Low Dose HSP90 Inhibition with AUY922 Blunts Rapid Evolution of Metastatic and Drug Resistant Phenotypes Induced by TGF-β and Paclitaxel in A549 cells

Nickolas A. Bacon¹, Isabel Larre¹,², Abdalla A. Lawag¹, Carlen Merritt II¹, Mackinzie Smith¹, Matthew Rosolen¹, Vincent E. Sollars¹,+¹
¹Department of Biomedical Sciences, Joan C. Edwards School of Medicine at Marshall University
²Department of Clinical and Translational Sciences, Joan C. Edwards School of Medicine at Marshall University

Abstract

Objectives—Despite advances in cancer treatment, drug resistance and metastasis continue to contribute to treatment failure. Since drug resistance and metastasis in cancer are features that often occur toward the late stages in the disease after withstanding numerous selective pressures, they may rely on a shared adaptive mechanism in order to persist. The heat shock response is one of the most well conserved adaptive responses to cellular stress found in nature. A major player in the heat shock response is HSP90, with some studies suggesting that it can facilitate the molecular evolution of drug resistance and metastasis in cancer. Non-small cell lung cancers (NSCLCs) are strongly associated with drug resistance and metastasis either at the time of diagnosis or early in the treatment process.

Materials and Methods—We explored the role of HSP90 in the evolution of metastatic and drug resistant features in NSCLC by treating A549 cells with AUY922, a clinically relevant HSP90 inhibitor, and inducing metastatic and drug resistant phenotypes via treatment with TGF-β and paclitaxel, respectively. We measured phenotypic plasticity in E-Cadherin, a marker for epithelial to mesenchymal transition and two ABC transporters associated with drug resistant lung cancers.

Results—We found that metastatic and efflux dependent drug resistant features negatively correlated with AUY922 treatment. We followed our results with functional assays relevant to metastasis and ABC transporters to confirm our results. Specifically we found the expression of E-
cadherin was significantly increased in A549 cultures pretreated with AUY922 prior to exposure to paclitaxel, while expression of the drug transporters ABCB1 and ABCC1 was significantly reduced under similar conditions.

**Conclusion**—Together our data indicates that HSP90 inhibition with AUY922 can limit the acquisition of metastatic and drug resistant phenotypes in A549 cells at low, clinically appropriate doses.

**Graphical Abstract**

**Keywords**
HSP90 inhibitors; lung cancer; drug resistance; metastasis; molecular evolution; phenotypic plasticity

**1. INTRODUCTION**

Lung cancer is the most commonly diagnosed cancer and is the leading cause of cancer mortality worldwide regardless of sex[1]. Eight five percent of lung cancers are non-small cell lung cancers (NSCLC)[2], with metastasis and ABC transporter driven chemotherapeutic resistance common features of these cancers either at the time of diagnosis or manifesting during the treatment process[3, 4] [5–8]. Therefore, discovering ways to limit metastasis and chemotherapeutic resistance in NSCLCs will benefit many cancer patients.

Several lines of recent evidence indicate that pharmacological inhibition of Heat Shock Protein 90 (HSP90) can limit molecular pathways involved in epithelial to mesenchymal transition (EMT), migration and metastasis *in vitro* and in animal xenografts of different cancer cell lines of various tissue types[9, 10]. Whether newer, clinically relevant HSP90 inhibitors can limit these processes in NSCLC cell lines has not been documented. Furthermore, certain HSP90 inhibitors have demonstrated a synergistic anticancer effect...
with conventional cytotoxic chemotherapies such as paclitaxel (PTX, [11, 12]) and doxorubicin[12]. While the synergistic action of these drugs and HSP90 inhibitors was attributed to mechanisms related to cell signaling, PTX is a known substrate for ABCB1 and doxorubicin is a known substrate for both ABCB1 and ABCC1 [13, 14]. This leaves open the possibility that perhaps some of the synergistic actions of HSP90 inhibition is due to intracellular accumulation of PTX and doxorubicin as a result of reduced ABC transporter expression. However, no relationship has been shown between HSP90 inhibition and reduced expression of ABC transporters in the context of multi drug resistant cancers to date.

In the present study, we investigate whether treatment with the clinically relevant HSP90 inhibitor, AUY922, given at the same dose and exposure time could limit the rapid acquisition of invasive phenotypes and ABC transporter driven drug resistance in A549 cells, a common NSCLC cell line. AUY922 is unique among HSP90 inhibitors as it is readily soluble in ethanol and can inhibit both the inducible (HSP90α) and constitutive (HSP90β) isoforms of HSP90 at low nanomolar concentrations[15, 16]. We were guided by the work of others who used relatively low doses of HSP90 inhibition to limit the emergence of tamoxifen resistance in MCF-7 breast cancer cells[17]. If successful, our work would further strengthen the case for other clinically relevant HSP90 inhibitors, and AUY922 in particular, to be deployed in the clinic at low doses as an adjunct to prevent molecular evolution of drug resistant and metastatic phenotypes that can be associated with cytotoxic chemotherapies [18, 19]. Furthermore, successfully utilizing smaller doses of chemotherapy can be expected to improve the quality of life for cancer patients undergoing chemotherapy. We have found that inhibition of HSP90 through treatment with AUY922 at very low dosages (10–20 nM) prevents EMT. These dosages are remarkably below the maximum tolerated dosage (once/week 70 mg/m², 215 μM in serum after 10 hours) found using dose escalation studies in clinical trials of AUY922[10]. Thus, it is possible to affect phenotypic plasticity at dosages that will have minimal adverse events as none were detected at doses as high as 8 mg/m² (190 μM in serum after 10 hours).

