An angiotensin system inhibitor (Losartan) potentiates anti-tumor efficacy of cisplatin in a murine model of non-small cell lung cancer

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Title: An angiotensin system inhibitor (Losartan) potentiates anti-tumor efficacy of cisplatin in a murine model of non-small cell lung cancer

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GLOSSARY OF ABBREVIATIONS

ASI = Angiotensin system inhibitor
AT1R = Angiotensin II type 1 receptor
ARB = Angiotensin receptor binders
E = Epithelial
M, Mesenchymal
iE = Intermediate epithelial
iM = Intermediate mesenchymal
NSCLC = Non-small cell lung cancer
EMT = Epithelial-mesenchymal transition
RAAS = Renin angiotensin aldosterone system
VEGF = Vascular endothelial growth factor
PBS = Phosphate buffered saline
RT-qPCR = Reverse transcription quantitative real-time polymerase chain reaction
PDL1 = Programmed death-ligand
CENTRAL PICTURE

Improved anti-tumor efficacy of chemotherapy with Losartan

CENTRAL MESSAGE

Losartan potentiates the therapeutic efficacy of cisplatin in a murine model of NSCLC via downregulation of epithelial to mesenchymal signaling pathways.
PERSPECTIVE STATEMENT

RAAS signaling affects tumorigenesis and treatment susceptibility in solid tumors. Through a series of *in vitro* and *in vivo* experiments we found that losartan potentiates the anti-tumor efficacy of cisplatin in a murine model of NSCLC via attenuation of the EMT pathway. Angiotensin system inhibition should be further evaluated in a phase I/II study when combined with neoadjuvant chemotherapy for locally advanced NSCLC.
ABSTRACT

Objective: Previous studies have demonstrated synergistic anti-tumor effects of angiotensin system inhibition (ASI) combined with cisplatin therapy in pancreatic cancer. This study examines whether synergistic anti-tumor effects occur with combination ASI and cisplatin treatment in lung cancer, and whether ASI-induced changes in epithelial-mesenchymal transition play a role in the mechanism of this anti-tumor phenomenon.

Methods: A set of lung cancer cell lines representing a spectrum of epithelial to mesenchymal phenotypes were identified and characterized. Response of EMT markers to losartan was characterized. Cell culture models of lung cancer were next treated with losartan, cisplatin, or combination of both. Markers of epithelial-mesenchymal transition or surrogates of other signaling pathways (AKT, Stat3, PD-L1), and cell viability were quantified. Findings were confirmed in both allogenic and syngeneic in vivo murine flank tumor models.

Results: Losartan treatment significantly increased E-cadherin and reduced Vimentin in human lung cancer cell lines. Combination treatment with losartan and cisplatin 1) enhanced epithelial markers, 2) reduced mesenchymal markers, 3) inhibited pro-mesenchymal signaling mediators, and 4) reduced cell viability. Findings were confirmed in vivo in a murine flank tumor model with transition from
mesenchymal to epithelial phenotype and reduced tumor size following combination
losartan and cisplatin treatment.

Conclusions: Combination losartan and cisplatin treatment attenuates the EMT
pathway and enhances the cytotoxic effect of chemotherapy with in vitro and in vivo
models of NSCLC. This study suggests an important role for ASI therapy in the
treatment of lung cancer.

Keywords: Losartan, Lung cancer, Epithelial-Mesenchymal Transition,
Cisplatin

INTRODUCTION

Lung cancer is among the most commonly diagnosed cancers and the leading
cause of cancer mortality worldwide. The role of angiotensin system inhibition (ASI) in
modifying the risk of developing lung cancer has been an area of intense study, with
important implications should this common class of drugs be found to promote lung
cancer 1, 2, 3, 4. Comparatively little study has been done into the impact ASI may have
on treatment of an established lung cancer. Several small retrospective studies have
demonstrated a 40% increase in survival in patients incidentally receiving ASI vs those
not exposed to ASI and a 47% increase in progression free survival with ASI use which
was most pronounced with ARB therapy. In each of these studies, chemotherapy included standard platinum-based therapy.

Significant effort has gone into understanding the effects of the Renin angiotensin aldosterone system (RAAS) on the tumor microenvironment. Angiotensin II type 1 receptor (AT1R) overexpression has been associated with poor prognosis in patients with bladder cancer, operable breast cancer, ovarian cancer, and intestinal type gastric cancer, suggesting that local dysregulation of RAAS signaling may occur in or drive malignancy. Activation of the RAAS axis has been associated with enhanced tumor proliferation, invasion, and metastasis. RAAS signaling may create an immunosuppressive tumor microenvironment by activating pro-fibrotic signaling pathways leading to a desmoplastic environment. Tumor angiogenesis and vascular permeability are enhanced by RAAS via VEGF, promoting tumor growth. Inflammation and immune cell signaling are also impacted by RAAS in ways which can impact the response to malignancy. ASI appears to mitigate many of these effects, suggesting several mechanisms by which ASI could improve outcomes in cancer therapy.

