PI3K (13, 14). In a phase I clinical trial, treatment with AUY922 was well tolerated at weekly
doses up to 70mg/m² with prolonged disease stabilization in some patients (15). More recently, it has been reported that responses to HSP90 inhibitors have been achieved in patients who had previously progressed on trastuzumab therapy (16, 17).

In pre-clinical models, it has been reported that the combination of HSP90 inhibitors and trastuzumab has shown synergistic behavior in HER2 amplified breast cancer cell lines (18-20). Recent clinical data has also suggested that targeting the HER2 axis with both a direct inhibitor as well as anti-HSP90 agent may improve clinical outcomes (16, 21-23). Furthermore, it was recently reported that a geldanamycin analogue had anti-tumor activity in trastuzumab-resistant breast cancer models but there was no observed synergy when combined with trastuzumab (19). Our hypothesis was that the combination of an HSP90 inhibitor currently in clinical development and trastuzumab would be particularly synergistic in trastuzumab-resistant breast and gastric cancer models. To explore this, we performed a pre-clinical evaluation comparing the biologic effects of AUY922 in a large panel of both breast and gastric cancer cell lines with a particular emphasis on those with HER2 amplification. We analyzed the growth inhibitory effects of this agent and its effects on expression of HER2, the downstream signaling molecules AKT and ERK and the pharmacodynamic marker, HSP70 (24). We conditioned models of trastuzumab resistance in both breast and gastric cancer and compared the effects of AUY alone, trastuzumab or the combination of these two agents in these models.

In summary, this is the first report to describe the synergy between an HSP90 inhibitor and trastuzumab in trastuzumab-resistant gastric cancer. Because of its improved pharmacokinetics and predictable toxicity profile, AUY922 is now being investigated in several phase II clinical trials. Therefore, the studies described herein were designed to provide a rationale to test AUY922 in combination with trastuzumab in patients with metastatic gastric cancer that have progressed on trastuzumab therapy.
MATERIALS AND METHODS

Cell lines, cell culture, and reagents

The effects of NVP-AUY922 and trastuzumab on malignant cell growth were studied in a panel of 16 established human gastric and 45 breast cancer cell lines. The human gastric cancer cell lines AGS, NCI-N87, KATO III, SNU-1, SNU-5, and SNU-16 were obtained from the American Type Culture Collection (Rockville, MD, USA). The human gastric cancer cell lines NUGC4, NUGC3, FU97, IM95, IM95m, AKN45, MKN74, MKN1 were obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan). The cell line OE19 and OE33 was obtained from the European Collection of Cell Cultures (Sigma-Aldrich, Germany). SNU-1, SNU-5, AGS, N87, Kato III, SNU-16, MKN45, MKN74, MKN1, NUGC-3, NUGC-4, OE-19 was cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum and PSF (Irvine Scientific, Santa Ana, CA, USA). AGS, FU-97, IM95 and IM95m were cultured in MEM Eagles medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal bovine serum and Insulin 10 mg/ml (Sigma Aldrich, St Louis, MO). Breast cancer lines were obtained and cultured as previously described (25). All lines were incubated under standard conditions (5% CO₂; 37°C). Cells were routinely assessed for mycoplasma contamination using a multiplex PCR method. Mitochondrial DNA from the cells was sequenced to confirm their correct identity. PCR products were sequenced using a 3730 DNA analyzer (Applied Biosystems).

AUY922 was provided by Novartis Pharmaceuticals (Cambridge, MA). It was prepared as a 10-mM stock solution in dimethyl sulphoxide (DMSO) (Fisher Scientific, Pittsburgh, PA). Trastuzumab was obtained from UCLA pharmaceutical services and was prepared from a stock concentration of 21 mg/ml.

PI3 Kinase PCR, sequencing, and mutational analysis

Assays were performed as previously described in O’Brien et al (26). The NCI-N87 and NCI-N87TR cell lines were sequenced to assess the PIK3CA and PTEN mutation status. PI3KCA was sequenced using known primers for exon 9 and exon 20 regions of the PIK3CA gene (27). PTEN was sequenced using known primers for exon 1 to 9 (28). PCR products were sequenced
using the 3730 DNA Analyzer. Sequences were analyzed and compared using Geneious version 5.6 created by Biomatters.