2. MATERIALS AND METHODS

2.1 CELL CULTURE

A549 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC)(Account #: 86012804; Lot: 165012) and cultured as recommended by ECACC. All experiments were performed between passages 5 and 20 from low passage frozen stocks from the originally purchased vial.

2.2 CELL VIABILITY ASSAY (XTT)

The viability of A549 cells under AUY922 and PTX treatment was assessed in parallel using the XTT assay (Biotinium; catalog #: 30007) per manufacturer recommended protocol. Plates were read on Spectramax i3x® microplate reader at wavelength 450nM. Each experiment was performed three times.
2.3 WESTERN BLOT

A549 cells were seeded in six-well plates at 4.0 × 10^5 cells/well and allowed to attach overnight. The next day, medium was aspirated and replaced with 1.0 mL fresh growth medium and treated with indicated drug. After 6 hours, medium was aspirated and the cells were harvested, quantified, and transferred to nitrocellulose membranes as described[20]. Primary antibodies were used at a dilution of 1:1500 mouse anti-HSP70 (Enzo, ADI-SPA-810-D, clone: C92F-3A-5) and 1:10000 mouse anti-GAPDH (Fitzgerald Industries®, cat# 10R-G109a). Species-specific europium-conjugated secondary antibody (ScanLater goat anti mouse; part#: R7562; Molecular Devices®) was used. Afterward, membranes were washed with TBS-T and analyzed via Spectramax i3x® western blot cartridge (Molecular Devices®). Two independent experiments were performed in triplicate (n=6).

2.4 FOUR DAY EMT INDUCTION

A549 cells were seeded into six-well plates (50,000 cells/well) and allowed to attach overnight. Medium was aspirated and replaced with 2.0 mL fresh growth medium and treated with drug for 48 hours. After, medium was aspirated from each well and replaced with 2.0 mL of fresh growth medium. One plate from each treatment group was treated with 10 ng/mL TGF-β (Peproptech, Cat#:100–21), while the others remained in untreated medium for an additional 48 hours. Afterward, cells were detached with trypsin for 15 minutes placed into flow tubes, washed, stained for viability (Zombie-NIR, 77184, Biolegend), counted via trypan blue, normalized to cell numbers and blocked as previously described[21]. Samples were stained with the following antibodies: Alexafluor®647 anti-human E-Cadherin (BD Biosciences, 56371, clone: 67A4), Alexafluor®488 anti-human ABCC1 (BioLegend, 370306, clone: QCRL-3), and Brilliant Violet®421 ABCB1 (BD Biosciences, 566015, clone: UIC2) per sample for 30 minutes on ice protected from light. Three independent experiments were performed in triplicate for each treatment group. (n=9)

2.5 WOUND HEALING ASSAY

A549 cells were seeded into six well plates (50,000 cells/well) and allowed to attach overnight. Cultures were treated with drug for 48 hours. After, cells were detached with trypsin, collected and spun at 300g for 5 minutes. Supernatant was decanted and pellets were resuspended in 1.0 mL fresh growth medium. 3.0 × 10^5 cells were collected from each tube and resuspended in 400 μL of fresh growth medium. We empirically determined that this cell concentration would yield a confluent monolayer when seeded into 20 μL drops and allowed to attach overnight (15,000 cells/drop). Three 20 μL drops were seeded into the top row of three six-well plates, one plate per treatment group (9 drops per treatment group in total) and allowed to attach overnight. Cells were then scratched with a 200 μL pipette tip and 1.0 mL of fresh growth medium was added to each well. Pictures and measurements were taken at time 0 hours and time 18 hours over the middle of the colonies by drawing a single straight line through the nuclei of cells on the left side of the migration front and dropping three perpendicular lines across the viewing field (one at the top, one through the middle and one at the bottom) to the migration front on the right. Migration index was calculated by dividing average starting gap width by the average gap width at 18 hours. Two independent experiments were performed (n=18 for each treatment group).
2.6 TRANS EPITHELIAL RESISTANCE (TER) ASSAY

A549 cells were seeded into 6 transwell dishes (10,000 cells/well) and allowed to grow for 72 hours. At this time point, fresh medium was given and three wells were treated with 20 nM of AUY922. TER measurements were taken at 24 hours within 5 minutes of removing from the incubator so as to eliminate the influence of temperature on our measurements. Final values were obtained by subtracting the resistance of the bathing solution and an empty support. Results are expressed as ohms per square centimeter ($\Omega \cdot \text{cm}^2$). To compare effects obtained in different monolayers on different days, we normalized data as described[22]. Two independent experiments were performed in triplicate (n=6).