Epithelial-mesenchymal transition (EMT) is a change from a polar and immobile epithelial cell to a mesenchymal cell, often accompanied by downregulation of E-cadherin and upregulation of Vimentin. Tumor cells that develop EMT take on characteristics that can permit cellular escape from primary tumor, invade the microcirculation through the basement membrane, and form distant metastases. These cells also have increased proliferation and have been associated with drug
This phenomenon is understood to be a significant component of tumor progression in many tumors, including lung cancer\(^\text{18}\). The RAAS has been shown to drive EMT\(^\text{19}\), and ASI inhibited metastasis in preliminary experiments\(^\text{18}\).

To further explore the interaction between the observed effects of both ASI and EMT on cancer treatment, we first developed an \textit{in vitro} model of lung cancer featuring a range of cell types across the EMT spectrum and quantified the response of each cell type to ASI. We next established a series of tests to quantify malignant potential of the cells: cellular proliferation, proliferation markers, and cell migration. We then tested the response of cells across the EMT spectrum to cisplatin, the most common chemotherapeutic agent in lung cancer. Findings were confirmed in a murine model.

**METHOD AND MATERIALS**

\textit{In Vitro} Studies:

To create a library of cancer cell types representative of the EMT spectrum, we divided 12 candidate human lung cancer cell lines into four types based on surface expression of EMT markers: E (Epithelial), iE (Intermediate E), iM (Intermediate M), M (Mesenchymal)\(^\text{20}\). The lung cancer cell lines: H292, H358 (human squamous cell), H441, H460, H1437, H1299, H1703, H1975, H2228, Calu-3, SW1573, A549, which are commonly used in lung cancer research, were initially classified by epithelial (E-cadherin+) and mesenchymal (Vimentin+) phenotype. In both E-cadherin+ and Vimentin+ cell lines, Pan-Cytokeratin (PCK) expression was also assayed. The lung cancer cell lines were further divided into E (Epithelial), iE (Intermediate E), M (Mesenchymal) 4 subtypes (Figure 1 A & B, Figure S1 A)\(^\text{20}\). A
A representative lung cancer cell line was selected for each EMT classification for subsequent study (Figure 1C,D): E, H441; iE, H358; iM, H1299; M, SW1573. Human cell lines were originally obtained from the American Type Tissue Culture Collection (Gaithersburg, MD) in 2016. The mouse-derived lung cancer cell line, TC-1 (received as a gift from Sunil Singhal, MD, University of Pennsylvania) was selected for murine studies. All cell lines were cultured in a 37°C cell culture incubator with RIPA-1640 medium, containing 10% fetal bovine serum and 1% penicillin, as previously described. All cell lines were tested for mycoplasma.

Prior to proceeding with AT1R inhibition experiments, all 5 cancer cell lines were found to have constitutive expression of AT1R (Supplement Fig 1B). The cell lines used in this study were exquisitely sensitive to cisplatin, so we chose a less than 60% lethal dose to proceed with subsequent in vitro and in vivo experimentation (Figure 4C).

To test cell migration as a proxy for metastatic potential, a scratch assay was performed as previously described, briefly cell lines were seeded into 6 well plates (2×10^6 cells/mL) after being serum starved for 12 hours prior. 5 scratch lines per well were created prior to application of either: 1) PBS control, 2) Losartan 0.5μM. Wells were photographed at 0h, 24h, 48h after seeding. The number of migratory cells was quantified and compared between groups (Figure 3A-B).
Cell proliferation assay, immunofluorescence, immunohistochemistry, western blotting, RT-qPCR, and screening for AT1R expression are described in **Supplementary Methods**.

**In Vivo Studies:**

All animal experiments were performed in compliance with IACUC approved protocols at MGH (protocol #: 2010N000005, 11/11/2018). Nude mice and C57BL/6 (male, 8-12 weeks) (25g), were purchased from the Jackson Laboratory (Barr Harbor, ME) and kept in a pathogen free facility. Mice were anesthetized (Ketamine, AH01VGG, Henry Schein Putney, USA; Xylazine, 061716A, AKORN Animal Health, USA) and injected with 100 μL (5 million cells) of SW1573 (nude mice) or TC-1 (C57BL/6) cell line preparation (mixed with Matrigel in a ratio of 1:1) on both flanks. Mouse body weight and tumor volume changes were recorded every 3 days. After 4 weeks, mice were randomly divided into 4 treatment groups: PBS control (100 μL via daily gavage), Losartan (30mg/Kg/day via daily gavage), Cisplatin (5mg/kg in 125 μL volume via IP injection every 3 days for 5 total doses), Losartan +Cisplatin. Flank tumors were harvested, and tumor weight and volume were obtained (Figure 6). The tumors were then divided in half, with one half fixed in formalin for histology and the other half were flash frozen in liquid nitrogen and then stored at -80°C for biochemical analysis.

