

RESVERATROL BINDS NUCLEAR RECEPTOR 4A1 (NR4A1) AND ACTS AS AN NR4A1 ANTAGONIST IN LUNG CANCER CELLS

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Running Title: Resveratrol acts as an NR4A1 antagonist in lung cancer.

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Abbreviations: CDIM, bis-indole derived compound; DIM-3,5-Cl₂, 1,1-bis(3'-indolyl)-1-(3,5-dichlorophenyl)methane; EGFR, epidermal growth factor receptor; IDH-1, isocitrate dehydrogenase-1; ITC, isothermal titration calorimetry; LBD, ligand binding domain; NR4A, nuclear receptor 4A; TXNDC5, thioredoxin domain-containing 5.

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ABSTRACT

Resveratrol is a polyphenolic phytochemical found in fruits, nuts and vegetables that contributes to the remarkable dietary effects of polyphenolic as inhibitors aging and multiple aging related diseases. In addition, resveratrol has been extensively investigated as an inhibitor of inflammatory diseases including cancer, however, the underlying mechanisms of these chemotherapeutic effects of resveratrol are not completely understood. In cancer cells resveratrol inhibits cell growth, survival, migration and invasion, and many of the effects of resveratrol resemble those observed for bis-indole derived (CDIM) compounds that bind the pro-oncogenic nuclear receptor 4A1 (NR4A1, Nur77) and act as receptor antagonists. Using an isothermal titration calorimetry binding assay, we observed that resveratrol bound to the ligand binding domain of NR4A1 with a K_D value of 2.4 μ M and a ΔG of -32.2 kJ/mol. Resveratrol also inhibited NR4A1-dependent transactivation in H460 and H1299 lung cancer cells suggesting that resveratrol is an NR4A1 antagonist. This observation was confirmed in a series of functional (cell proliferation, survival, migration and invasion) and gene expression assays in H460 and H1299 cells showing that treatment with resveratrol mimicked the effects of NR4A1 knockdown and were similar to results of previous studies using CDIM/NR4A1 antagonists. These data indicate that applications of resveratrol may be more effective in patients that overexpress NR4A1 which is a negative prognostic factor for patients with some solid tumor-derived cancers.

SIGNIFICANCE STATEMENT

We have examined the mechanism of action of resveratrol and show binding to NR4A1 ($K_D = 2.4 \mu$ M) and inhibition of NR4A1-dependent transactivation in lung cancer cells. Treatment of H460 and H1299 lung cancer cells with resveratrol inhibits cell growth, survival, migration/invasion and related genes, and acts as an NR4A1 antagonist. Resveratrol can now

be used more effectively in cancer chemotherapy by targeting patients that overexpress NR4A1 in lung cancer.

INTRODUCTION

Dietary polyphenolics produced in vegetables, fruits and nuts have been associated with multiple beneficial health effects including longer lifespans and other age-related diseases (**Liu et al., 2019; Wu et al., 2021; Hano et al., 2020**). Polyphenols associated with these health benefits include phenolics acids, coumarins, flavonoids, lignans and stilbenes including 3,4',5-trihydroxy-trans-stilbene (resveratrol) which is enriched in foods such as blueberries, grapes, peanuts and red wine and exhibits prototypical polyphenolic health benefits (**Saiko et al., 2008; Zhou et al., 2021; Raj et al., 2021; Santana et al., 2021; Koh et al., 2021**). Resveratrol has been extensively investigated as a therapeutic agent for treatment of multiple diseases in both in vitro cell culture and animal models. Resveratrol inhibits proliferation and inflammation in cell culture models of endometriosis (**Kolahdouz et al., 2017; Rudzitis-Auth et al., 2013**) and this includes inhibition of NF κ B and other kinases (**Ergenoglu et al., 2013; Bruner-Tran et al., 2011**) downregulation of ESR1 (**Amaya et al., 2014**), activation of SIRT1 (**Taguchi et al., 2014**) and decreased MMP9 and MMP2 activities (**Bayoglu et al., 2014**). Resveratrol also has multiple effects on neuronal cells in culture and in vivo and this includes inhibition of brain inflammation and damage, and enhanced memory (**Teertam et al., 2020; Le et al., 2019; Tang et al., 2021**). For, example, in rat brain resveratrol upregulates Sirt1/microRNA 149-Sp to protect against ischemia (**Teertam et al., 2020**) whereas resveratrol protection in neonatal hypoxic-ischemic brain injury involves SIRT-1-regulated inhibition of HMGB1 and downstream NF κ B signaling (**Le et al., 2019**). Moreover, induction of inflammation in BV2 cells enhances TLR4, MyD88, NF κ B and multiple cytokines and these responses are inhibited by resveratrol (**Le et al., 2019**). Resveratrol also activates a SIRT1/NF κ B to protect against sevoflurane-induced cognitive impairment in mice (**Tang et al., 2021**).

Resveratrol is a highly effective inhibitor of cancer cell and tumor growth, migration and invasion in multiple cell lines (**rev. in Saiko et al., 2008**) and in most of these cell lines and this was accompanied by altered regulation of pathways/gene products associated with these anticancer activities. The results of many studies on the anticancer activities of resveratrol have been variable and cell context-specific, however, some pathways and genes such as resveratrol-dependent inhibition of the mTOR pathway have been reported in many different cancer cell lines (**Khan et al., 2020; Bian et al., 2020; Wang et al., 2018; Liu et al., 2018; Chang et al., 2017; Selvaraj et al., 2016; Wu and Liu, 2013; Rashid et al., 2011; He et al., 2011; Alayev et al., 2015**).

Research in our laboratory has been focused on the orphan nuclear receptor 4A1 (NR4A1, Nur77) and its functions in cancer and non-cancer cell lines and animal models (**Selvaraj et al., 2016**). Nr4a1 and other members of this family (Nr4a2 and Nr4a3) are immediate early genes that are induced by diverse stressors to maintain cellular homeostasis and NR4A1 is overexpressed in solid tumor-derived cancers and other inflammatory diseases (**Safe and Karki, 2020; Pearen and Muscat, 2010**). Results primarily of NR4A1 knockdown studies show that this receptor regulates cancer cell growth, survival, migration and invasion, and this include mTOR signaling pathways on multiple cancer cell lines. NR4A1 regulates prosurvival and growth promoting genes such as epidermal growth factor receptor (Egfr), survivin and bcl-2 as well as β 1-integrin and other integrins in many different cancer cell lines. Isocitrate dehydrogenase-1 (Idh-1) and thioredoxin domain containing 5 (Txndc5) are also NR4A1-regulated genes in solid tumor-derived cell lines and these genes serve to maintain high reductant levels in cancer cells (**rev. in Safe and Karki, 2020**). Bis-indole derived compounds (CDIMs) have been characterized as ligands that bind NR4A1 and act as NR4A1 antagonists that inhibit cancer cell growth, survival, migration and invasion (**Safe and Karki, 2020; Lee et al., 2014**). CDIM-NR4A1 antagonists also inhibit mTOR in lung and other cancer cell lines (**Safe and Karki, 2020; Lee et al., 2012; Lacey et al., 2016**) and many of the effects of these NR4A1

antagonists have also been observed for resveratrol (Saiko et al., 2008; Koh et al., 2021; Wang et al., 2018; Lacey et al., 2016; Wright et al., 2017). We hypothesize that one of the underlying mechanisms of action of resveratrol in cancer cells is that of an NR4A1 ligand that acts as an antagonist and this study demonstrates for the first time that resveratrol is an NR4A1 ligand.