Proliferation assays

Cells were seeded at densities ranging from 2-5x10^5 in a 24-well plate. Cells were treated in cell line-specific media with AUY922 starting at 100nM decreasing in two-fold dilutions over six to twelve concentrations. The same cell lines were treated with trastuzumab starting at 10 μg/ml decreasing in two-fold dilutions over six concentrations. Cells were harvested by trypsinization on day 6 and counted using a particle counter (Z1; Beckman Coulter Inc., Fullerton, CA, USA). Growth inhibition was calculated as a percentage of the untreated controls. Experiments were performed in duplicate for each cell line (Microsoft Excel, Redmond, WA).

To evaluate for speed of recovery, 1x10^6 cells were plated and treated at 60% confluence in 10 cm dishes for 24 hours with media control, AUY (100nM), trastuzumab (10ug/mL), or the combination of AUY and trastuzumab. Cells were washed 2x with PBS and given fresh media for 0’, 24 hours, 48 hours, or 72 hours.

The Combination Index (CI) was calculated using the Chou-Talalay method (29). Data were analyzed using the Calcusyn software (Biosoft, Ferguson, MO, USA) to score synergistic relationships for combination treatments. CI<1, CI=1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively. The p-values were obtained using a one-sided t test compared to a hypothetical value of 1.

Western blots and immunoprecipitation

Cells in log-phase were washed in ice cold PBS and lysed at 4 degrees in 400uL of Mild Lysis Buffer. Insoluble material was cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Protein was quantitated using BCA (Pierce Protein Research Products, Rockford, IL, USA), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Life Technologies, Carlsbad, CA, USA). AKT, ERK, HER2 and HSP90 expression were detected, respectively, by anti-AKT and anti-ERK (Cell Signaling Technology, Beverly, MA, USA), anti-HER2 (EMD Biosciences Gibbstown, NJ), and anti-HSP70/HSP72 (Enzo Lifesciences, Farmingdale, NY, USA). Phospho-AKT, phospho-ERK and phospho-HER2 were
detected by polyclonal anti-pAKT (Ser473), anti-pERK (Thr202/Thr204), and monoclonal anti-pHER2 (Tyr1221/1222) antibodies (Cell Signaling, Beverly, MA, USA). BIM, HER3, and IGF1-R were detected by anti-BIM, anti-HER3, and anti-IGF1-R (Cell Signaling Technology, Beverly, MA, USA). Phospho-HER3 and phosphor-IGF1-R were detected by monoclonal anti-pHER3 and anti-pIGF1-R antibodies (Cell Signaling Technology, Beverly, MA, USA).

Cell Cycle analysis and apoptosis studies

The effects of AUY922 on the cell cycle were evaluated using Nim-Dapi staining. Cells were plated evenly in control and experimental wells and allowed to grow to log-phase. Cells were then treated with concentrations of AUY922 (10 nM), 10 μg/ml of trastuzumab, or the combination of these two drugs for 24 hours or 5 days for cell cycle or apoptosis, respectively. At the time of analysis, supernatant was collected for apoptosis or aspirated off for cell cycle, cells were washed with PBS, and trypsin was applied to release cells, which were then centrifuged at 3000 rpm for 5 minutes. Supernatant was aspirated and cells were then resuspended in 100 μL of Nim-Dapi (NPE Systems, Pembroke Pines, FL) and gently vortexed. Cells were analyzed with UV using a Cell Lab Quanta SC flow cytometer (Beckman-Coulter, Fullerton, CA). For apoptosis assays, after centrifugation, the supernatant was aspirated and cells were then resuspended in 150 μL binding buffer, and stained with 10 μL annexin V-FITC and 10 μL PI at room temperature for 5 min (Medical & Biological Laboratories Co., Woburn, MA) (30, 31). After incubation cells were processed as directed in the kit and analyzed using a FITC signal detector and propidium iodide (PI) detector using a Cell Lab Quanta SC flow cytometer.