**Drug dosing:**
In vitro losartan dosing was determined by dose ranging study (Sup Fig 2B). The maximum dose of losartan which produced ≥80% cell viability was determined to be 0.1μM, which was then used for in vitro experiments. Angiotensin II dose ranging study was also performed in a similar manner (Sup Fig 2A). Both losartan and angiotensin II dosing was consistent with previous studies\textsuperscript{25}. In vivo dosing was based on previous work\textsuperscript{26}. In humans, a chronic dose of 100 mg losartan daily produced trough serum drug levels of 0.82 μmol/L and peak levels of 1.88 μmol/L – a concentration range which is similar to the exposure dosing in this study\textsuperscript{27}. Cisplatin dose ranging was performed (Sup Fig 2C) and 2μM was the maximum dose which produced >60% cell viability was used for in vitro experiments. In vivo dosing of cisplatin (5mg/kg in 125 μL volume via IP injection every 3 days for 5 total doses), was based on previous studies\textsuperscript{28}.

**Statistical Analysis:**

For *in vitro* and animal *in vivo* experiments, One-Way ANOVA (P < 0.05 is considered significant) was performed to evaluate intergroup differences.
RESULTS

Losartan attenuated EMT in lung cancer cell lines

Following establishment of the cell library, we sought to test whether previously reported effects of RAAS signaling on EMT also occurred in lung cancer. We tested the effect of losartan on EMT markers in lung cancer cell lines. Losartan exposure resulted in a dose-dependent reduction in Vimentin in mesenchymal cell lines, with a significant reduction in vimentin detected at the 0.1 uM dose (p<0.05) (Figure 1 C). Losartan exposure resulted in a dose-dependent increase in E-cadherin in epithelial cell lines with a significant increase in E-cadherin detected at the 0.01 uM dose with H358 and the 0.05 uM dose for H441 (p<0.05) (Figure 1 D). These findings were confirmed with immunofluorescence (Figure 2), demonstrating a dose-dependent reduction in vimentin in mesenchymal cell lines. An increase in E-cadherin in epithelial lines was also observed. Co-exposure with losartan and angiotensin II negated this effect and returned levels of vimentin and E-cadherin to baseline levels.

The effect of losartan treatment on cell motility was assessed via scratch assay (Figure 3). The migratory cell number was not significantly changed by losartan treatment at 24 hours, but at 48 hours losartan treatment significantly reduced migratory cell number in all cell lines. In total, these findings suggest that the effect of losartan on EMT previously observed with other cancer types also occurs in lung cancer.

Combined treatment with losartan and cisplatin attenuated EMT response
To model the interaction between ASI and platinum-based chemotherapy observed on outcomes in patients with lung cancer, we exposed lung cancer cell lines to either losartan, cisplatin, or a combination of the two. In epithelial cells exposed to combination treatment, an increase in E-cadherin was observed with cisplatin and with losartan, however combination treatment resulted in a significant increase in E-cadherin over that of losartan or cisplatin treatment alone (p<0.05) (Figure 4 A). In mesenchymal cell lines vimentin was reduced by cisplatin and losartan, and combination treatment resulted in a significant decrease in vimentin over losartan or cisplatin treatment alone (p<0.05) (Figure 4 B). The anti-tumor effect of combined losartan and cisplatin treatment was assessed and resulted in significantly reduced cancer cell viability than treatment with either losartan or cisplatin alone (p<0.05) (Figure 4 C).

The effect of combined losartan and cisplatin treatment on established EMT mediators Zeb1 (Figure 3 C), Twist 1(Figure 3 D), and Snail 1 (Figure 3 E) was then evaluated. Treatment of either losartan or cisplatin reduced expression of all three mediators. Combined treatment with losartan and cisplatin reduced expression levels of all three mediators below that of cells treated with losartan or cisplatin alone, irrespective of cell line phenotype. AKT and Stat3 are signaling mediators with important cell cycle functions which can drive tumor replication and inhibit apoptosis. Both AKT and Stat3 levels were not significantly changed by either losartan or cisplatin alone, however, combination treatment resulted in significant reduction of both AKT, Stat3 and their phosphorylated states (p<0.05) (Figure 5). The effect of combination
therapy resulted in almost complete suppression of these mediators and may point to a mechanism for the effects of losartan and cisplatin on EMT.

**Treatment with losartan and cisplatin reduced tumor size and attenuated EMT**

To test the effects of combined losartan and cisplatin treatment on a human lung cancer cell line in vivo, we utilized an allogenic tumor model. Nude mice received bilateral subcutaneous flank injections of SW1573 (100μL, 5 million cells, N=4) (Figure 6A). Combination losartan and cisplatin treatment reduced tumor volume and tumor weight significantly compared to control or single drug therapy (Figure 6 A-D). EMT markers were assessed via immunohistochemistry and PCR (Figure 6 I-L) and demonstrated a significant elevation of E-cadherin and decrease in vimentin with combined treatment vs PBS control and single drug therapy (p<0.05), demonstrating the transformation of the mesenchymal tumor phenotype typically expressed by SW1573 into a more epithelial phenotype. Zeb 1, IL-6, and PDL-1 were all significantly reduced only with combination treatment (p<0.05) (Figure L, M). We also tested the interaction between losartan and cisplatin in a syngeneic flank model in C57BL/6 with TC-1(Figure 6 E-H, K, N, O and Figure S4 F), and the results were consistent with the observations made with the allogenic model above.