MATERIALS AND METHODS

Ligand – receptor binding assays

Isothermal titration calorimetry (ITC) was used to determine the ligand binding constant (K_d) to NR4A1 utilizing an Affinity ITC (TA Instruments, New Castle, DE). Briefly, the experimental setup was as follows. The ITC sample cell contained 250 μ L of NR4A1 protein (ligand binding domain, LBD) at a concentration of 20 μ mol/L in buffer containing 20 mmol sodium phosphate/L (pH 7.4), 5% glycerol, and 1.0% ethanol. The ligand titrant was prepared in the same buffer as above at a ligand concentration of 66.6 μ mol/L. The ligand titration into protein was performed at 25 °C with a stir rate of 125 rpm. Each ligand injection volume was 5 μ L followed by 200 sec to measure the total heat flow required to maintain constant temperature. A total of twenty injections were done for each ligand/NR4A1 combination. Each ligand titration into protein experiment was repeated for a total of three separate and independent experiments to generate the curves shown in the figure. In a separate set of injections, the same ligand was injected into buffer only (no protein) in order to determine heat flow as a result of ligand dilution into buffer. The ligand/buffer values were subtracted from the ligand/protein values prior to data analysis using the Affinity ITC manufacturer-supplied data analysis software package. Sigmoidal curve fitting was performed using the Affinity ITC

manufacturer-supplied data analysis software package in order to determine the following binding parameters: K_d , the equilibrium binding dissociation constant ($\mu\text{mol/L}$); n , the equilibrium ligand-to-protein binding stoichiometry (mol ligand per mol NR4A1); ΔG , the equilibrium free energy of ligand binding (kJ/mol). The resulting data are plotted as heat flow/area data (μJ) versus the cumulative resveratrol concentration ($\mu\text{mol/L}$) present in the sample cell. Statistical analysis of the triplicate data was performed utilizing SigmaPlot 14.5 (Systat Software, Inc.) to determine the parameter mean (K_d , n , ΔG) and standard deviation. In addition, we also used a direct binding assay by determining the loss of fluorescence of a tryptophan residue in the LBD as previously described (Lee et al, 2014).

Computation-based molecular modeling

Molecular modeling studies were conducted using Maestro (Schrödinger Release 2020-1, Schrödinger, LLC, New York, NY, 2020). The version of Maestro used for these studies is licensed to the Laboratory for Molecular Simulation, a Texas A&M University core user facility for molecular modeling and is associated with the Texas A&M University High Performance Research Computing facility. All Maestro-associated applications were accessed via the graphical user interface (GUI) VNC interactive application through the HPRC Ada OnDemand portal. The crystal structure coordinates for human orphan nuclear receptor NR4A1 ligand binding domain (LBD) (Zhan et al., 2012) were downloaded from the Protein Data Bank (<https://www.rcsb.org>; PDB ID 3V3Q). The human NR4A1 LBD crystal structure was prepared for ligand docking utilizing the Maestro Protein Preparation Wizard; restrained minimization of the protein structure was performed utilizing the OPLS3e force field. Each ligand (resveratrol or DIM-3,5-CI2) three-dimensional structure was prepared for docking utilizing the Maestro LigPrep, again using the OPLS3e force field. Maestro Glide (Friesner et al., 2006, Halgren et

al., 2004) was utilized with the default settings to dock each prepared ligand to the prepared protein, predict the lowest energy ligand binding orientation, and calculate the predicted binding energy in units of kcal/mol.

Cell culture, reagents, and antibodies

H460 and H1299 lung cancer cells are purchased from American Type Culture Collection (Manassas, VA). Both cell lines were derived from male patients with non-small cell lung cancer (H1299) or large cell lung cancer (H460). Cells are cultured in RPMI1640 medium with 10% FBS at 37°C in the presence of 5% CO₂. The details of antibodies used for Western blots and for CHIP assays are summarized in Supplemental Table 1.

Cell proliferation assay

Cell proliferation was investigated using XTT Cell Viability Kit (Cell Signaling Biotechnology) according to the manufacturer's instructions. cells (1.5×10^4 /well) were plated in 100 μ l of plating medium (as above) on 96-well plates and allowed to attach for 24 hours. The medium was then changed to RPMI 1640 containing 2.5% charcoal-stripped FBS, and either vehicle (dimethyl sulfoxide [DMSO]) or different concentrations of compounds in DMSO were added. After 24, 48 and 72 hours of culture, 25 μ l of XTT reaction solution (sodium 3'-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate (mixed in proportion 50:1) was added to the each well. The optical density was read at 490 nm wavelength in a plate reader after incubation for 4 hours. All determinations were replicated in at least three separate experiments.

Transfection and luciferase assay

Cells were plated on 12-well plates at 5×10^4 /well in RPMI 1640 medium supplemented with 2.5% charcoal-stripped FBS. After 24 h growth, various amounts of DNA [i.e., UASx5-Luc

(400 ng), GAL4-NR4A1 (50 ng) and β -gal (50 ng)] were co-transfected into each well by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 6 hours of transfection, cells were treated with plating media (as above) containing either solvent (DMSO) or the indicated concentration of compound for 18 hours. Cells were then lysed using a freeze–thaw protocol and 30 μ L of cell extract was used for luciferase and β -gal assays. LumiCount (Packard, Meriden, CT) was used to quantify luciferase and β -gal activities. Luciferase activity values were normalized against corresponding β -gal activity values as well as protein concentrations determined by Bradford assay.

Annexin V staining assay

Annexin V staining assay was performed using Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry (Invitrogen, Carlsbad, CA). Briefly, cells were seeded in 6 well plates followed by various drug treatments. The cells were then washed with ice cold PBS and 5 μ L Alexa Fluor® 488 Annexin V with 100 μ g/mL PI (as per the manufacturer instructions) were added to the cells and incubated for 15 min and the cells were determined by Accuri flow cytometer.

Boyden chamber invasion assay and scratch migration assay

Attached cells (2.0×10^5) were treated with DMSO or with different concentration of resveratrol in medium supplemented with 2.5% charcoal stripped FBS for 24 hours or transfected with different siRNA with RNA imax transfection for 72 hours as manufacturer's protocol. Then, for Boyden chamber invasion assay, 1.0×10^5 cells from each treatment condition were allowed to invade through the Boyden Chamber for 48 hours. Cells that invaded into the Boyden Chamber were fixed using formaldehyde, stained, and then counted. For scratch migration assay, cells were grown to 90% confluency in 6-well plates then scratched with a 200 μ L sterile pipette tip and washed with PBS to remove detached cells from the plates.

Cells were kept in incubator with DMSO or indicated treatments for 48 hours. After 48 hours, cells were fixed with 4% formaldehyde and stained with crystal violet solution. The wound gap was observed under AMG EVOS fl microscope. At least 3 replicates were performed for each treatment group.

Western blot analysis

Cells (3.0×10^5) were seeded on 6-well plate and after various treatments, whole cell lysates were obtained by treating them with high salt lysis buffer RIPA (Thermo Scientific, Waltham, MA) that contained protease and phosphatase inhibitors (GenDEPOT, Baker, TX). The total protein in the lysates was quantified by Bradford assay. Equal amounts of protein from each lysate were then loaded on SDS polyacrylamide gel. The proteins on the gel were transferred to a PVDF membrane, then blocked for an hour using 5% skimmed milk. The membranes were then incubated with primary antibody for overnight at 4 °C. It was then washed with Tris-buffered saline and Polysorbate 20 (TBST) and incubated with HRP-linked secondary antibody for 1 hour at room temperature. The membranes were further washed with TBST and treated with Immobilon western chemiluminescence HRP-substrates to detect the protein bands using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR, USA). Protein levels in various treatment groups were normalized to β -actin.