2.7 EIGHT DAY ABC TRANSPORTER INDUCTION

A549 cells were treated with AUY922 during the first 48 hours of the experiment as in 2.4, but instead of inducing changes in E-cadherin with TGF-$\beta$, we induced changes in ABC transporters with PTX. We extended the experimental timeline from four days to eight days to provide cells pretreated with AUY922 an additional two days to expand in untreated media after their initial treatment during the first 48 hours of the experiment and after induction of ABC transporters with PTX during days 4–6. A549 cells were collected from stock flask and thirty, 20 $\mu$L drops (8,000 cells/drop, 2.4 $\times$ 10$^5$ cells/petri dish) were seeded into 100 mm petri dishes and allowed to attach overnight. This method of seeding provided reproducible circular colonies over the course of the experiment that limits the stress to cells associated with trypsinization and reseeding. The following day, cells were treated with AUY922. After 48 hours, treated medium was aspirated and replaced with fresh untreated growth medium. After an additional two days, medium was aspirated and replaced with fresh growth medium. At this time point some petri dishes were treated with 10 nM of PTX (ABC transporter induction), while the others remained untreated (no ABC transporter induction). Petri dishes were allowed to grow for an additional two days. On day six, medium was aspirated from each petri dish and replaced with fresh untreated growth medium and allowed to grow for an additional two days. On day eight, cells were harvested. Cells were detached with trypsin/4 mM EDTA for 20 minutes at 37°C. Cells were washed, stained for viability, counted, normalized to cell numbers, and blocked as previously described[21]. Each sample was stained with antibodies: Alexafluor®488 anti-human ABCC1 (BioLegend, 370306, clone:QCRL-3), and Brilliant Violet ®421 ABCB1 (BD Biosciences, 566015, clone:UIC2). At least two independent experiments were performed with at least two biological replicates per treatment. (No MDR induction n=5, MDR induction n=8)

2.8 DOXORUBICIN EFFLUX ASSAY

A549 cells were seeded as described in 2.7. On day eight, cells were harvested and 2.5 $\times$ 10$^5$ cells were placed into flow tubes, washed with growth medium and centrifuged at 300g for 5 minutes. Supernatant was decanted and pellets were gently resuspended in 500 $\mu$L of warm growth medium treated with 10 $\mu$M of doxorubicin. Cells were incubated at 37°C for 30 minutes to load with doxorubicin. Samples were washed in growth medium and centrifuged at 300g for 5 minutes. Supernatant was decanted and pellets resuspended in 1.0 mL of growth medium. Samples were incubated at 37°C with agitation for 2 hours. Afterward,
samples were washed with 2.0 mL of room temperature PBS followed by centrifugation at 300g for 5 minutes. Supernatant was decanted and pellets were resuspended in 200 μL of Zombie-NIR viability reagent was applied per manufacturer’s instructions. After the final wash and decant, cells were gently resuspended in residual cold FACS buffer with a pipette tip to generate a homogenous single cells suspension and analyzed via flow cytometry. This method in using doxorubicin to study efflux activity of ABC transporters has been used by other investigators [23, 24] and we developed our assay in accordance with these studies. Three independent experiments were performed in at least duplicate (n=8).

2.9 FOUR, SIX AND EIGHT DAY CULTURE GROWTH

A549 cells were collected from stock flask, seeded as described in 2.7. On day four and six, cells were washed and detached. Cells were resuspended in 1.0 mL of fresh growth medium and a 10 μL aliquot was taken from each sample to count via hemacytomter and 0.4% typan blue staining. For samples without MDR induction with PTX, a single experiment was performed in triplicate for day four and day six (n=3 for each day). For samples with MDR induction with PTX, two independent experiments in triplicate were performed on days four and six (n=6 for each day). Day eight counts were a combination of counts gathered on day eight from MDR induction immunophenotyping and doxorubicin efflux studies.

2.10 FLOW CYTOMETRIC ANALYSIS

All flow cytometric analysis were performed via Novocyte ® 3000 flow cytometer (ACEA Biosciences, Inc., San Diego, California). 50,000 single cell events were analyzed. All data analysis was performed using NovoExpress Software version 1.3.0 (ACEA Biosciences, Inc., San Diego, California). For all antibodies, we used fluorescence minus one (FMO) and corresponding isotype controls to determine positive staining background. All data analysis was performed using NovoExpress Software version 1.3.0 (ACEA Biosciences, Inc., San Diego, California).