**DISCUSSION**

In this study, we created a library of cancer cell lines based on their epithelial vs mesenchymal characteristics and quantified the effects of losartan treatment on these
cell lines. We identified a synergistic effect of combined losartan and cisplatin exposure on EMT phenotype, which was associated with enhanced cytotoxicity. The synergistic effect of co-treatment with losartan and cisplatin was evaluated in vivo with both allogenic and syngeneic models with significant tumor volume reduction.

This study reports the novel finding that combination therapy with losartan and cisplatin results in enhanced tumor toxicity – a finding that was confirmed in both in vitro and in vivo models. This finding is consistent with observational studies in humans reporting increased response to chemotherapy with ASI in lung cancer\(^3\). In these studies, cisplatin was a consistent element of therapy. Subgroup analysis of bevacizumab, a VEGF inhibitor did not show an interaction with concurrent ASI therapy\(^6\). While testing the interaction between additional classes of chemotherapeutic agents was beyond the scope of this study, establishing an explicit link between cisplatin and ASI offers an exciting new direction in the investigation of the anti-tumor effects of ASI therapy. The 40% increase in survival reported by observational studies evaluating ASI and therapeutic regimens including cisplatin suggest that patients could realize significant benefit if this synergistic effect can be harnessed and implemented\(^3\).

Classically, loss of an epithelial cellular phenotype and adoption of a mesenchymal phenotype has been associated with enhanced tumorigenesis. We utilized a set of cancer cell types across the EMT spectrum and confirmed that ASI treatment drives cells towards the epithelial phenotype with increased E-cadherin and decreased vimentin. EMT has previously been shown to be induced by RAAS signaling in a model of colorectal cancer\(^19\), where ASI had similar effects – increasing E-cadherin
and reducing Vimentin. No studies have previously explored the relationship between EMT and ASI in lung cancer.

Surprisingly, cells exposed to both losartan and cisplatin demonstrated an exaggerated shift toward an epithelial phenotype with mediators of EMT – Twist 1, Snail 1, and Zeb 1 all highly suppressed. This finding was observed both in vitro and in vivo models. Previous consideration of the role of RAAS in potentiating the effect of chemotherapy was centered around RAAS effects on the tumor microenvironment- altering blood flow, potentiating local immune function, or altering local fibrosis. While the anti-tumor effects of ASI likely involve these mechanisms, the current findings suggest that there may also be an intracellular interaction between losartan and cisplatin which is capable of 1) influencing the phenotype of cancer cell and 2) directly altering the cytotoxic effects of cisplatin.

This study has several important limitations. This study used representative cell lines across the EMT spectrum, and these select cell lines may not represent the average lung cancer. While it appears that increased losartan doses correspond with enhanced epithelialization of tumor phenotype, the losartan dosing was not fully optimized and limited by toxicity in the in vivo experiments. Additional studies are needed to find the optimal dosing regimen to balance drug toxicity with anti-tumor effects. In this study, work was done in cell culture and murine models. The effects of ASI on the tumor microenvironment are complex, necessitating confirmation with in vivo human studies. Identifying the specific mechanism by which losartan and cisplatin interact to enhance cytotoxicity is beyond the scope of this paper. Additional studies
are required to further explore this finding. Finally, while combination therapy was seen to have effects that were beyond individual therapy conditions, more work will be needed to determine whether the observed effect is synergistic or additive.