Transfection and small interfering RNAs

For RNA interference experiment, cells were seeded on 6-well plate at 3×10^5 /well then allowed 24hrs to attach and grow. Then, they were transfected with siRNA of 100 nmol each/well for 6-well plate using 6.5 μ L/well RNA iMax transfection reagent for 72 hours. Small interfering RNAs (siRNAs) targeting NR4A1 (siNR4A1), Sp1 (siSp1), and Sp4 (siSp4) were purchased from Sigma-Aldrich. Negative Control Ig L2 siRNA were purchased from Qiagen. The oligonucleotides used were as follows:

siNR4A1_1, SASI_Hs02_00333289; siNR4A1_2, SASI_Hs02_00333290; siSp1_1: SASI_HS01-00070994; siSp1_2: SASI_Hs02_00333289; siSp4_1: SASI_HS01-00114420; siSp4_2: SASI_HS01-00114421.

ChIP assay

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. All cells (3×10^7) were treated with DMSO or indicated concentration of resveratrol for 3 hours. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG (Cell signaling), NR4A1 (Abcam), Sp1 (Abcam), Sp4 (Santa Cruz), or RNA polymerase II (pol II; GeneTex) antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The primers for detection of the $\beta 1$ integrin promoter region were 5'-TCA CCA CCC TTC GTG ACA C-3' (sense) and 5'-GAG ATC CTG CAT CTC GGA AG-3' (antisense). PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide (EtBr).

RT-PCR

RNA was isolated using Qiagen RNeasy Mini kit (Irvine, CA). Quantification of mRNA ($\beta 1$ -integrin) was performed using Bio-Rad iTaq Universal SYBR Green 1-Step Kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. Human GAPDH mRNA was used as a control to determine relative mRNA expression. The primers for detection of the $\beta 1$ integrin

mRNA were 5'-GAA GGG CGT GTT GGT AGA CA-3'(Forward) and 5'-GTT GCA CTC ACA CAC ACG AC-3' (Reverse).

Statistical analysis

Each assay was performed in triplicate and the results were presented as means with standard deviation (SD). The statistical significance of differences between the treatment groups was determined by Dunnett's multiple comparison test in ordinary one-way ANOVA. Analysis of Western blotting was done using ImageJ (1.53K) software. GraphPad Prism 8 (Version 8.4.3) software was used for analysis of variance and determine statistical significance. Data with a *P* value of less than 0.05 were considered statistically significant and indicated with "*" in figures.

RESULTS

1. *Binding and transactivation.*

Based on the similarities between the effects of resveratrol and NR4A1 antagonists on solid tumor derived cancer cells we initially investigated the binding of resveratrol (**Fig. 1A**) to the ligand binding domain of human NR4A1 using an isothermal titration calorimetry (ITC) assay procedure. The results showed that resveratrol bound to NR4A1 with a calculated K_D value of $2.4 \pm 0.7 \mu\text{M}$ (SD, 3 determinations) and a ΔG value of $-32.2 \pm 0.8 \text{ kJ/mol}$ (**Fig. 1B**). The stoichiometry of binding (*n*) is 0.82 mol ligand bound/mol protein. The direct interaction of resveratrol with NR4A1 was also confirmed in a fluorescence quenching assay of a Trp in the NR4A1 binding pocket (**Lee et al., 2014**) and the K_D value was $1.4 \mu\text{M}$. We also used a computer modeling approach and compared the predictive interactions of resveratrol in the ligand binding pocket of NR4A1 (**Fig. 1C**) to that observed for the bis-indole NR4A1 ligand bis(3'-indolyl)-1-(3,5-dichlorophenyl)methane (DIM-3,5-Cl₂) (**Karki et al, 2021**) (**Fig. 1D**). The simulations predicted that both compounds interact with common amino acids side chains

Ser110, Glu114, Arg184 and Thr236; in addition, they also interacted uniquely with amino acid side chains Leu113, Leu239 and Ile260 (resveratrol), and Arg232 (DIM-3,5-Cl₂) demonstrating some ligand structure-dependent differences in binding of resveratrol and DIM-3,5-Cl₂ to NR4A1.

The activity of resveratrol as an NR4A1 ligand was confirmed in transactivation assays in H460 and H1299 lung cancer cells transfected with a yeast GAL4-NR4A1 chimera construct and a UAS-luc reported gene containing 5 tandem yeast GAL4 binding elements. Resveratrol decrease transactivation in both cell lines (**Figs. 1E and 1F**) indicating NR4A1 antagonist activity which has previously been observed for the CDIM/NR4A1 ligands in lung and other cancer cell lines (**Safe and Karki, 2020; Lee et al., 2014; Lacey et al., 2016; Lee et al., 2010**).

2. Resveratrol and NR4A1 knockdown inhibit lung cancer cell growth, survival, migration and invasion.

Treatment of lung cancer cells with resveratrol decreased H460 and H1299 cell growth by approximately 40-50% (**Figs. 2A and 2B**) and also decreased NR4A1 protein expression (**Fig. 2C**). Knockdown of NR4A1 also decreased lung cancer cell growth by 40-65% (**Figures 2D and 2E**) and NR4A1 protein (**Fig. 2F**) and levels of NR4A1 protein are quantitated (**Supplemental Figure 1A**). Cell growth inhibition was observed over a range of concentrations (50-150 μ M) and previous studies in lung cancer cells used concentrations of 100 or 200 μ M to investigate effects of resveratrol on multiple endpoints (Wright et al., 2017; Wang et al., 2018). Based on preliminary studies we used two concentrations (125 and 150 μ M) of resveratrol which changed most pathways and levels of gene products investigated in this study. We also observed that resveratrol (125 and 150 μ M) induced markers of apoptosis (PARP and caspase-3 cleavage; bcl-2 downregulation) (**Fig. 2G**) and similar results were observed after knockdown of NR4A1 (**Fig. 2H**) and quantitation of the western blots (G and H) are summarized in (**Supplemental Figure S1B and S1C**). Resveratrol (125 and 150 μ M) also induced Annexin V

staining in H460 and H1299 cells (by >12-fold) (**Fig. 2I**). In addition, we also observed that resveratrol inhibited cell migration in a scratch assay by > 45% at the high dose and similar results were observed after NR4A1 knockdown (approximately 25% inhibition) (**Figs. 3A and 3B**). Resveratrol (125 and 150 μ M) inhibited cell invasion by 75% in a Boyden chamber assay by >25% after receptor knockdown (**Figs 3C and 3D**). These results demonstrate that the functional inhibitory effects of resveratrol on H460 and H1299 cell growth, survival and migration and invasion mimic those obtained after knockdown of NR4A1 suggesting that the anticancer activity of resveratrol is due, in part, to its activity as an NR4A1 ligand.