2.11 STATISTICAL ANALYSIS

All statistical analysis was performed using Prism v8.0a software (GraphPad Software Inc., San Diego, California). Appropriate statistical tests were performed with corresponding significance values indicate in figure legends. For the E-cadherin flow cytometry studies and wound-healing assay, analysis was performed using one-way ANOVA with Tukey’s test for multiple comparisons. For trans-epithelial resistance, eight-day flow cytometry studies without MDR induction, eight-day growth curves and doxorubicin efflux studies, analysis was performed using Student’s t-test for unpaired data. For ABC transporter flow cytometry studies with/without EMT and with MDR induction, analysis was performed using one-way ANOVA with Dunnet’s test for multiple comparisons.

3. RESULTS

3.1 AUY922 AND PACLITAXEL HAVE SIMILAR EFFECTS ON A549 CELL VIABILITY AFTER 48 HOURS EXPOSURE

Before investigating metastatic and drug resistant properties of A549 cells, we had to evaluate their sensitivity to AUY922 and PTX. PTX was used in many of our studies as it is
currently used in combination with other chemotherapies in NSCLC and has been associated with development of drug resistance through ABC transporters[25] and metastasis[18, 19]. Intriguingly, A549 cells displayed almost identical viability curves to increasing doses of either drug at 48 hours of exposure (Fig. 1A). Since we were most interested in evaluating how A549 cells change in response to these drugs, not necessarily how they are killed, we wanted to refrain from using high doses of either drug in our experiments to avoid selecting for a particular metastatic or drug resistant phenotype in the A549 cell population. We decided we could achieve this by focusing on doses below the EC₅₀ (as measured by viability with an XTT assay), which in this case would be doses less than 25.4 nM for AUY922 (95% CI 22.3 to 28.5 nM) and less than 30.2 nM for PTX (95% CI 25.0 to 36.8 nM) (Fig. 1A).

Since PTX can exhibit additional growth inhibition beyond 48 hours (Supplemental Figure 1), we decided that 10 nM treatment with PTX would be appropriate to minimize selection for any particular phenotype, as our planned experimental endpoints were to extend well beyond 48 hours. We chose 20 nM of AUY922 as this dose was enough to demonstrate up-regulation of HSP70 (Fig. 1B), and is below the EC₅₀. Up-regulation of HSP70 is considered to be a reliable indicator of successful pharmacological HSP90 inhibition[17]. We used Cmax values gathered from clinical trials[26–28] to confirm that 20 nM of AUY922 is well within a clinically relevant treatment range. We have found that inhibition of HSP90 through treatment with AUY922 at very low dosages (10–20 nM) prevents EMT (Fig. 1). These dosages are remarkably below the maximum tolerated dosage (once/week 70 mg/m², 215 μM in serum after 10 hours) found using dose escalation studies in clinical trials of AUY922[10]. Thus, it is possible to affect phenotypic plasticity at dosages that will have minimal adverse events as none were detected at doses as high as 8 mg/m² (190 μM in serum after 10 hours).

3.2 AUY922 PROMOTES E-CADHERIN SURFACE EXPRESSION IN A549 CELLS, WHICH REMAINS DURABLE AFTER EMT INDUCTION WITH TGF-β

Induction of EMT is a well-known method to study metastatic properties in vitro and is thought to drive metastatic changes in vivo[29, 30]. A hallmark of EMT is loss of E-cadherin and studies have demonstrated reduced E-cadherin expression in NSCLC tumors derived from clinical samples correlates with poor tumor differentiation and invasion of local structures including vasculature[31]. Therefore, we reasoned that in our studies E-cadherin would be a suitable marker to evaluate changes related to metastatic progression that would translate to the clinic.

We exposed A549 cells to 20 nM of AUY922 or 10 nM of PTX for 48 hours, followed by 48 hours in untreated media, and captured phenotypic changes on day four via flow cytometry. This protocol was found to induce A549 cells to switch from a cuboidal epithelial state to an elongated mesenchymal state. Treatment with AUY922 promoted the E-cadherin positive fraction while PTX reduced E-cadherin positive fraction relative to the control (Fig. 1C, D). Additionally, there was a significant increase in median fluorescence intensity (MFI) in AUY922 treated group cultures relative to either PTX treated cultures or control cultures, an indication that the positive fraction had an overall increase in E-cadherin expression per cell.
(Fig. 1E). Overall, these changes indicate that A549 cells exhibit phenotypic plasticity in their ability to adjust E-cadherin levels.

Transforming Growth Factor-β (TGF-β) is a well-known and potent inducer of EMT in A549 cells[32]. To evaluate the durability of the increase in E-cadherin expression caused by AUY922, we decided to follow the initial 48 hour treatment period with 48 hours in TGF-β treated media. While 20 nM of AUY922 did not completely desensitize A549 cells to TGF-β driven EMT in these cultures, both the percent positive fraction and the MFI were significantly higher than either the PTX treated or control cultures (Fig. 1F–H). The E-cad+ population was similar in both untreated A549 cells and those pretreated with AUY922 and then given TGF-β (Fig. 1C and F), suggesting that AUY922 conditioned the cells to resist EMT induction. Together, this data indicates that AUY922 generates changes in A549 cells that promote E-cadherin expression that remains durable even in the presence of strong drivers of the metastatic cascade such as TGF-β, relative to PTX treatments or control cultures.