318 In summary, we confirm the dose dependent cytotoxic effect of losartan in lung cancer models and report a novel synergistic effect of combination losartan and cisplatin treatment, which appears to be due to modulation of the EMT phenotype by losartan. More research is needed to elucidate the mechanism of this drug interaction, and to further explore its application in clinical medicine.
Figure 1 (A) Cell lines were characterized by expression levels of E-cadherin and vimentin. H441 and H358 expressed an epithelial phenotype – high E-cadherin and low vimentin. Lines H1299 and SW1573 expressed a mesenchymal phenotype – low E-cadherin and high vimentin. (B) The lung cancer cell lines were separated into 4 subtypes: 1) Epithelial (E) (H441), 2) intermediate Epithelial (iE) (H358), 3) intermediate Mesenchymal (iM) (H1299), 4) Mesenchymal (M) (SW1573). TC-1 was selected as a syngeneic cell line to balance differences of lung cancer cell phenotypes. Cell lines were exposed to losartan and significant decreases in vimentin (C) and E-cadherin (D) were observed (p<0.05).
**Figure 2.** (A) Representative images of immunofluorescence staining for E-cadherin and Vimentin in each cell line. Cells were treated with losartan and Ang II (200X). (B) Quantification of staining with image analysis. Losartan treatment significantly increased E-cadherin or reduced vimentin in each cell line (p<0.05). Treatment with combination losartan and angiotensin II returned vimentin and E-cadherin levels to baseline.
Figure 3. Representative image of cell migration assessed via scratch assay in the presence of losartan for each cell line (A), quantification of migratory cell number (B). At 24 hours there was no significant difference in migratory cell number between losartan treated and control cells. At 48 hours, losartan treatment had significantly reduced the migratory cell number for each cell type (p<0.05).
Figure 4. (A) E-cadherin incrementally increased with cisplatin treatment, demonstrated dose dependent increase with losartan, and significantly increased with combination losartan and cisplatin treatment over PBS or single drug treatment (p<0.05). (B) Vimentin decreased with cisplatin and showed a dose dependent decrease with losartan. Combination losartan and cisplatin significantly reduced vimentin expression over PBS or single drug treatment (p<0.05). (C) Cell viability was reduced with cisplatin and losartan individually, and combination treatment significantly reduced cell viability below single drug treatment (p<0.05). (D-F) EMT mediators Twist 1, Snail 1, and Zeb 1 were significantly reduced with cisplatin and losartan monotherapy and demonstrated significant reduction with combination treatment below single agent treatment result (p<0.05).
Figure 5. (A-D) Expression of proliferation markers AKT, Stat3 and their phosphorylated molecules in all 5 lung cancer cell lines treated with losartan, cisplatin, or combined losartan and cisplatin. No treatment effect on AKT, AKT-p, Stat3, or Stat3-p levels were observed with single agent cisplatin or losartan. Combination losartan and cisplatin significantly reduced AKT, AKT-p, Stat3, and Stat3-p in all cell lines (p<0.05). PDL1 was not significantly affected by either losartan or cisplatin, combination treatment significantly reduced PDL1 in all cell lines (p<0.05).
Figure 6. (A-D) Allogeneic flank tumor model: Human SW1573 cells were injected into the flanks of nude mice. (E-H) Syngeneic flank tumor model: Murine TC-1 cells were injected into the flank of C57BL/6 mice. In both flank tumor models, losartan did not affect tumor volume or weight. Cisplatin significantly reduced tumor weight and volume in both models *. Combination treatment with losartan and cisplatin significantly reduced tumor size and volume below that of PBS control, losartan or cisplatin alone *. (I-K) Immunohistochemical quantification of EMT markers E-cadherin and vimentin in allogeneic and syngeneic flank tumor models. Losartan and cisplatin each increase E-cadherin separately, and the combination of losartan and cisplatin is associated with an increase in E-cadherin over individual treatment *. Vimentin is reduced by losartan and cisplatin individually, and combination treatment reduced vimentin below either individual treatment *. (L,O) E-cadherin and vimentin expression changes confirmed via PCR. (L-O) Zeb 1, PDL1, IL-6 expression unchanged by losartan or cisplatin alone but were significantly reduced with combination treatment *. * = p<0.05.
Concomitant use of angiotensin system inhibitors with platinum-based chemotherapy has been associated with improved outcomes. Epithelial – mesenchymal transformation (EMT) has been associated with tumorigeneses. We model the effects of losartan on EMT in lung cancer cell lines and demonstrate inhibition of EMT markers. We combine losartan and cisplatin and demonstrate significant inhibition of EMT markers and observe enhanced cytotoxicity with combination therapy. These findings were confirmed via in vivo flank tumor models.
Figure S1. (A) We assessed EMT makers (E-cadherin, Vimentin, Zeb 1, CYR-61) and identified primarily epithelial (H441, H358) and mesenchymal cell lines (H1299, SW1573). (B) AT1R expression in all 5 lung cancer cell lines (magnification is 200). (C-E) AT1R expression in all 5 lung cancer cell lines treated with losartan or losartan combined cisplatin. D, H441, * P=0.001; H358, * P=0.000; H1299, * P=0.001; SW1573, * P=0.003. E, H441, * P=0.022, # P=0.000; H358, * P=0.000, # P=0.013; H1299, * P=0.043, # P=0.029; SW1573, * P=0.025, # P=0.028. (F) Zeb 1 expression with increasing losartan (H441, * P=0.022; H358, * P=0.014; H1299, * P=0.001; SW1573, * P=0.003; TC-1, * P=0.001). (G) Twist 1 expression with increasing losartan (H441, * P=0.002; H358, * P=0.024; H1299, * P=0.020; SW1573, * P=0.012; TC-1, * P=0.001). (H) Snail 1 expression with increasing losartan (H441, * P=0.003; H358, * P=0.001; H1299, * P=0.001; SW1573, * P=0.028; TC-1, * P=0.024).
Dose ranging studies: The 5 lung cancer cell lines were treated with increasing doses of Ang II (A) and losartan (B) with cell viability measured. The first statistically significant point on the cell survival curve was shown by *. H441 * P=0.014; H358 * P=0.012; H1299 * P=0.000; SW1573 * P=0.019; TC-1 * P=0.012. (H) H441 *

P=0.018; H358 * P=0.010; H1299 * P=0.000; SW1573 * P=0.000; TC-1 * P=0.005.