Trastuzumab-conditioned models

The trastuzumab-sensitive breast cancer cell lines BT-474 and SK-BR-3 and the gastric cancer cell line N87 were grown in the presence of trastuzumab over the course of greater than one year. Anti-proliferative activity of trastuzumab was compared in these cells to their parental controls. HER2 amplification was tested on these conditioned models by FISH as previously described (32).
In Vivo Experiments

Female athymic nude mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously on the right flank with N87- or N87-TR\textsuperscript{C} cells (5x10\textsuperscript{6} cells) in 50\% matrigel. When tumors reached approximately 100 mm\textsuperscript{3}, animals were randomized into 1 of 3 or 1 of 4 groups for N87- and N87-TR\textsuperscript{C}, respectively (\(n=8\)/group). Treatment arms included: (1) vehicle control (i.p. twice weekly); (2) trastuzumab (10mg/kg, i.p., twice weekly); (3) AUY922 (50mg/kg; i.p.; 5 days on, 2 days off); or (4) a combination of trastuzumab and AUY922 at the above concentrations and schedules. Tumors were measured thrice per week. Volumes were calculated from L x H x W in mm\textsuperscript{3}. Animal weights were taken twice per week. Data was analyzed using StudyLog software from StudyDirector (San Francisco, CA). Results are presented as means; error bars represent standard error (SE) of the mean.

Immunohistochemistry

Paraffin embedded tissue sections (4 µm) were deparaffinized in xylene and rehydrated in ethanol gradient prior to antigen retrieval in citrate (pH 6) buffer. Immunohistochemistry was performed using an anti-HER2 antibodies (CB11, Biogenex) and detected using Horseradish peroxidase (HRP) labeled secondary antibodies (DAKO). Sections were counterstained with hematoxylin, dehydrated and mounted with Permount. Slides were scanned with ScanScope CS system (Aperio, Vista, CA, USA) and HER2 staining intensity was scored following guidelines on a scale of 0 to 4+ as follows: 0 is negative, or membrane staining in < 10% of the tumor cells; 1+ is a faint/barely perceptible membrane staining detected in > 10% of the tumor cells; 2+ is a weak to moderate complete membrane staining in > 10% of the tumor cells; and 3+ is a strong complete membrane staining in > 10% of the tumor cells (\(n=10\)).

Statistical methods

Associations between biomarker expression and IC\textsubscript{50} values were analyzed using a two-sample Wilcoxon rank-sum (Mann-Whitney) test: significant if p-value < 0.05. If the p-value is between 0.1-0.05 it is considered leaning towards statistically significant. Data were calculated using Stata/MP 11.1 (StataCorp, College Station, TX).
RESULTS

Activity of AUY922 in human breast and gastric cancer cell lines
To evaluate the effects of AUY922 on human breast and gastric cancer cells, we used a panel of 45 breast and 16 human gastric cancer cell lines that express variable levels of relevant client proteins of HSP90 including EGFR, AKT and HER2, including several cell lines known to be trastuzumab resistant in vitro (Table 1, Supplemental Table 1). The breast lines represent a diverse spectrum of the disease including subsets of basal, luminal, and HER2 amplified cells. As has been demonstrated with other HSP90 inhibitors, AUY922 had potent in vitro activity in the nanomolar range, but the HER2 amplified cell lines in both breast and gastric cancer panels were particularly sensitive. The IC_{50}s of HER2 expressing breast cancer cell lines and those not expressing HER2 were different (p=0.01). Among the gastric cancer cell lines, there was a trend towards statistical significance (p=0.05) (Figure 2). Interestingly, even among the HER2 amplified cells that were resistant to trastuzumab in vitro, there was a remarkable anti-proliferative effect seen with the addition of AUY922. Some non-HER2- amplified cell lines were also sensitive to AUY922, which likely reflects dependencies on other client proteins of HSP90 besides HER2.

AUY922 induces growth arrest and affects downstream signaling in HER2 amplified breast and gastric cancer cells
Having determined that this HSP90 inhibitor had very selective growth inhibition on the HER2 amplified breast and gastric cancer cell lines, its effects on cell cycle were analyzed. Two HER2 amplified gastric cancer lines (OE19, N87) were incubated for either 48 hours or 5 days with either control, 10nM AUY, or 100nM AUY and cells were analyzed by flow-cytometry using NimDAPI staining. G2 arrest was seen in these two sensitive cell lines that was concentration-dependent (Fig. 3A). Additionally, effects on apoptosis were evaluated using the same treatment concentrations. After 5 days of treatment, cells showed an increase of both early and late apoptosis (Figure 3B).