3.3 AUY922 LIMITS MOTILITY OF A549 CELLS

If E-cadherin loss is associated with increased invasion and metastasis, then it is reasonable that the increase in E-cadherin we observed with AUY922 treatment could influence the migratory capacity of A549 cells. To investigate this, we exposed A549 cells to 20 nM of AUY922 and 10 nM of PTX for 48 hours, and performed the wound-healing assay as described. As expected, AUY922 treated cells had drastically limited migration capacity compared to PTX treated or control cultures (Fig. 2 A, B) that was robust enough for the gaps to remain open even after 48 hours (Supplemental Figure 1).

3.4 AUY922 PROMOTES TIGHT JUNCTION FORMATION IN A549 CELLS

The increase in E-cadherin expression as measured by MFI (Fig. 1E and H) under AUY922 treatment prompted us to find additional details to identify a more definitive cause for the lack of migration in the scratch assay. Increased E-cadherin expression may indicate the promotion of tight junctions between cells. To investigate this, we plated A549 cells in transwell plates and measured tight junction formation via trans-epithelia resistance (TER) in our previous treatment conditions. At 24 hours, there was a substantial increase in TER in AUY922 treated cultures relative to control cultures (Fig. 2C).

This observation helps explain the lack of migration of AUY922 treated A549 cells in the scratch assays. By promoting tight junction formation, the increased intensity in cell-cell adhesion makes it less likely for the cells at the edge of the scratch to migrate into the gap. This places AUY922 as a potential cell-cell adhesion enhancer, which has benefits in the context of cancer metastasis as increases in cell-cell adhesion discourages cancer cells from invading surrounding tissue.

3.5 AUY922 TREATMENT DOES NOT INDUCE SURFACE EXPRESSION OF ABCB1 OR ABCC1 COMPARED TO PACLITAXEL TREATMENT

During the EMT induction experiments where we exposed A549 cells to AUY922 or PTX for 48 hours and followed with 48 hours of untreated medium or TGF-β treated medium to
measure changes in E-cadherin expression, we also probed for expression of ABCB1 (P-glycoprotein, P-gp) and ABCC1 (multidrug resistance protein 1, MRP1). In agreement with others [33, 34], PTX treatment in A549 cells promoted a slight increase in ABCB1\(^+\) fraction relative to the control cultures when captured on day four (Fig. 3A–C). Induction of EMT with TGF-β in the final 48-hour time period enhanced this ABCB1\(^+\) fraction. PTX treated cultures also had similar increases in ABCC1\(^+\) fraction relative to the control, with or without TGF-β in the final 48 hours before capture (Fig. 3D–F).

In contrast, there was no increase in ABCB1 or ABCC1 in either case relative to the control in AUY922 treated cultures (Fig. 3). While the changes in AUY922 cultures were of little statistical significance compared to control cultures, considered as a whole there was a general downward trend, especially in AUY922 treated cultures exposed to TGF-β in the final 48 hours before capture (Fig. 3C, F). This was interesting to us since A549 cells treated with AUY922 received a chemical stimulus at twice the molar dose of PTX cultures, yet remained insensitive to induction to part of the xenobiotic defense response for which ABC transporters evolved to carry out\[35\]. Additionally, if we analyze A549 cells treated with 20 nM AUY922 for 48 hours followed by fresh media for the remaining 6 days we found an enduring response of reduced fraction of the ABCB1\(^+\) or ABCC1\(^+\) (Figure 4 A–C) relative to control cultures that received fresh untreated media every 2 days for 8 days (Figure 4 A–C). This indicates that treatment with AUY922 at these levels will not induce a ABC transporter response and will reduce transporter expression over extended timeframes.

### 3.6 SINGLE TREATMENT WITH AUY922 DESENSITIZES A549 CELLS TO INDUCTION OF MDR PHENOTYPE VIA PACLITAXEL TREATMENT

While treatment with AUY922 did not induce transporter activity, we wished to determine if it would inhibit phenotypic plasticity of cells when presented with a stimulus that would induce transporter activity. With this information, we formed a new strategy to maximize the resolution of our putative observations of the reduced fraction of ABCB1\(^+\) and ABCC1\(^+\) cells under AUY922 treatment (Fig. 4). In order to maximize the resolution of our observations of putative reduction in the MDR phenotype with AUY922 treatment, we altered the protocol as described in the methods under 8-day experiments. This experimental timeline allows the assay of longer responses and provides a window for triggering a drug response by PTX.