3. Resveratrol and NR4A1 knockdown modulate expression of several gene products (proteins) and mRNAs in common.

Previous studies in multiple solid tumor derived cell lines have identified several NR4A1-regulated genes and proteins, and they include thioredoxin containing domain 5 (TXNDC5), isocitrate dehydrogenase-1 (IDH-1) and inhibition of phospho-AMPK α (p-AMPK α) (**Safe and Karki, 2020; Lee et al., 2014; Lee et al., 2012; Lacey et al., 2016**). Treatment of H460 and H1299 cells with resveratrol (125 and 150 μ M) decreased expression of TXNDC5, IDH-1 and induced p-AMPK α proteins (**Figs. 4A and 4B**) and similar results were obtained in H460 and H1299 cells transfected with oligonucleotides targeted to NR4A1 (#1 siNR4A1, #2 siNR4A1) (**Figs. 4C and 4D**) confirming comparable effects of resveratrol and NR4A1 knockdown. Previous studies show that resveratrol and CDIM/NR4A1 antagonists inhibit mTOR and downstream pathways and results in **Figures 5A and 5B (quantitation)** show that resveratrol (125 and 150 μ M) inhibited phosphorylation of mTOR and the downstream kinases S6RP and 4E-BP1 in H460 and H1299 cells. These results are consistent with activation of AMPK by resveratrol (**Fig. 4**), however, in this study resveratrol also downregulated the kinase proteins (mTOR, S6RP and 4E-BP1) and this would also contribute to their decreased phosphorylation. The effects of NR4A1 knockdown in H460 and H1299 cells (**Figs. 5C and 5D**) also resulted in

decreased expression of mTOR, S6RP and 4E-BP1 and their phosphorylated forms and thus resembled effects observed in cells treated with resveratrol.

NR4A1 not only directly binds promoter DNA but also acts as a nuclear cofactor that activates expression of several genes and proteins in cancer cells through protein-protein interactions with Sp1 or Sp4 bound to GC-rich promoter sites (**Safe and Karki, 2020; Lacey et al., 2016; Lee et al., 2010; Hedrick, Li et al., 2017; Hedrick et al., 2016; Hedrick et al., 2017; Shrestha et al., 2021**). This is commonly observed for other nuclear receptors which act as ligand-dependent nuclear cofactors (**Safe and Kim, 2008**). β 1-integrin is regulated by NR4A1/Sp1 and NR4A1/Sp4 in rhabdomyosarcoma, breast colon and pancreatic cancer cells and interactions with Sp1 or Sp4 is cell context dependent (**Lacey et al., 2016; Hedrick, Li et al., 2017; Hedrick et al., 2016; Hedrick et al., 2017; Shrestha et al., 2021**). Figure 6A illustrates that treatment of H460 or H1299 cells with resveratrol or knockdown of NR4A1 by RNA interference (RNAi) decreases levels of β 1-integrin protein (quantitation in **Supplemental Figure 1D**) and resveratrol also decreased β 1-integrin mRNA levels (**Fig. 6B**) confirming that β 1-integrin is an NR4A1-regulated gene. Knockdown of Sp1 in H460 and H1299 cells decreased Sp1 but only minimally affected Sp4 expression and this was accompanied by decreased levels of β 1-integrin protein confirming a role for NR4A1/Sp1 (**Fig. 6C**). In contrast, knockdown of Sp4 by RNAi decreases expression of both Sp1 and Sp4 proteins and also β 1-integrin protein (**Fig. 6D**). Thus, it is not possible to demonstrate unambiguously if NR4A1/Sp4 regulates expression of β 1-integrin and quantitation of these data is illustrated in **Supplemental Figures S1E and S1F**. CHIP analysis shows that in H460 cells treated with DMSO (control) or resveratrol that NR4A1, Sp1 and Sp4 were associated with the GC-rich promoter region of the β 1-integrin gene and treatment with resveratrol resulted in some loss of NR4A1, Sp1 and Sp4 binding (**Fig. 6E**) to the promoter. Similar results were observed in previous studies with CDIM/NR4A1 antagonist (**Lacey et al., 2016; Hedrick, Li et al., 2017; Hedrick et al., 2016; Hedrick et al., 2017; Shrestha et al., 2021**) demonstrating that the anticancer activity of

resveratrol in H460 and H1299 lung cancer cells is due, in part, to the activity of resveratrol as an NR4A1 antagonist.

DISCUSSION

Polyphenolic compounds including resveratrol are enriched in diets containing fruits, nuts and vegetables and their consumption is associated with numerous health benefits including longer lifespans, protection from aging-related and inflammatory diseases including cancer (**Liu et al., 2019; Wu et al., 2021; Hano et al., 2020; Saiko et al., 2008; Zhou et al., 2021; Raj et al., 2021; Santana et al., 2021; Koh et al., 2021**). These compounds act as antioxidant, anti-inflammatory and antiviral agents and also regulate multiple pathways and genes that contribute to diverse disease states and serve as an important class of dietary chemo-preventive agents. Resveratrol has been extensively investigated in preclinical cell culture and in vivo models and exhibits impressive cancer chemotherapeutic properties which have been attributed to the effects of this compound on expression of multiple genes. This also includes activation of AMPK and subsequent inhibition of mTOR signaling as well as the effects of resveratrol on activation of SIRT1 and other histone and non-histone deacetylase which are two pathways linked to the chemotherapeutic effects of resveratrol (**Koh et al., 2021; Khan et al., 2020; Bian et al., 2020; Wang et al., 2018; Liu et al., 2018; Chang et al., 2017; Selvaraj et al., 2016; Wu and Liu, 2013; Rashid et al., 2011; He et al., 2011; Alayev et al., 2015**). Results of preliminary studies showed that effects of resveratrol on SIRT1 expression in H460 and H1299 cells were cell context dependent and highly variable (data not shown).

Despite the remarkable activities of resveratrol, the effects of this compound in human clinical trials have not matched the promise of results from preclinical cell culture and animal models of disease (**Berman et al., 2017; Ramirez-Garza et al., 2018; Singh et al., 2019; Jazirehi et al., 2004**). Although resveratrol is generally well tolerated and provides some indications of benefits, poor bioavailability has been a problem and detrimental effects have

been observed for some cancers. For example, resveratrol inhibited several kinases in models of multiple myeloma (**Popat et al., 2013**) however, treatment of drug-resistant multiple myeloma patients with resveratrol resulted in several toxic side effects including renal failure.

It is also possible that the modest results obtained for resveratrol in human clinical trials may be due, in part, to unknown mechanisms of action which prevent a more targeted or precision medicine approach. Studies in this laboratory have identified NR4A1 as a pro-oncogenic factor in solid tumor-derived cells and animal models (**Safe and Karki, 2020**). NR4A1 also regulates cancer cell growth, survival, migration/invasion and this includes inactivation of AMPK, activation of mTOR and these responses can be reversed by bis-indole derived (CDIM) NR4A1 antagonists. Many of the effects of CDIMs are similar to those caused by resveratrol in cancer.