(C)Cell viability with increasing doses of cisplatin. The first statistically significant point on the cell survival curve was shown by *. H441, * P=0.000; H358, * P=0.002; H1299, * P=0.000; SW1573, * P=0.003; TC-1, * P=0.008. Differences observed in the cell viability following cisplatin treatment between Figure S2C and Figure 4C were likely due to normal experimental variation, as these experiments were conducted at different times.
**Figure S3.** (A) IL-6 expression with increasing losartan (H441, * P=0.001; H358, * P=0.000; H1299, * P=0.000; SW1573, * P=0.000; TC-1, * P=0.000). (B) PD-L1 expression with increasing losartan (H441, * P=0.000; H358, * P=0.000; H1299, * P=0.043; SW1573, * P=0.000; TC-1, * P=0.020). (C) PD-L2 expression with increasing losartan (H441, * P=0.012; H358, * P=0.000; H1299, * P=0.001; SW1573, * P=0.002; TC-1, * P=0.000). (D) IL-6 expression with increasing losartan and losartan combined cisplatin (H441, * P=0.032, # P=0.044; H358, * P=0.021, # P=0.025; H1299, * P=0.042, # P=0.042; SW1573, * P=0.033, # P=0.036; TC-1, * P=0.001, # P=0.013). (E) PD-L1 expression with increasing losartan and combined cisplatin (H441, * P=0.000, # P=0.000; H358, * P=0.000, # P=0.023; H1299, * P=0.000, # P=0.000; SW1573, * P=0.000, # P=0.000; TC-1, * P=0.005, # P=0.007). (F) PD-L2 expression with increasing losartan and combined with cisplatin (H441, * P=0.000, # P=0.000; H358, * P=0.002, # P=0.002; H1299, * P=0.001, # P=0.008; SW1573, * P=0.046, # P=0.033; TC-1, * P=0.004, # P=0.011).
Figure S4. (A) P-stat3 expression with increasing losartan (H441, * P=0.024; H358, * P=0.000; H1299, * P=0.000; SW1573, * P=0.000; TC-1, * P=0.032). (B) Stat3 expression with increasing losartan (H441, * P=0.030; H358, * P=0.032; H1299, * P=0.015; SW1573, * P=0.040; TC-1, * P=0.036). (C) P-AKT expression with increasing losartan (H441, * P=0.033; H358, * P=0.047; H1299, * P=0.002; SW1573, * P=0.000; TC-1, * P=0.024). (D) AKT expression with increasing losartan (H441, * P=0.003; H358, * P=0.032; H1299, * P=0.000; SW1573, * P=0.005; TC-1, * P=0.043). (E) PD-L1 expression with increasing losartan (H441, * P=0.034; H358, * P=0.005; H1299, * P=0.038; SW1573, * P=0.005; TC-1, * P=0.005). (F) Body weight of nude mice with SW1573. (G) Body weight of C57BL/6 mice with TC-1. (H) Vimentin, P-stat3, Stat3, P-AKT, AKT, and PD-L1 expression in mice with lung cancer.
REFERENCES


with losartan results in sufficient serum levels of the metabolite EXP3179 for PPARgamma activation. Hypertension 54: 738-43.

Figure 1

(A) Western blot images of E-cadherin, Vimentin, and Beta actin expression in various lung cancer cell lines.

(B) Diagram of lung cancer cell lines categorized by E-cadherin (E-cad +) and Vimentin (Vim +) expression:
- E-cad +, Vim +: E441, H358, H1299, SW1573
- E-cad +, Vim -: PCK + (E), iE (iE), iM (iM), M (M)
- E-cad -, Vim -: PCK - (E), iE (iE), iM (iM), M (M)

(C) Optical density graph showing the effect of varying Los concentrations on different cell lines.

(D) Optical density graph showing the effect of varying Los concentrations on different cell lines.
Figure 2

A

DAPI/E-cadherin/Vimentin (X200)

<table>
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<th>H358</th>
<th>H1299</th>
<th>SW1573</th>
<th>TC-1</th>
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B

- **H414**
  - E-cadherin
  - Vimentin

- **H358**
  - E-cadherin
  - Vimentin

- **H1299**
  - E-cadherin

- **SW1573**
  - Vimentin

- **TC-1**
  - Vimentin
Figure 3

(A)

<table>
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<tr>
<th>(µM)</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
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<td><img src="image5" alt="Image" /></td>
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H441

(B)

- **H441**
  - 24h
  - 48h

- **H358**
  - ![Image](image7)

- **H1299**
  - ![Image](image8)

- **SW1573**
  - ![Image](image9)

- **TC-1**
  - ![Image](image10)

![Image](image11)
Figure 5

(A) Stat3

(B) P-Stat3

(C) P-AKT

(D) AKT

(E) PD-L1

Legend:
- H441
- H358
- H1299
- SW1573
- TC-1
Figure 6

A. Nude mice-SW1573

B. SW1573

C. Start to treat

D. Tumor Weight (g)

E. C57BL6 mice-TC-1

F. TC-1

G. Start to treat

H. Tumor Weight (g)

I. SW1573

J. E-cadherin Vimentin

K. TC-1

L. E-cadherin Vimentin Zeb1

M. SW1573

N. IL-6 PD-L1

O. TC-1

P. IL-6 PD-L1
LOSARTAN IMPROVES THE THERAPEUTIC EFFECT OF CISPLATIN VIA INHIBITION OF EMT

**BACKGROUND**
RAAS activation is associated with enhanced tumor proliferation, invasion, and metastasis.

Inhibition of this pathway with ASI can mitigate these effects.