Because of the known role of HSP90 as an important chaperone of HER2, we also sought to examine the effect of HSP90 inhibition on HER2, AKT and MAP Kinase phosphorylation in gastric cancer cells. Two gastric HER2 amplified cell lines were treated with AUY922 at a concentration of 100 nM
as previously described. In the HER2 amplified cell lines (N87 and OE19), AUY decreased both total HER2 levels as well as AKT and HER2 phosphorylation when compared to untreated controls (Figure 3C). This observation in gastric cancer has been similarly described in selected HER2-amplified breast cancer cell lines suggesting that AUY may be blocking HER2-driven signaling through MAP Kinase and AKT (14). In the cells that were resistant to AUY, there were no significant changes in any of these candidate markers (Supplemental Figure 1). These studies demonstrate that AUY922 can suppress the phosphorylation of key pathways that are downstream of HER2 at clinically achievable concentrations. We also checked the effects of AUY922 on these cells by expression of the pharmacodynamic marker, HSP70. As anticipated, little change is seen after 30 minutes, but HSP70 is reliably increased in all sensitive cell lines after 18 hours (Figure 3C).

We then evaluated the speed of recovery of client proteins, AKT, ERK, and HER2 after treatment with AUY922 in the N87 cell line. Beginning at 48 hours after washout of AUY, we began to see recovery of pERK and HER2 but not AKT, suggesting that the effects on AKT may be more dependent on HSP90 inhibition. Moreover, we demonstrated increased expression of the pro-apoptotic protein BIM up to 72 hours, consistent with increased apoptosis (Supplemental Figure 2).

**Trastuzumab and AUY are synergistic in HER2 amplified breast and gastric cancer cells.**

We have previously demonstrated that the combination of trastuzumab and lapatinib is synergistic in HER2 amplified breast and gastric cancer cells (21, 32). To evaluate the combination of an HSP90 inhibitor and trastuzumab in these cancers, we treated two HER2 amplified breast cancer cell lines (BT474 and SKBR3) and two gastric cancer lines (N87 and OE19) with various clinically relevant concentration ranges of AUY922 and trastuzumab. Multiple drug effect analysis were performed to determine the nature of the interaction occurring in the combination treatment (synergy, additive, or antagonism). When these 2 agents were combined, we saw significant synergy in all HER2 amplified cell lines (Figure 4A). Mean combination indices (CI) were obtained ranging from 0.078 ± 0.043 in BT474 to 0.281 ± 0.066 in OE19 (Figure 4B). While synergy was clearly seen in all four HER2 amplified lines, the effect was most pronounced in
BT474. When we examined the effects of this combination on downstream markers (AKT, ERK, and HER2), in both breast and gastric cancer cell lines, we saw the expected decrease in both pAKT and pERK as well as a decrease in HER2 levels (Figure 4C). However, the combination had no significant decrease on either AKT or ERK phosphorylation when compared to AUY alone (Figure 4C).

We then evaluated the effects on apoptosis of the combination of AUY and trastuzumab when compared to either agent alone and found increased levels of cleaved caspase 3 and the pro-apoptotic molecule BIM, suggesting that the synergy is partially mediated through increased apoptosis (Figure 4D).

**The combination of AUY922 and trastuzumab is synergistic in HER2 amplified trastuzumab-conditioned breast and gastric cancer in vitro.**