In this study we used H460 and H1299 lung cancer cells as models and treatment with resveratrol decreased lung cancer cell growth, enhanced apoptosis and decreased migration and invasion (**Figs. 2 and 3**). These results have previously been observed in lung cancer cells treated with resveratrol (**Wang et al., 2018; Wright et al., 2017**) and our studies also show that comparable effects have been observed in H460 and H1299 cells after NR4A1 knockdown (**Figures 2 and 3**) and after treatment with CDIM/NR4A1 antagonists (**Lee et al., 2012**). These data suggested that resveratrol may be an NR4A1 ligand and this was confirmed in direct binding and ITC assays where the K_D value for binding was in the low μM range (**Fig. 1**). Docking resveratrol to the NR4A1 LBD (**Fig. 1C**) utilizing the Schrodinger Maestro modeling approach resulted in several favorable interactions (yellow dotted line) between resveratrol and specific amino acid residues of NR4A1 LBD (Ser110, Gul114, Arg184, Thr236 (aromatic, Leu239, and Ile260). Two unfavorable interactions (orange dotted line) between resveratrol and the NR4A1 LBD were also predicted (Leu239 and Ile260). Docking studies with the newly developed high affinity CDIM ligand bis(3'-indolyl)-1-(3,5-dichlorophenyl)methane (DIM-3,5-Cl₂) (**Karki et al., 2021**) to the NR4A1 LBD (**Fig. 1D**) resulted in similar favorable interactions (yellow

dotted line) as predicted for resveratrol including specific interactions with Ser110, Glu114 (halogen bond), Arg184, Arg232 (aromatic), and Thr236 side chains but also some differences. These differences in the interactions of resveratrol and DIM-3,5-Cl₂ with amino acids in the ligand binding domain of NR4A1 are consistent with designation of these compounds as selective receptor modulators. The binding results coupled with the inhibitory effects of resveratrol on NR4A1-dependent transactivation (**Fig. 1**) demonstrate for the first time that resveratrol is an NR4A1 ligand which acts as a receptor antagonist and inhibits NR4A1-dependent transactivation in lung cancer cells.

We also examined a number of gene products previously shown to be regulated by CDIM/NR4A1 antagonists in cancer cells (**Safe and Karki, 2020**) and these include decreased expression of TXNDC5, IDH1, mTOR, β 1-integrin and induction of apoptosis gene products and activation of pAMPK (**Figs. 4-6**). Responses observed for resveratrol and NR4A1 knockdown were comparable and β 1-integrin was regulated by NR4A1 through interactions of NR4A1 as a cofactor of Sp1 bound to the GC-rich sites of the β 1-integrin gene. Since knockdown of Sp4 in H1299 and H460 cells also decreases Sp1 expression it was not possible to determine unequivocally a role for Sp4 in regulating β 1-integrin gene expression via NR4A1/Sp4. The ChIP assay shows that both Sp1 and Sp4 bind the GC-rich integrin promoter and it is possible that NR4A1 may coactivate both Sp1 and Sp4, and this process is blocked by resveratrol as previously observed for CDIM/NR4A1 ligands (**Lacey et al., 2016; Hedrick, Li et al., 2017; Hedrick et al., 2016; Hedrick et al., 2017**). Thus, like CDIM/NR4A1 antagonists' resveratrol also inactivates NR4A1/Sp – regulated genes such as β 1-integrin and this further confirms that the mechanisms and functions of resveratrol are due, in part, to its activity as a NR4A1 antagonist.

This study demonstrates for the first time that resveratrol binds with high affinity to NR4A1 and acts as an NR4A1 antagonist in lung cancer cell lines. Although the KD value for resveratrol is in the low μ M range indicating strong ligand-receptor interactions, the dose-

response functional effects of resveratrol are in the 100-200 μM range in lung cancer cells (**Wright et al., 2017; Wang et al., 2018**) and this is several orders of magnitude higher than the KD value. This difference may be due to several factors including the effectiveness of the bound receptor complex to interact with nuclear cofactors, cellular uptake of resveratrol and rapid metabolism (conjugation) which is commonly observed for other polyphenolics. Like many solid tumors NR4A1 is overexpressed in many solid tumors and is a negative prognostic factor for patient survival (**Lee et al., 2012**). This suggests that clinical applications of resveratrol in lung cancer chemotherapy may be more effective in treating patients with tumors that overexpress NR4A1. It should also be noted that there is a long list of potential targets of resveratrol that include kinases, cytokines, cell signaling molecules, key genes involved in cancer cell proliferation, survival and migration/invasion (**Saiko et al., 2008; Zhou et al., 2021; Raj et al., 2021; Santana et al., 2021; Koh et al., 2021; Kolahehdouz et al., 2017; Rudzitis-Auth et al., 2013; Ergenoglu et al., 2013; Bruner-Tran et al., 2011; Amaya et al., 2014; Taguchi et al., 2014; Bayoglu et al., 2014; Teertam et al., 2020; Le et al., 2019; Tang et al., 2021**). This list also includes interactions with other receptors including nuclear receptor superfamily members. This study highlights the contribution of resveratrol as an NR4A1 ligand (antagonist) in lung cancer cells and the effectiveness and contributions of this response to the overall anti-cancer activity of resveratrol may also be tumor-type specific and needs to be further investigated.

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Authors' Contributions

Participated in Research Design: Zhang, Lei; Safe, Stephen

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Contributed to Analytical Tool: Hampton, Joshua Trae; Liu, Wenshe Rayi

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FIGURE CAPTIONS

Figure 1: Resveratrol as an NR4A1 ligand. A. Structure of resveratrol B. Binding of resveratrol to NR4A1 (LBD) by isothermal titration calorimetry (ITC) (B) as outlined in the Methods. Molecular modeling of the interaction of resveratrol (C) and 1,1-bis(3'-indolyl)-1-(3,5-dichlorophenyl)methane (DIM-3,5-Cl₂) (D) with NR4A1 (LBD) was carried out using Maestro and crystal structure coordinates for the human orphan nuclear receptor NR4A1 ligand binding

domain as outlined in the Methods. Effects of resveratrol on luciferase activity in H460 (E) and H1299 (F) cells transfected with GAL4-NR4A1 and UAS-luc as outlined in the Methods. Results are expressed as means \pm SD for at least 3 replicate determinations for each treatment group and significant ($p < 0.05$) effects compared to control are indicated (*). The ITC binding assay was repeated (3X) and the means K_D and ΔG values \pm SD are indicated in panel 1B.

Figure 2. Resveratrol and NR4A1 knockdown (siNR4A1) inhibit growth and induce apoptosis in H460 and H1299 cells. H460 (A) and H1299 (B) cells were treated with resveratrol for up to 72-hours and effects on cell proliferation and NR4A1 protein expression (C) were determined use an XTT assay. H460 and H1299 cells were transfected with siNR4A1 (2 oligonucleotides) and effects on proliferation of H460 (D) and H1299 (E) cells and NR4A1 protein expression (F) was determined. G. H460 and H1299 cells were treated with resveratrol or whole cell lysates were analyzed by western blots and bands were quantitated. H460 and H1299 cells were transfected with siNR4A1 (H) and whole cell lysates were analyzed by western blots and bands were quantitated. I. H460 and H1299 cells were treated with resveratrol and effects on Annexin V staining were determined as outlined in the Methods. Results are expressed as means \pm SD for at last 3 separate determinations for each treatment group and significant ($p < 0.05$) changes compared to control (DMSO) are indicated. Calculations of changes in intensity of protein bands are also normalized to the β -actin loading control for each treatment group. Quantitation of blots in 2C/2F, and 2G and 2H are summarized in Supplemental Figures 1A, 1B and 1C respectively.