**ANGIOTENSION SYSTEM INHIBITION**

ASI CAN ATTENUATE EMT

**IN VITRO: ANALYSIS IN HUMAN NSCLC**

LOSARTAN ↑ E-CAD and ↓ VIMENTIN

↓ EMT

↓ IL-6, AKT, PD-L1

**IN VIVO: MURINE MODEL OF NSCLC**

LOSARTAN

↑ CHEMO CYTOTOXICITY

↓ CELL PROLIFERATION

![Tumor volume trend graph](image)
LOSARTAN IMPROVES THE THERAPEUTIC EFFECT OF CISPLATIN VIA INHIBITION OF EMT

BACKGROUND
RAAS activation is associated with enhanced tumor proliferation, invasion, and metastasis.

Inhibition of this pathway with ASI can mitigate these effects.

ASI CAN ATTENUATE EMT

IN VITRO: ANALYSIS IN HUMAN NSCLC

LOSARTAN \(\uparrow\) E-CAD and \(\downarrow\) VIMENTIN

\(\downarrow\) EMT

\(\downarrow\) IL-6, AKT, PD-L1

IN VIVO: MURINE MODEL OF NSCLC

LOSARTAN
\(\uparrow\) CHEMO CYTOTOXICITY
\(\downarrow\) CELL PROLIFERATION

SW1573

Start to treat

Tumor volume (mm³)

Days

Control
Losartan
Cisplatin
Losartan + Cisplatin
Angiotensin System Inhibitors are associated with improved survival in locally advanced NSCLC and exhibit anti-tumor synergism with chemotherapy in a murine model of NSCLC

Hexiao Tang, Mozhdeh Sojoodi, Yongtao Wang, Derek J. Erstad, Sarani Ghoshal, Gunisha Arora, Hao Li, Zenan Lin, Eric Abston, Kenneth K. Tanabe, Bryan C. Fuchs, and Michael Lanuti
Supplementary Material Figure S1.

A

B

C

D

E

F

G

H

Supplementary Figure S1.

A

B

C

D

E

F

G

H
Supplementary Material Figure S2.

(A) Viability (%) vs. Ang II (µM)

(B) Viability (%) vs. Los (µM)

(C) Viability (%) vs. Cis (µM)

- H441
- H358
- H1299
- SW1573
- TC-1
Supplementary Material Figure S3.

[A] IL-6

[B] PD-L1

[C] PD-L2

[D] IL-6

[E] PD-L1

[F] PD-L2

Legend:
- H441
- H358
- H1299
- SW1573
- TC-1

Y-axis: RQ
X-axis: Los(μM)

Table:

<table>
<thead>
<tr>
<th>Los(μM)</th>
<th>Cis(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Significance:
- * P < 0.05
- ** P < 0.01
- *** P < 0.001
- ### P < 0.0001
Supplementary Material Figure S4.

A

P-Stat3

Optical density vs. Log (μM)

B

Stat3

Optical density vs. Log (μM)

C

P-AKT

Optical density vs. Log (μM)

D

AKT

Optical density vs. Log (μM)

E

PD-L1

Optical density vs. Log (μM)

F

SW1573

Body Weight (g) vs. Days

Start to treat

G

TC-1

Body Weight (g) vs. Days

Start to treat

H

Vimentin

PD-L1

Stat3

P-Stat3

P-AKT

SW1573-Nude mice

TC-1-C57BL/6 mice

PBS | Los | Cis | Los + Cis
---|---|---|---
PBS | Los | Cis | Los + Cis
METHODS (Supplementary)

Cell Proliferation Assay

Cell proliferation assay was performed as previously described (24). Briefly, H441, H358, H1299, SW1573, TC-1 cell lines were maintained as above. Cells were seeded onto 24 well plates and were cultured to an exponential growth level prepared about 2×10^4 cells/mL, 0.5 mL per well, each group repeated with 4 wells. Cells underwent serum starvation for 12 hours, and then wells were assigned to receive 48 hours of treatment in one of the following groups: 1) PBS control, 2) Angiotensin II (A9525, Sigma, USA) at 0.01, 0.1, 0.5, and 1μM doses 3) Losartan (180220, American Health, USA) at 0.01, 0.1, 0.5, 1μM doses, 4) Cisplatin (NDC 16729-288-38, Accord Healthcare, USA) at 0.1, 1, 5, 10, 50μM doses (28), 5) Cisplatin 2μM combined with Losartan 0.5μM.