In order to assess the effects of AUY in trastuzumab-conditioned models of breast and gastric cancer, we exposed the breast cancer cell lines BT474 and SKBR3 and the gastric cancer cell line N87 to increasing concentrations of trastuzumab in culture over a one-year period. We established that these cells remained HER2 amplified and were indeed trastuzumab-conditioned (TRC) as previously described (21, 33). We then treated these cells with both trastuzumab and AUY. Figure 5A demonstrates that these cells were relatively resistant to trastuzumab (when compared to their parental controls) but maintained a high degree of sensitivity to AUY. When AUY and trastuzumab were combined, there was continued synergy with mean combination indices of 0.726 ± 0.131 (N87-TRC), 0.397 ± 0.180 (SKBR3-TRC) and 0.941 ± 0.041 (BT474-TRC) (Figure 5A). We then selected the two most trastuzumab resistant lines (N87-TRC and BT474-TRC) and assessed downstream markers as above. We found that the combination continued to decrease pERK and pAKT. Interestingly, when comparing the effects of this combination to AUY as a single agent, there was increased degradation of total HER2, a finding not observed in the corresponding parental lines (8% for N87-TRC and 20% for BT474-TRC compared to their untreated controls) (Figure 5B). These findings would suggest that the combination of AUY and trastuzumab affects HER2 degradation when its chaperone, HSP90 is being inhibited. Furthermore, levels of HSP70 were elevated to a similar degree with both single AUY and the combination but not with trastuzumab alone (Supplemental Figure 3).
To further evaluate the mechanism of resistance of our conditioned N87 cell line, we examined multiple proteins that have been linked to trastuzumab resistance. We found no mutations in PI3 Kinase in these lines. However, in an effort to identify any other client proteins that are distinct in these trastuzumab-resistant lines, we then investigated levels of HER3 and IGF1-R, two other clients of HSP90 that have been linked to trastuzumab resistance. As can be seen in figure 5C, levels of HER3 and IGF1-R (two clients of HSP90) are increased in the trastuzumab conditioned N87 line when compared to its parental counterpart. Treatment with the combination of AUY and trastuzumab resulted in a proportionally greater decrease on HER3 and IGF1-R levels in the conditioned line when compared to the parental line. This suggests a novel mechanism for synergy in this trastuzumab-conditioned model. We then evaluated the effects on apoptosis of the combination of AUY and trastuzumab when compared to either agent alone and found increased apoptosis and BIM levels as previously described (Figure 5D).

The combination of AUY922 and trastuzumab is synergistic in HER2 amplified trastuzumab-conditioned gastric cancer in vivo.

To expand on our in vitro observations and with a particular emphasis on gastric cancer, we injected the N87 parental line and the N87 cells that were conditioned to trastuzumab (N87-TR\textsuperscript{C}) into nude mice and when tumors were established, these mice were randomized into treatment with control, AUY922 or trastuzumab. As can be seen in Figure 6A, there was some tumor inhibition seen with trastuzumab in the parental N87 model (corresponding with our in vitro observations) but significantly less efficacy was seen with trastuzumab in the N87-TR\textsuperscript{C} (trastuzumab-conditioned) mice when compared to its parental N87 counterpart (Figure 6B). In order to see if cells that were conditioned to trastuzumab would be sensitive to the combination in vivo, we treated these N87-TR\textsuperscript{C} xenografts with either trastuzumab alone, AUY922 alone, the combination of trastuzumab and AUY, or excipient control. As expected, single-agent AUY caused some tumor regression in N87-TR\textsuperscript{C} xenografts. However, the combination of AUY and trastuzumab showed near complete tumor regression by day 15 (Figure 6B). Unlike previous reports with other HSP90 inhibitors, the combination of AUY and trastuzumab was significantly better than either drug alone. Furthermore, as can be seen in figure 6C, the effects by immunohistochemistry on HER2 staining confirmed greater decreases in HER2 staining with the combination of AUY and trastuzumab than with...
AUY alone (figure 6D). This conditioned model retained nearly the same degree of total HER2 and HER2 phosphorylation. All these observations were statistically significant, confirming the added benefit of a combined approach to targeting HER2.

DISCUSSION

Over the last several years, HSP90 has become a target for drug development and a number of HSP90 inhibitors are in clinical studies in several different malignancies (34). Many developmental candidates exploit the ATP-dependence of chaperone proteins by promoting ubiquitin-mediated proteosome degradation (35-38). HER2 is widely considered among the most sensitive and certainly the most therapeutically relevant clients of HSP90 and numerous HSP90 inhibitors have shown their best potential activity in HER2 amplified cells (39, 40). Because of the increased interest in HER2 as a novel target in gastric cancer, we sought to evaluate the combination of an HSP90 inhibitor (that is currently in phase II clinical trials) with or without trastuzumab in breast and gastric cancer cells. In this paper, we demonstrate that among the sixteen gastric cancer and forty-five breast cancer lines evaluated, the anti-proliferative effects of AUY922 are more selective in the HER2 amplified subset. In addition, no correlation was shown between the activity of AUY922 and any other client protein we evaluated (mutations of KRAS, PI3 kinase and c-MET). We show that significant synergy exists with AUY and trastuzumab in all HER2 models tested and that this synergy is maintained when these models become conditioned to trastuzumab. Our results suggest that the inhibition of HSP90 may be a useful strategy in patients with HER2 amplified diseases, even among those that have progressed on trastuzumab-based therapy.