Figure 3. Resveratrol inhibits cell migration and invasion. A. H460 and H1299 cells were treated with resveratrol (A) or transfected with siNR4A1 oligonucleotides (B) and effects on cell migration were determined in scratch assays. Cells were treated with resveratrol (C) or transfected with siNR4A1 oligonucleotides (D) and effects on cell invasion were determined in a

Boyden chamber assay. Results of the effects of resveratrol (A and C) and knockdown (B and D) have been quantitated and appear in the same panels (right side). Quantitative results are expressed as means \pm SD for at least 3 separate determinations per treatment group and significant ($p < 0.05$) inhibition is indicated (*). Resveratrol (125 and 150 μ M) decreased migration ability in H460 cells by 23.22% with a 95% CI of 13.37 to 33.06% and 44.19% with a 95% CI of 34.34 to 54.04% respectively. Resveratrol (125 and 150 μ M) decreased cell migration ability in H1299 cells by 41.76% with a 95% CI of 25.16 to 58.35% and 40.10% with a 95% CI of 23.50 to 56.70% respectively. Resveratrol (125 and 150 μ M) decreased invasion ability in H460 cells by 41.43% with a 95% CI of 25.94 to 56.92% and 68.78% with a 95% CI of 53.29 to 84.27% respectively. Resveratrol (125 and 150 μ M) decreased invasion ability in H1299 cells by 27.30% with a 95% CI of 17.34 to 37.26% and 66.46% with a 95% CI of 56.50 to 76.41% respectively.

Figure 4. Effects of resveratrol and NR4A1 knockdown on selected NR4A1-regulated gene products. H460 and H1299 cells were treated with resveratrol (A; B-quantitation of bands in A) or transfected with siNR4A1 oligonucleotides (C; D-quantitation of bands in C) and whole cell lysates were analyzed by western blots as outlined in the Methods. Quantitative results (B and D) are expressed as means \pm SD for at least 3 separate gels per treatment group and significantly ($P < 0.05$) induced changes in band densities compared to CTL (DMSO or empty vector) are indicated (*). Levels for all proteins were normalized to β -actin.

Figure 5. Effects of resveratrol and siNR4A1 on mTOR signaling. H460 and H1299 cells were treated with resveratrol (A; B-quantitation of bands in A) or transfected with siNR4A1 oligonucleotides (C; D, quantitation of bands in C) and whole cell lysates were analyzed by western blots as outlined in the Methods. Results (B and D) are expressed as means \pm SD for at least 3 separate gels per treatment group and significantly ($p < 0.05$) induced changes in band

intensities compared to CTL (DMSO or empty vector) are indicated (*). Levels for all proteins were normalized to β -actin.

Figure 6. Mechanism of β 1-integrin regulation by resveratrol. H460 and H1299 cells were treated with resveratrol for 24-hours and whole cell lysates were analyzed by western blots or transfected with siNR4A1 oligonucleotides (A). B. H460 and H1299 cells were treated with resveratrol for 24-hours and β 1-integrin mRNA levels were determined by real-time PCR as outlined in the Methods. H460 and H1299 cells were transfected with oligonucleotides that target Sp1 (siSp1) (C) or Sp4 (siSp4) (D) expression and whole cell lysates were analyzed by western blots. E. Effects of resveratrol on interactions of NR4A1 Sp1, Sp4 and polIII with the GC-rich region of the β 1-integrin promoter were determined in a ChIP assay as outlined in the Methods. Quantitative results (B) are means \pm SD for at least 3 replicate determinations and significant ($p < 0.05$) changes relative to untreated control values are given (*). Quantitation of western blots in A, C and D are summarized in Supplemental Figures 1D, 1E and 1F respectively.

Figure 1.

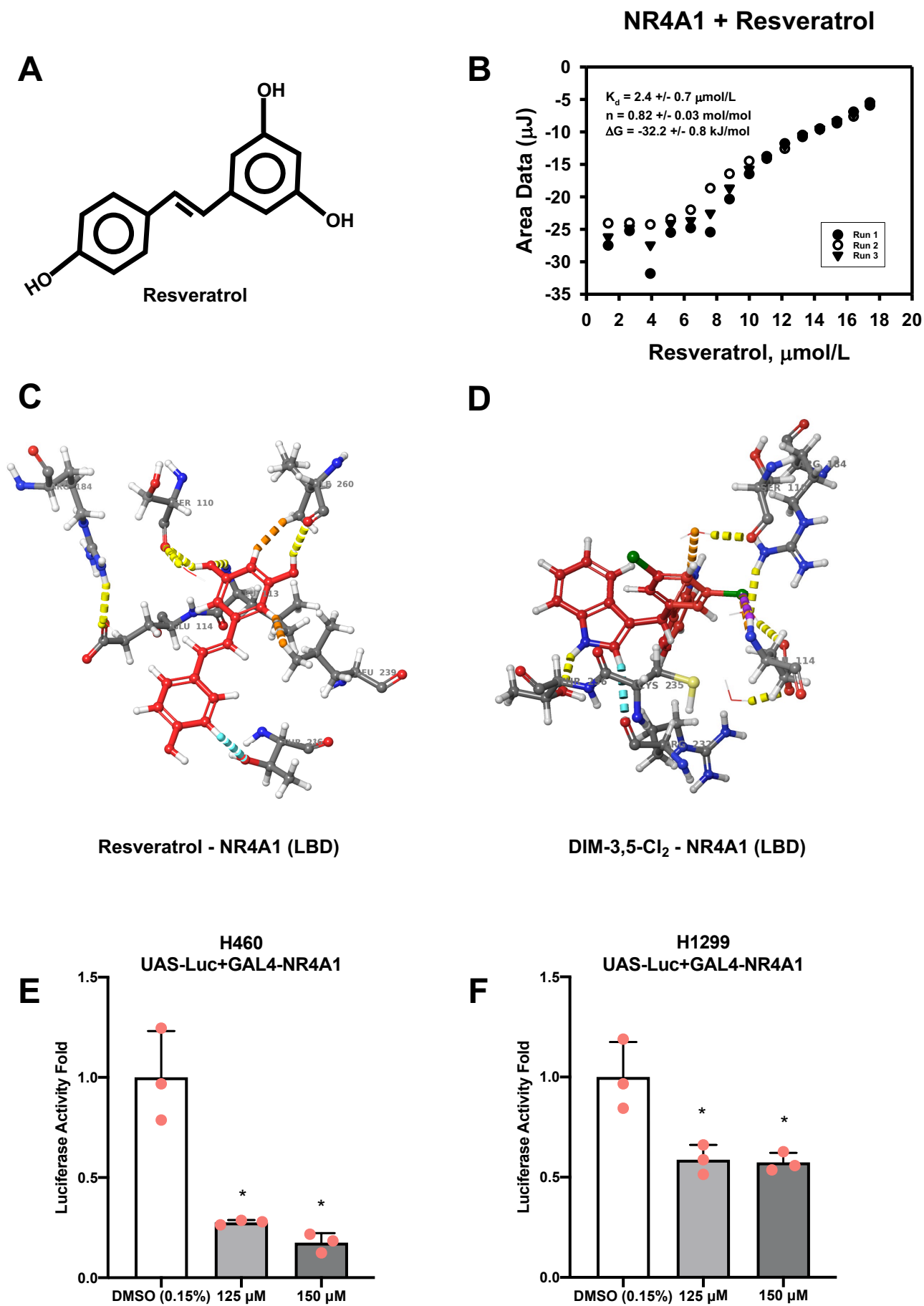


Figure 2.