After 48 hours of treatment, the culture medium was aspirated, and 0.5 mL of MTT (C18H16BrN5S (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (298-93-1, Sigma, USA) was added to each well. After 3 hours, the supernatant was aspirated and discarded. After adding 200 μL of Dimethyl sulfoxide (DMSO), the solution was thoroughly shaken and dissolved. 100 uL of each well was added to 96-well plates, and then analyzed in a Spectrophotometer at 540 nm (Emax Precision Microplate Reader, Molecular Devices, San Jose, CA).

Immunofluorescence (IF)

H441, H358, H1299, SW1573, and TC-1 cell lines were seeded into 24 well plates at 6×10^4 cells/mL. Cells underwent serum starvation for 12 hours, and then wells were
assigned to receive 48 hours of treatment in one of the following groups: 1) PBS control, 2) Losartan 0.5μM, 3) Losartan 5μM, 4) Ang II, 5) Losartan 5μM + Ang II 1μM. Next, primary antibody was applied (E-cadherin, 1:400, ab76055, Abcam, USA; Vimentin 1:400, 5741S, Cell Signaling Technology, USA), followed by second antibody (Cy3-AffiPure Donkey Anti-Mouse IgG, 1:500, 715-165-150, Jackson ImmunoResearch, USA; Donkey Anti-Rabbit IgG, 1:1000, ab150073, Abcam, USA) and DAPI (1:500, ab104139, Abcam, USA).

**Immunohistochemistry (IHC)**

Human lung adenocarcinoma cancer tissue and healthy lung tissue sections were obtained as above. Tissue was processed as previously described (29). The primary antibody (E-cadherin, 1:300, 3195S, Cell Signaling Technology, USA; Vimentin, 1:300, 5741S, Cell Signaling Technology, USA; AT1R, 1:200, ab124505, Abcam, USA) was added dropwise. The secondary antibody (2668240, M IHC Select, USA) was then added per protocol. The above-mentioned steps also performed on mouse tumor tissue.

**Western blotting**

H441, H358, H1299, SW1573, and TC-1 cell lines were seeded into 6 well plates at 2×10^5 cells/mL. Cells underwent serum starvation for 12 hours, and then wells were assigned to receive 48 hours of treatment in one of the following groups: 1) PBS control, 2) Losartan 0.01, 0.05, 0.1, 0.2, 0.5μM, 3) Cisplatin 2μM and Losartan 0.1μM, 4) Cisplatin 2μM and Losartan 0.5μM. The primary antibodies (E-cadherin, 1:1000, 3195S, Cell Signaling Technology, USA; Vimentin, 1:1000, 5741S, Cell Signaling Technology,
USA; Stat3, 1:1000, 9132, Cell Signaling Technology, USA; AKT, 1:1000, 9272, Cell Signaling Technology, USA; P-Stat3 1:1000, 9145, Cell Signaling Technology, USA; P-AKT 1:1000, 13038, Cell Signaling Technology, USA; AT1R, 1:1500, ab124505, Abcam, USA; PD-L1, 1:1000, ab233482, Abcam, USA) and secondary anti-rabbit antibodies (7074s, Cell Signaling Technology, USA) were applied.

RT-qPCR
H441, H358, H1299, SW1573, and TC-1 cell lines were prepared using the same treatment groups as described above in the Western blot section. RNA was extracted, quantification and cDNA prepared via standard protocol. All primers (18S Hs03003631_g1, IL-6 Hs00174131_m1, PD-L1 Hs00204257_m1, PD-L2 Hs00228839_m1, Zeb1 Hs01566408_m1, Twist1 Hs01675818_s1, Snail1 Hs00195591_m1) were purchased from Thermo Fisher (USA), and processed per the company's official website instructions.

Animal experiments
23 male nude mice of 10 weeks old (25g) were purchased from the Jackson Laboratory (Barr Harbor, ME) and kept in a pathogen free facility in accordance with an approved animal IACUC (protocol # 2010N000005). All mice were housed at the Their Animal Laboratory at the Massachusetts General Hospital. Mice were anesthetized (Ketamine, AH01VGG, Henry Schein Putney, USA; Xylazine, 061716A, AKORN Animal Health, USA) and injected with 100 μL (5 million cells) of SW1573 cell line preparation (mixed with Matrigel in a ratio of 1:1) on both flanks. Mouse body weight and tumor volume
changes were recorded every 3 days. After 4 weeks, mice were randomly divided into 4 treatment groups: PBS control (100 μL via daily gavage), Losartan (30mg/Kg/day via daily gavage), Cisplatin (125 μL via IP injection every 3 days for 5 total doses), Losartan +Cisplatin. The above procedure was repeated in C57BL/6 (male, 8-12 weeks, 25g, Jackson Laboratory) using TC-1 cells. Treatment was initiated 3 days after TC-1 cell injection. Following 2 weeks of treatment, the mice were sacrificed via cervical dislocation. Flank tumors were harvested, and tumor weight and volume were obtained. The tumors were then divided in half, with one half fixed in formalin for histology and the other half were flash frozen in liquid nitrogen and then stored at -80°C for biochemical analysis.