Since the initial description of clinical activity of trastuzumab in HER2 amplified cancers, there has been a growing interest in both the mechanisms of resistance to anti-HER2 therapies as well as therapeutic strategies to overcome this resistance. Proposed mechanisms of resistance have included activation of phosphoinositide 3-kinase (PI3 kinase) signaling, the presence of truncated forms of HER2 that lack the antibody binding epitope, or enhanced signaling through ErbB-3 or other receptor tyrosine kinases in the
HER family pathway (26, 41). Regardless of the precise mechanism of resistance to anti-HER2 agents, ongoing clinical data would suggest that trastuzumab-resistant tumors continue to depend on HER2 signaling. Observations have been made by our group and others that significant synergy exists when combining lapatinib and trastuzumab in breast and gastric cancers (21, 32). Furthermore, recent clinical trials have shown that combinations of trastuzumab with either lapatinib or the monoclonal antibody pertuzumab in HER2 amplified breast cancer is well tolerated and can lead to improved clinical outcomes (42, 43). Thus, developing strategies with which to partner anti-HER2 agents in both breast and gastric cancer is of considerable clinical importance.

While several groups have explored the combination of HSP90 inhibitors with anti-HER2 agents in breast cancer models, to our knowledge, this is the first to examine this combination in gastric cancer. A recent report by Lee et al did evaluate the role of AUY922 in gastric cancer cell lines, however our study adds additional data to the role of HSP90 inhibition in gastric cancers in several respects (44). First, we showed more selectivity of AUY in the HER2 amplified cells (as opposed to the non-HER2 amplified group) but more importantly, we demonstrated that significant synergy exists between AUY and trastuzumab in all HER2 amplified models studied. Our data also support those of Lee et al by demonstrating that the effects of AUY may be through AKT signaling and in this study, we demonstrate that the synergy seen with this combination is a consequence of both effects on apoptosis and cell signaling. This synergy may have clinical implications, as was recently demonstrated in breast cancer patients treated with another HSP90 inhibitor that had progressed on trastuzumab, in that these women seemed to have higher clinical responses that what would be expected when treated with trastuzumab alone (45).

A recent report did demonstrate that a geldanamycin HSP90 inhibitor did have anti-tumor activity in trastuzumab-resistant breast cancer models (19). However, the most important observation in our paper is that in cell lines and xenografts that have been conditioned to the effects of trastuzumab, the synergy between this HSP90 inhibitor and trastuzumab was maintained. In both the breast and gastric trastuzumab-conditioned models, there is minimal effect of single agent trastuzumab even at higher concentrations. However, when trastuzumab was combined with AUY in gastric cancer, greater anti-tumor activity and greater effects on HER2 degradation are seen than with
either drug alone. Mechanistically, our data suggest that inhibition of the HER2 pathway in these two distinct ways may augment the effects on downstream signaling by decreasing HER2, especially in trastuzumab resistant models, reinforcing the rationale behind dual mechanisms of blockade in HER2 amplified diseases. Furthermore when compared to its parental counterpart, our conditioned model demonstrated increased expression of total HER3 and IGF1-R levels suggesting increased dependence on these pathways as a mechanism of trastuzumab resistance. Other reports have shown that HER2 can dimerize with additional cell surface receptors (including HER3 and IGF1-R) which may partially explain a mechanism of trastuzumab resistance (46, 47). Additionally, both HER3 and IGF1-R are known client proteins of HSP90, making these new dimer complexes susceptible to inhibition with AUY922. Although these results are preliminary, they support the ongoing investigation of AUY922 in combination with trastuzumab in HER2 amplified disease. Clinical trials based on these studies are currently ongoing in both breast and gastric cancers [Clinical trials.gov NCT01271920, NCT01402401].

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REFERENCES


Table 1. NVP-AUY922 IC<sub>50</sub> concentrations in gastric cancer cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Histology</th>
<th>HER2 Amplification Status</th>
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<td>NCI N87*</td>
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<td>Gastric</td>
<td>Amplified</td>
<td>6.64 ± 0.91</td>
</tr>
<tr>
<td>AGS§</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>7.71 ± 1.08</td>
</tr>
<tr>
<td>SNU 5</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>7.75 ± 1.22</td>
</tr>
<tr>
<td>SNU 1</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>8.37 ± 0.62</td>
</tr>
<tr>
<td>IM95^</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>9.40 ± 3.96</td>
</tr>
<tr>
<td>KATO III</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>17.14 ± 5.03</td>
</tr>
<tr>
<td>IM98m</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>23.08 ± 0.22</td>
</tr>
<tr>
<td>SNU 16</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>78.60 ± 27.06</td>
</tr>
<tr>
<td>NUGC 4</td>
<td>Gastric</td>
<td>Not amplified</td>
<td>148.13 ± 48.55</td>
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*NCI-N87 and OE19 had 23.88±1.77 and 40.92±1.17 % growth inhibition when treated with 10μg/mL of trastuzumab. OE33 had no growth inhibition when treated with the same concentration of trastuzumab.