Cells pretreated with 20 nM AUY922 for the first 48 hours appeared insensitive to induction of the MDR phenotype with 10nM PTX on days 4–6 compared to control cultures that only received 10 nM of PTX on days 4–6 and showed dramatic induction of the MDR phenotype (Fig. 5A–C). The observation of reduced induction of the MDR phenotype under AUY922 treatment appeared to be dose dependent, as pretreatment with 10 nM of AUY922 also demonstrated a proportional decrease in the MDR phenotype (Fig. 5A–C). It appears that just as AUY922 treatment desensitized A549 cells to acquisition of a metastatic phenotype through TGF-β driven EMT, they also appear insensitive in attaining the MDR phenotype. Furthermore, MDR induction with PTX generated a robust double positive ABCB1 and ABCC1 population, which was not present in cultures that did not receive MDR induction with PTX (data not shown), and this was where the greatest reduction in the MDR
phenotype as a result of AUY922 treatment appeared to occur (Supplemental Figure 2). This is a significant finding in that ABCB1 and ABCC1 have considerable substrate overlap in chemotherapies commonly used to treat lung cancer[13], and experimental evidence suggests expression of multiple ABC transporters are required for cancers to achieve multidrug resistance[36, 37] [38]. This means that HSP90 inhibition with AUY922 has the potential to limit phenotypic plasticity of lung cancer cells to an MDR phenotype that can generate the greatest efflux of chemotherapies across their cell membrane.

3.7 LOSS OF MDR PHENOTYPE FROM AUY922 TREATMENT HAS A FUNCTIONAL CONSEQUENCE THAT IMPACTS A549 CELL SURVIVAL

Our results indicating that treatment with AUY922 desensitizes A549 cells to induction of the MDR phenotype with PTX suggest that these cells are more sensitive to cell death via PTX treatment than controls treated with PTX alone on day 4. However, given that ABC transporters are only one of several methods in which cancer cells can generate MDR, and the wide range of signaling pathways that may be affected by HSP90 inhibition, it is possible that we could be reducing one mechanism of multidrug resistance with AUY922 while reinforcing another.

To investigate if AUY922 altered the viability of A549 cells over the 8-day period, we began tracking the culture growth at important time points in the experiment, in particular days four, six and eight. A549 cells that received fresh medium every two days for eight days showed classical unimpeded exponential growth, while A549 cells that received 20 nM of AUY922 for the first two days followed by fresh medium every two days for the next 6 days showed slowing of culture growth at each time point throughout the experiment, but an overall positive slope to the growth curve (Fig. 6A).

In the treatment groups that received 10 nM of PTX on day 4 followed by fresh medium on day 6, more complex changes in culture growth were observed. A549 cells that remained untreated until they received 10 nM of PTX on day 4 showed a slowing of growth from days 4 to 6, but on days 6 to 8 demonstrated accelerated growth (Fig. 6B black line). In contrast, A549 cells treated with 10 nM and 20 nM AUY922 showed a similar slowing of culture growth from days 4 to 6, but a dose dependent decrease in the slope of the growth curve from days 6 to 8 (Fig. 6B orange and green lines). In the case of 20 nM of AUY922 treatment during the first two days, there was a negative slope to the growth curve from days 6 to 8.

These results may be explained by our previous findings of reduced MDR phenotype in A549 cells pretreated with AUY922 captured on day 8 via flow cytometry (Fig. 5). When A549 cells treated with AUY922 throughout the first 48 hours of the experiment are unable to access the MDR phenotype during PTX treatment during days 4 to 6, they cannot efflux PTX when they receive fresh medium on days 6–8, thus promoting A549 cell death and a decrease in the slope of the growth curve during this time (Fig. 6B, C). These results are consistent with ABCB1 being able to efflux PTX well. However, ABCC1 effluxes PTX poorly [39].
To further test the functional consequences of the MDR phenotype, we performed drug efflux studies to ascertain the ability of A549 cells to remove drugs. To account for both ABCB1 and ABCC1 in the MDR phenotype and the results of the growth curve in cultures that received PTX on day four, we decided to perform an efflux assay using doxorubicin as a fluorescent substrate. Doxorubicin is a substrate for both ABCB1 and ABCC1 [13] and has intrinsic fluorescent properties that make it ideal to study drug efflux via flow cytometry. We performed the eight-day experiment just as before, but instead of immunophenotyping for ABCB1 and ABCC1, we loaded the cells with doxorubicin and provided a washout period to allow doxorubicin efflux, before analysis via flow cytometry. If our observation of the reduced MDR phenotype in AUY922 treated A549 cells has a functional significance, these cultures should accumulate doxorubicin to a greater extent than A549 cells that received PTX alone. Indeed, this was our result (Fig. 6D), with significant change occurring in A549 cells that received 20 nM of AUY922 (Fig. 6E). Our efflux assay did not indicate a significant change between cultures that only received MDR induction with PTX on day four compared to cultures treated with 10 nM AUY922 before MDR induction with PTX, even though there was a significant decrease in the MDR phenotype in these cells. This may be due to a threshold effect, where accumulation of drug in the A549 cell population does not occur unless transporter activity is reduced to a certain amount.