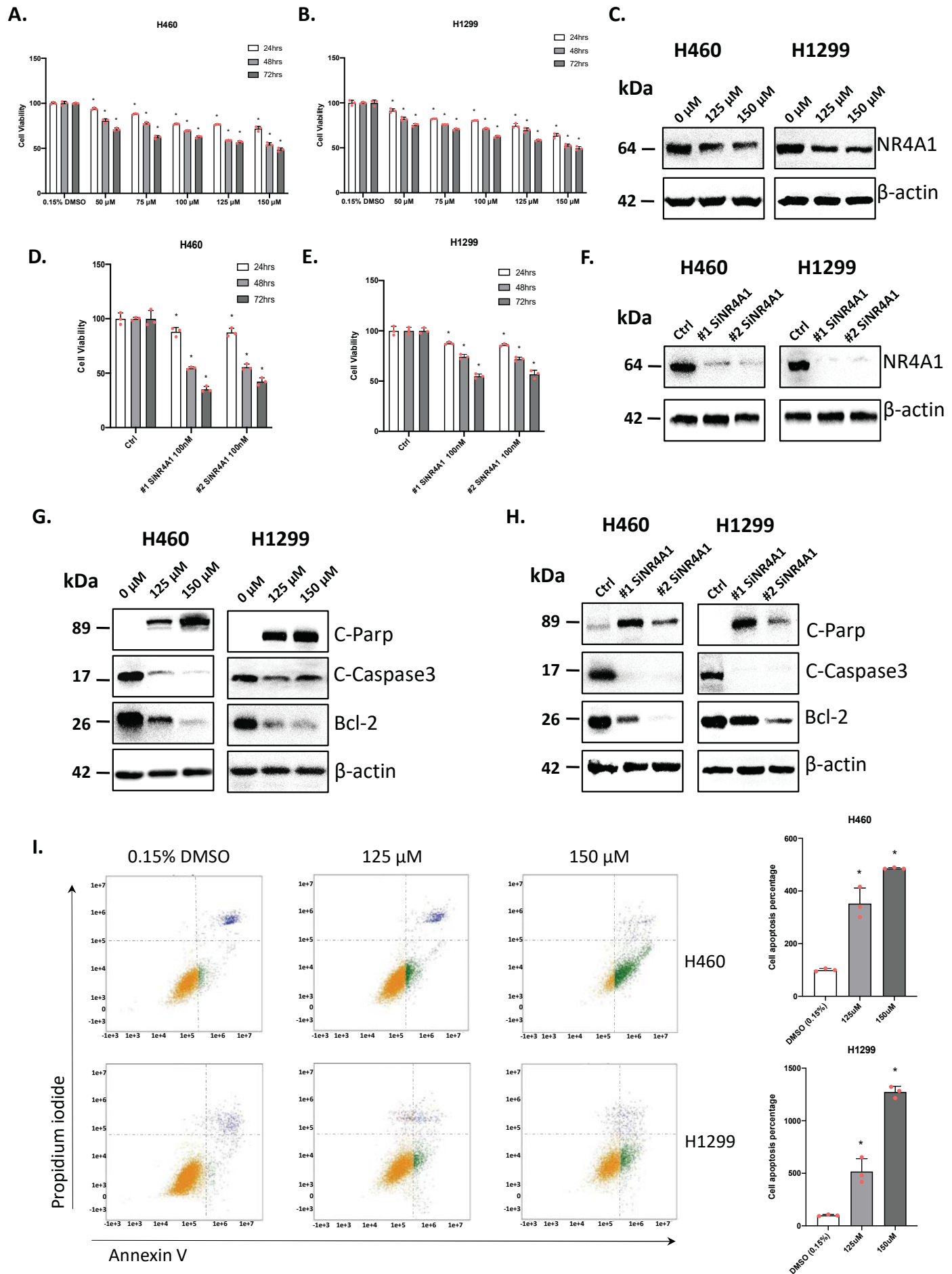


Figure 3.

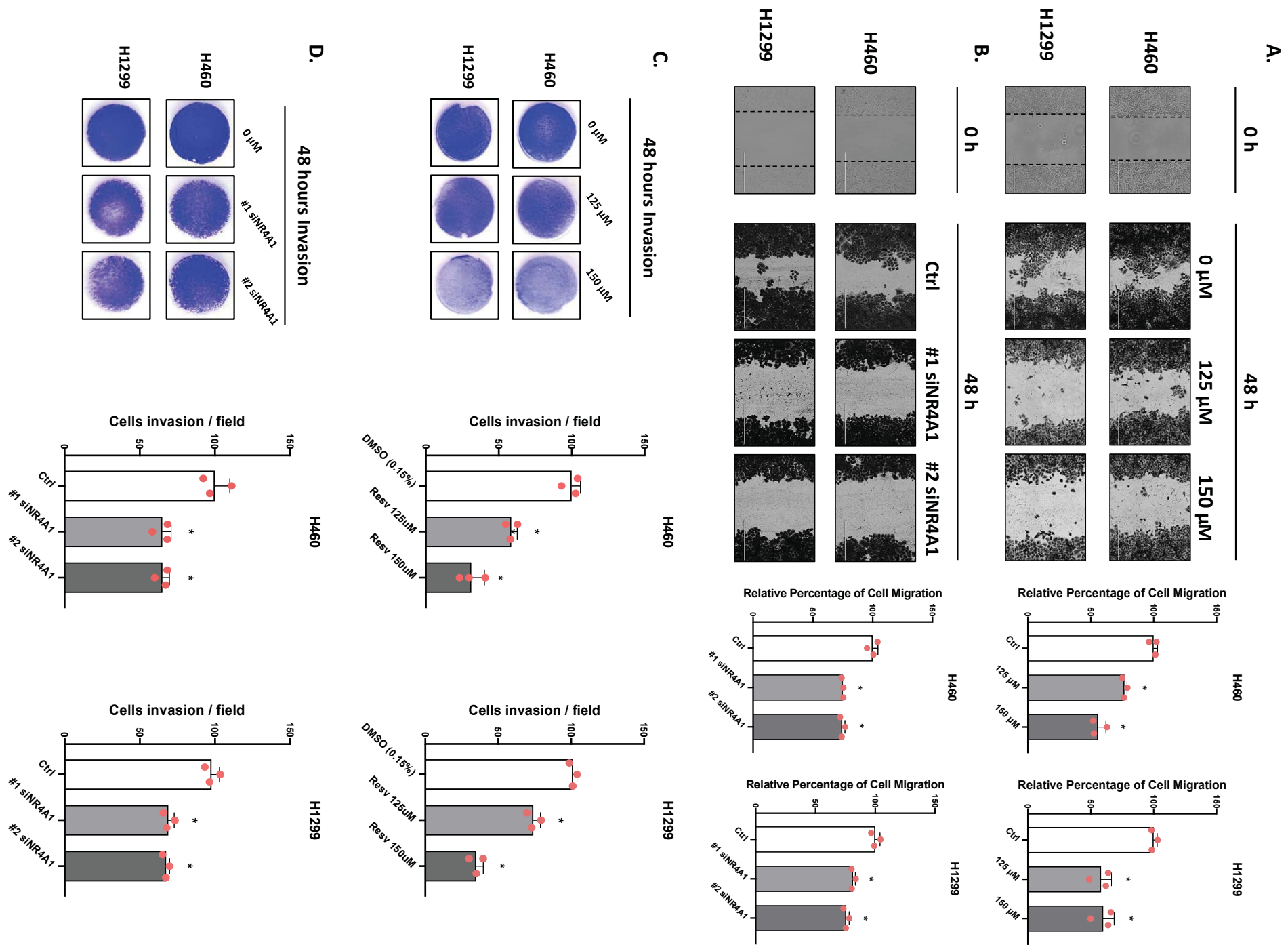


Figure 4.