** PI3K Mutation: c.1633G>A,
§PI3K Mutation: c.1357G>A,
^PI3K Mutation: c.1624G>A
LEGENDS

Figure 1. Structure of NVP-AUY922

Figure 2. Activity of AUY922 in human breast and gastric cancer cell lines
Cell proliferation assay was performed in a 5-day constant exposure. On day 1, cells were treated at a starting concentration of 100nM decreasing over at least 6 dilutions at a 1:2 ratio. IC$_{50}$ was calculated using day 1 and day 6 controls for cell line doubling time. The results represent at least two independent experiments. Out of the total of 61 cell lines tested, only three had IC$_{50}$ concentrations above 40nM. The IC$_{50}$s of HER2 expressing breast cancer cell lines and those not expressing HER2 were different (p=0.01). Among the gastric cancer cell lines, there was a trend towards statistical significance (p=0.05).

Figure 3. AUY922 induces growth arrest and affects downstream signaling in HER2 amplified breast and gastric cancer cells
A, N87 and OE19 cells were treated with 10nM and 100nM of AUY922 and analyzed for DNA content by FLOW cytometry. Both cell lines show concentration dependent increases in G2 arrest after 24 hours of treatment. B, AUY922 induces an increase in apoptosis in both N87 and OE19 after 5 days of treatment. C, Cells were plated and treated with 100nM for 30 minutes, 18 and 24 hours. Western blots showed that after treatment with AUY922, a decrease of at least 90% of pAKT as well as pHER2 was observed 24hrs after treatment in HER2-amplified gastric cell lines OE19 and N87. HSP70 is also increased in all evaluated cell lines beginning at 18 hours.

Figure 4. Trastuzumab and AUY are synergistic in HER2 amplified breast and gastric cancer cells
A, Cells were plated at 10$^5$ cells/well and treated with trastuzumab, AUY922, or a combination of trastuzumab and AUY922, at starting concentrations of 10-20mg/mL and either 5 or 10nM, respectively. The combination treatment resulted in an increased growth inhibition in vitro in all cell lines. B, Mean combination indices (CI) were calculated using CalcuSyn software and synergistic values are reported, as well as p-values. * For BT474, CI represents the average of the two higher concentrations. C, Phospho- and total proteins AKT, ERK, and total HER2, were evaluated after 24 hours of treatment in HER2 amplified cell lines. All cell lines
showed a significant decrease in pAKT and pERK, as well as total AKT and total HER2 when treated with AUY922 and the combination of AUY and trastuzumab, exhibiting decreases of 90% and 95% for pAKT and pERK, respectively. Total HER2 levels decreased 85% for N87 and 95% for BT474 compared to their untreated controls. Tubulin was used as a loading control.

D, Cells were treated with trastuzumab, AUY922, or a combination of trastuzumab and AUY922, at starting concentrations of 10mg/mL and 100nM, respectively. The combination showed a 8-fold increase in both cleaved caspase 3 and BIM levels compared to AUY alone.

**Figure 5. The combination of AUY922 and trastuzumab is synergistic in HER2 amplified trastuzumab-conditioned breast and gastric cancer in vitro and in vivo.**

A, Cell lines were conditioned by incrementally increasing concentrations of trastuzumab over a one-year period. Cells were plated 10^5 cells/well and treated with media, trastuzumab, AUY922, or a combination of trastuzumab and AUY922, at starting concentrations of 10mg/mL and 10nM, respectively. Similar to their respective parental cell lines, the combination treatment resulted in an increased growth inhibition *in vitro* in all cell lines. * For BT474-TR\(^C\), CI represents the average of the two higher concentrations.