Together, the growth curve analysis and the efflux assay serves to support the results of the decreased MDR phenotype in AUY922 treated cells captured via flow cytometry during the eight-day experiment. The growth curve analysis also shows that we are not engaging other drug resistance mechanisms that are sufficient to restore cell survival as a product of AUY922 treatment. Furthermore, the resultant reduction in ABCB1 and ABCC1 positive fraction in the culture is sufficient to generate a functional consequence that leads to intracellular accumulation of chemotherapy, promoting increased cell death.

4. DISCUSSION

Previous studies have implicated HSP90 in facilitating cellular mechanisms that drive drug resistance and the metastasis in several cancer types, and that pharmacological HSP90 inhibition can deconstruct these mechanisms[9, 10, 17]. However, these studies only evaluated drug resistance and metastasis separately. Here we demonstrate that HSP90 inhibition with AUY922 can limit both metastatic and drug resistant features in A549 NSCLC cells at the same clinically relevant dose. Moreover, these changes can be maintained even in the presence of strong inducers for metastatic and drug resistant phenotypes over a relatively long time frame.

Our findings support the work of others demonstrating HSP90 inhibition abrogates EMT in other cancers[9, 10]. However this is the first documentation of these observations in A549 NSCLC cells with AUY922. The increased E-cadherin expression in our flow cytometry studies combined with our scratch assays and TER measurements indicates that AUY922 treatment enhances cell-cell adhesion. This is pertinent to NSCLC, which is often locally advanced at the time of diagnosis[40, 41]. Immediate intervention with low dose AUY922 may be a useful treatment strategy in preventing further progression of the metastatic cascade in these cancers.
This is the first documentation of the relationship between HSP90 inhibition with AUY922 in A549 cells and reduced cell surface expression of ABCB1 and ABCC1 with any HSP90 inhibitor in any cancer cell line. A single treatment with AUY922 is enough to maintain a significant reduction in both ABCB1 and ABCC1 for up to 8 days. Moreover, pretreatment with AUY922 suppressed cell surface expression of ABCB1 and ABCC1, even when stimulated to induce ABC transporter expression with PTX. We were able to demonstrate a functional consequence of these findings by tracking cell growth over the eight-day experimental timeline and through efflux studies using doxorubicin, which is a substrate for both ABCB1 and ABCC1, as a fluorescent drug accumulation marker. Our findings and experimental design are clinically relevant since both ABCB1 and ABCC1 are thought to play a significant role in drug resistant NSCLCs\(^5\)–\(^8\), and PTX is still used to treat NSCLC. Additionally, ABC transporters, in particular ABCB1, can contribute to drug resistance in other cancers\(^{13, 42}\), potentially making our findings applicable to a wide variety of cancers.

The recent evidence that some treatment approaches may incidentally enable the metastatic cascade\(^{18, 19, 43}\) and the persistence of drug resistant relapse after current treatment methods\(^{44}\) demands new, rational, treatment strategies that can curtail the emergence of metastatic and drug resistant cancer phenotypes simultaneously. The work presented here points to AUY922 as a possible drug candidate to accomplish this in NSCLC. We demonstrate that a single treatment with AUY922 at a relatively low, clinically appropriate dose can reduce phenotypic changes associated with both drug resistance and metastasis in A549 cells and can be maintained even when potent inducers of metastatic and drug resistant phenotypes is applied. Additionally, we used AUY922 treatments in combination with drugs that are currently used to treat NSCLC, making a case that low dose pretreatment with AUY922 may prevent undesirable changes related to metastasis and drug resistance that has been associated with conventional cytotoxic therapy like PTX\(^{18, 19, 25, 43}\).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**ABBREVIATIONS**

- **EMT**: epithelial to mesenchymal
- **HSP90**: Heat Shock Protein 90
- **NSCLC**: non-small cell lung cancers
- **PTX**: paclitaxel
- **TGF-β**: Transforming Growth Factor- β
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Significance

Findings indicate that clinically relevant HSP90 inhibitor AUY922 can restrict evolution of metastatic and drug resistant phenotypes at low dosages.
### Highlights