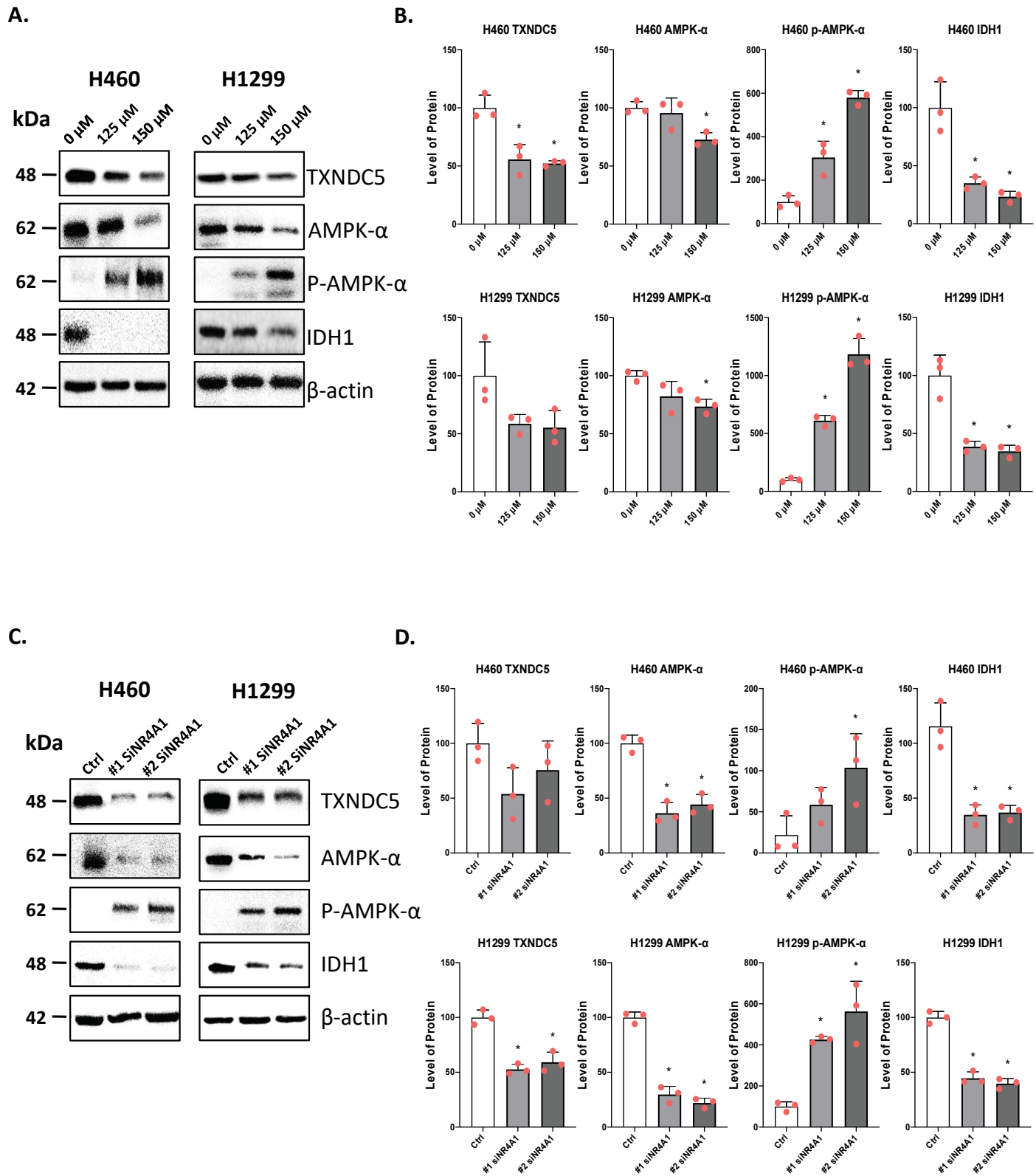


Figure 5.

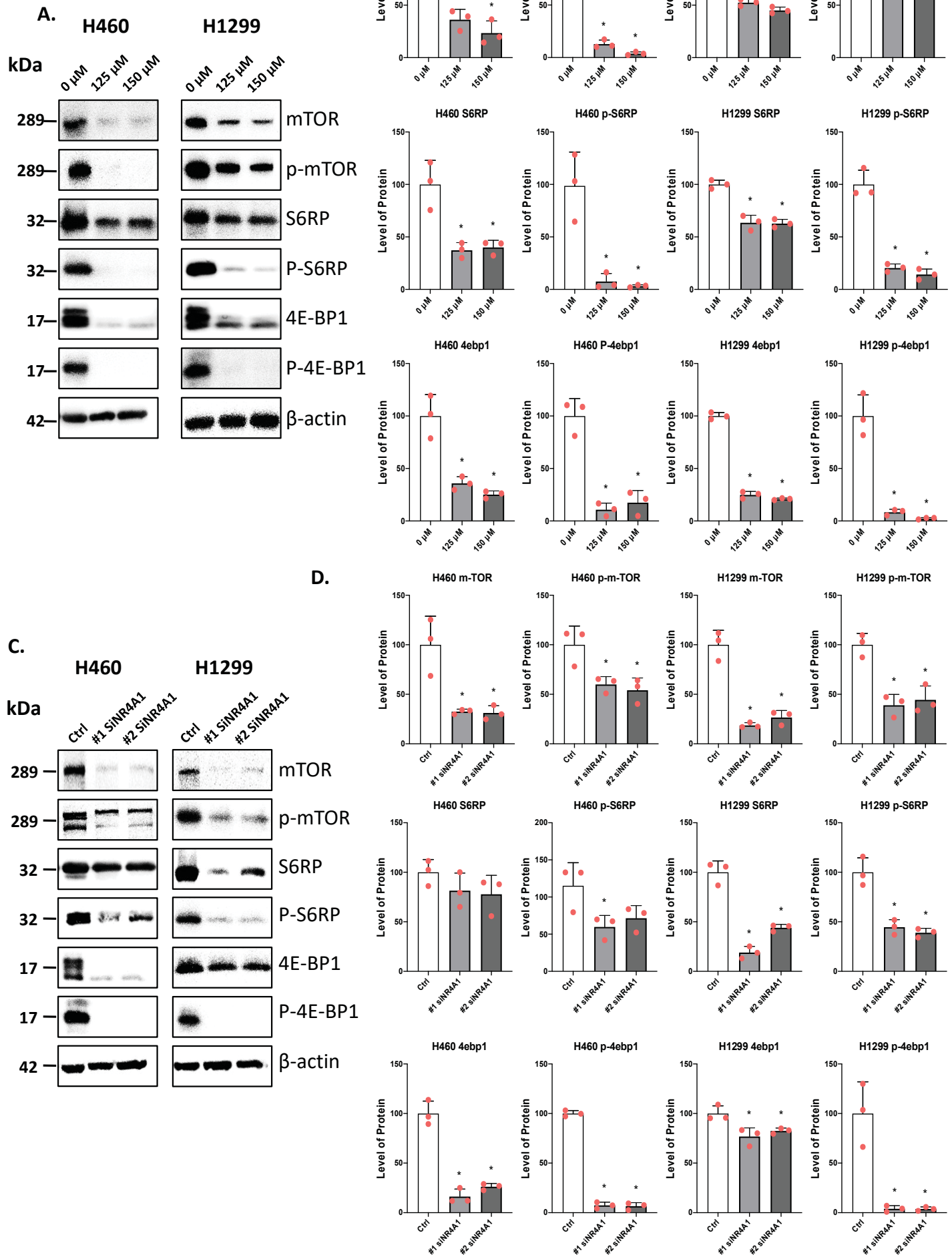


Figure 6.