B, Phospho- and total proteins AKT, ERK, and HER2, were evaluated after 24 hours of treatment. Tubulin was used as a loading control. All cell lines showed a significant decrease in pAKT and pERK, as well as total AKT and total HER2 when treated with AUY922 and the combination of AUY and trastuzumab. The combination resulted in 90% and 95% decrease for pAKT and pERK, respectively. The combination produced greater inhibition of total HER2 levels than with AUY alone (8% for N87-TR\(^C\) and 20% for BT474-TR\(^C\)) compared to their untreated controls.

C, The N87 TRc cells were plated 10^6 cells/dish and treated when 60% confluent for 24 hours with media, trastuzumab, AUY922, or a combination of trastuzumab and AUY922, at concentrations of 10mg/mL and 100nM, respectively. Expression was evaluated both at time 0’ and 24 hours after treatment was washed out. N87-TR\(^C\) expressed higher pHER3, HER3, pIGF1-R, and IGF1-R protein levels at baseline than in N87-Parental. In the combination treatment of AUY and trastuzumab, there was a decrease in HER3 levels which was proportionally greater than treatment with AUY alone.
D, The combination of AUY and trastuzumab showed increased apoptosis after 5 days of treatment as seen by flow cytometry and a 1.3-fold increase in BIM protein levels when compared to AUY alone.

**Figure 6. Efficacy of NVP-AUY922 on tumor growth inhibition on NCI-N87 and NCI-N87-TRC bearing xenografts**

A-B: NCI-N87 or NCI-N87-TRC cells (5x10^6 cells) were injected s.c. into female athymic nude mice. Drug treatments started when tumors reached 100mm^3 with vehicle control (i.p.; twice weekly), trastuzumab (10mg/kg, i.p., twice weekly), AUY922 (50mg/kg; i.p.; 5 days on, 2 days off), or a combination of trastuzumab and AUY922 (n=8/group).

A, Trastuzumab and AUY922 both caused approximately 50% tumor growth inhibition, as compared to the vehicle control. All comparisons were statistically significant between vehicle control versus T or AUY alone, p<0.007*.

B, N87-TRC bearing animals were used to assess the efficacy and synergistic effect of trastuzumab and AUY922, both as single agents and in combination. The combination treatment produced 90% tumor growth inhibition when compared to the vehicle control. All comparisons were statistically significant between vehicle control and combination (p=0.0063**), T or AUY and combination (p < 0.05***). The difference between the vehicle control and AUY is leaning towards significance with p=0.06.

C, Representative immunohistochemistry showing HER2 expression in N87-TRC tumors treated as indicated.

D, Quantification of the intensity of the completely stained cells (n=10 for each group). Student’s t-test: *p<0.0001, **p=0.054 versus control; # p=0.0074, AUY versus Combination.
Figure 1
Figure 2

A  Gastric Cancer Panel IC50 with NVP-AUY922 Treatment

B  Breast Cancer Panel IC50 with NVP-AUY922 Treatment

Cell Line
Figure 5

A

SKBR3-TR

CI=0.397±0.180

(p=0.07)

BT474-TR

CI=0.941±0.041

(p=0.36)

NCI-N87-TR

CI=0.726±0.131

(p=0.17)

% Growth inhibition

Concentration (AUY922 [0.00125uM]; Trastuzumab [2.5ug/mL])

0.00125 0.0025 0.005

2.5 5 10

B

NCI-N87-TR

C AUY T COMBO

pAKT

AKT

pERK

ERK

HER2

α-Tubulin

BT474-TR

C AUY T COMBO

pAKT

AKT

pERK

ERK

HER2

α-Tubulin

C

0' 24hr

pHER3

HER3

pIGF1R

IGF1R

α-Tubulin

NCI-N87-TR

C AUY T COMBO

0' 24hr

pHER3

HER3

pIGF1R

IGF1R

α-Tubulin
Figure 6

(A) Tumor Volume (mm$^3$) over Study Days for Vehicle Control, Trastuzumab, and NVP-AUY922.

(B) Tumor Volume (mm$^3$) over Study Days for Vehicle Control, Trastuzumab (T), NVP-AUY922 (A), and Combination.

(C) Histological images of Control, Trastuzumab, AUY, and Combination.

(D) H-score (+) for Control, AUY, Trastuzumab, and Combination in NCI-N87-TR$^c$. 
Molecular Cancer Therapeutics

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