- Low Doses of AUY922 can restrict phenotypic plasticity in lung cancer cells
- HSP90 inhibition prevents EMT and up-regulation of drug transporters cancer cells
- HSP90 inhibition of phenotypic plasticity has functional consequences
FIGURE 1. HSP90 inhibition prevents the induction of EMT in A549 cells.
(A): Chemosensitivity of A549 cells to 48 hour exposure to increasing concentrations of PTX (blue) and AUY922 (green) ranging from 0.001–2,000 nM. Viability was determined using the XTT assay. Each dose was performed in sextet per individual experiment. Data represents the mean ±95% CI of three independent experiments. EC\textsubscript{50} A549 v PTX: 30.2 (95% CI 25.0–36.8 nM); EC\textsubscript{50} A549 v AUY922: 25.4 (95% CI 22.3–28.5 nM) (B) Induction of HSP70 expression by HSP90 inhibition with AUY922 after only six hours of exposure. A representative blot of two independent experiments performed in triplicate is shown. (C)
Flow cytometric analysis of E-Cadherin expression after 48 hours of treatment followed by 48 hours in untreated medium (w/o EMT induction). (D) Quantification of the percentage of cells positive for E-Cadherin w/o EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (E): Quantification of the median fluorescence intensity of the E-cadherin positive fraction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (F) Flow cytometric analysis of E-Cadherin expression after 48 hours of treatment followed by 48 hours EMT induction in medium supplemented with 10 ng/mL of TGF-β. (G) Quantification of the percentage of cells positive for E-Cadherin with EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (H): Quantification of the median fluorescence intensity of the E-cadherin positive fraction with EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). **P<0.01; ****P<0.0001.
FIGURE 2. Effect of AUY922 treatment on migratory capacity and tight junction formation in A549 cells.

(A) After 48 hours of drug exposure cells numbers were normalized by reseeding in 20 μL drops (15,000 cells/drop) and allowed to attach overnight. The circular colonies that formed were then scratched with 200 μL pipette tip and pictures were taken at time 0 hours (top) and 18 hours (bottom). Two individual experiments were performed. Images are representative scratches from an individual colony. There were 9 total colonies per experiment per treatment group (n=18). (B) Quantification of mean migration index calculated by dividing starting gap width by the gap width at 18 hours. (C) Change in trans-epithelial electric resistance (TER) across confluent monolayers of A549 cells after 24 hours of 20 nM AUY922 treatment. Two independent experiments were performed in triplicate (n=6); All data is representative of the mean ±SEM of two independent experiments performed as described **P<0.01, ****P<0.0001.
FIGURE 3. Drug transporter expression in EMT induction experiments.
(A) ABCB1 expression without EMT induction (Top) and with EMT induction with TGF-β (Bottom) of indicated drug treatments. (B) Quantification of the percentage of cells positive for ABCB1 without EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (C) Quantification of the percentage of cells positive for ABCB1 and EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (D) ABCC1 expression without EMT induction (Top) and with EMT induction with TGF-β (Bottom) of indicated treatments. (E) Quantification of three independent experiments performed in triplicate (n=9). (F) Quantification of percentage of cells positive for ABCC1 with EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). *P<0.05, ***P<0.001, ****P<0.0001.
FIGURE 4. Flow cytometric analysis of ABC transporters captured at day 8 after indicated drug treatment.
(A) (Top) ABCB1 (Bottom) ABCC1 expression in A549 cells after a single treatment with 20 nM AUY922. (B) Quantification of the percentage of cells positive for ABCB1 captured at day 8. (C) Quantification of the percentage of cells positive for ABCC1 captured at 192 hours. All data is representative of the mean ±SEM of two independent experiments performed at least in duplicate (n=5). *P<0.05, **P<0.01.
FIGURE 5. Flow cytometric analysis of ABC transporter expression captured at day 8 for cultures treated with 10 nM PTX to induce MDR.

(A) (Top) ABCB1 (Bottom) ABCC1 expression in A549 cells after a single treatment of AUY922 followed by MDR induction with 10 nM PTX as described. (B) Quantification of the percentage of cells positive for ABCB1 captured at day 8. (C) Quantification of the percentage of cells positive for ABCC1 captured at day 8. PTX=10 nM PTX. Text before the slash indicates treatment conditions during hours 0–48. Text after the slash indicates treatment conditions from days 4 to 6. All data is representative of the mean ±SEM of three independent experiments performed at least in duplicate (n=8). *P<0.05, **P<0.01, ****P<0.0001.
FIGURE 6. Growth curve during 8-day ABC transporter experiments and doxorubicin efflux assay.

(A) Growth curve of A549 cultures during the 8-day experiment after a single 48 hour 20 nM treatment of AUY922 (n=3 per time point). (B) Growth curve of A549 cultures exposed to 10 nM of PTX to induce MDR as described after 48-hour pretreatment with indicated doses of AUY922 (n=6 per time point). (C) Comparison of viable cells at day 6 and day 8 n cultures pretreated with 20 nM AUY922 followed by MDR induction, indicating significant difference in viability. (D) Doxorubicin efflux assay representative flow cytometry histogram median fluorescence intensity of the total population of A549 cultures treated with 10 nM of PTX on day 4 (gray) and A549 cells pretreated with 20 nM of AUY922 before receiving 10 nM of PTX on day 4 (black). Light gray curve represents negative control. (E) Doxorubicin efflux assay quantification of the median fluorescence intensity of doxorubicin accumulation of the total cell population treated as indicated (n=8). In (B) and (C), text before the slash indicates conditions from hours 0–48, text after the slash indicates conditions from day 4 to day 6. All data is representative of the mean ±SEM of three
independent experiments performed at least in duplicate. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.