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Aryl hydrocarbon receptor is a target of 17-Allylamino-17-demethoxygeldanamycin and enhances its anticancer activity in lung adenocarcinoma cells

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; Hsp90, heat shock protein 90;

17-AAG, 17-Allylamino-17-demethoxygeldanamycin; 17-DMAG,

17-Dimethylaminoethylamino-17-demethoxygeldanamycin; NQO1,

NAD(P)H:quinone oxidoreductase; EGFR, epithelial growth factor receptor; ALK,

anaplastic lymphoma kinase; Dox., doxycycline; MTT,

3-(4,5)-Dimethylthiahiazo(-z-yl)-3,5-di- phenytetrazoliumromide; DMSO, dimethyl

sulfoxide; PBS, phosphate buffered saline; PI, propidium iodide; LDH, lactate

dehydrogenase; pRb, phosphorylated Rb; RT-PCR, Quantitative real-time reverse

transcription-polymerase chain reaction.

Abstract

We have demonstrated that aryl hydrocarbon receptor (AhR) is overexpressed in lung adenocarcinoma (AD). AhR is usually associated with heat shock protein 90 (Hsp90) in the cytoplasm. 17-Allylamino-17-demethoxygeldanamycin (17-AAG), an Hsp90 inhibitor, is currently under evaluation for its anticancer activity in clinical trials. Here, we investigated whether AhR plays a role in 17-AAG-mediated anticancer activity by functioning as a downstream target or by modulating its anticancer efficacy. AhR expression in lung AD cells was modulated by siRNA interference or overexpression. Tumor growth was determined with colony formation *in vitro* or *in vivo*. Anticancer activity of 17-AAG was determined by measuring cell viability, cell cycle distribution and expression of cell cycle regulators. Proteins and mRNA levels were examined by immunoblotting and the real-time reverse transcription-polymerase chain reaction, respectively. In this study, AhR overexpression positively modulated growth of lung AD cells, at least partially, via RelA-dependent mechanisms. Although treatment with 17-AAG reduced AhR levels and AhR-regulated gene expression in lung AD cells, AhR expression increased anticancer activity of 17-AAG. In addition, 17-AAG treatment reduced cell viability, CDK2, CDK4, cyclin E, cyclin D1 and phosphorylated Rb levels in AhR-expressing lung AD cells. NAD(P)H:quinone oxidoreductase (NQO1), which is regulated by AhR, was shown to increase anticancer activity of 17-AAG in cells. Knockdown of *NQO1* expression attenuated the reduction of cell cycle regulators by 17-AAG treatment in AhR overexpressed cells. We demonstrated that AhR protein not only functions as a downstream target of 17-AAG, but also enhances anticancer activity of 17-AAG in lung AD cells.

Introduction

Lung cancer is the most common cause of cancer death worldwide. The incidence of lung adenocarcinoma (AD) has increased recently, and AD has become the most common type of lung cancer (Devesa et al., 2005). Molecular biomarkers for lung cancer are dependent on histological type of cancer, history of smoking and ethnicity (Ladanyi and Pao, 2008; Thu et al., 2012). For example, incidences of mutation of epithelial growth factor receptor (EGFR) and translocation of anaplastic lymphoma kinase (ALK) are relatively high in lung AD and/or non-smoking lung cancer (Shaw et al., 2009; Sun et al., 2007). Thus, targeted therapy for various histological types of lung cancer is actively under investigation (Moran, 2011).

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is associated with heat shock protein 90 (Hsp90) in the cytoplasm of cells before activation by ligands (Pollenz et al., 1994). Some AhR ligands are known human carcinogens (McGregor et al., 1998; Smith et al., 2000). Blocking AhR activation is considered a preventive mechanism for carcinogenesis (Ciolino and Yeh, 2001). Regardless of ligand activation, AhR up-regulates expression of cytochrome P4501B1, fibroblast growth factor-9 and interleukin-6 in lung AD cells, and AhR expression is correlated with protein expressions of these regulated genes in lung AD (Chang et al., 2007; Chen et al., 2012; Wang et al., 2009). Due to the abundance of AhR in lung AD and its role in promoting growth of lung AD cells (Chang et al., 2007), we proposed that AhR is involved in the development of lung AD, raising the possibility of AhR as a therapeutic target for lung AD.

Hsp90 is an ATP-dependent molecular chaperone that regulates protein folding, stability and maturation of a diverse range of client proteins (Neckers and Workman, 2012). Hsp90 client proteins include kinases, receptors, and transcription factors,

many of which are involved in key pathways that are dysregulated in cancers (Katayama et al., 2011; Sawai et al., 2008; Schulte et al., 1995; Solit et al., 2002). In cancer cells, Hsp90 not only protects activated or mutated oncogenic client proteins, but also buffers cellular stress induced by malignancy (Trepel et al., 2010). Thus, targeting of Hsp90 for cancer therapy has been in development since 1990 (Neckers and Workman, 2012). 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is a derivative of geldanamycin that binds to the ATP-binding domain of Hsp90 and inhibits Hsp90 function (Georgakis and Younes, 2005). This is the first Hsp90 inhibitor to be evaluated in clinical trials (Banerji et al., 2005; Modi et al., 2011; Sausville et al., 2003). In lung cancer, mutated (rearranged) ALK and EGFR are Hsp90 client proteins that are sensitive to chaperone inhibition (Katayama et al., 2011; Sawai et al., 2008). Since 17-AAG induces degradation of mutant EGFR and ALK in lung cancer cells (Katayama et al., 2011; Sawai et al., 2008), it might overcome or prevent resistance to tyrosine kinase inhibitors in lung cancer.

Hughes et al. (Hughes et al., 2008) reported that 17-AAG reduces AhR protein levels and prevents its ligand-activated biological responses in cells. The data suggest that AhR, an Hsp90 client protein, is sensitive to 17-AAG. In our present study, we investigated whether AhR plays a role in 17-AAG-mediated anticancer activity by functioning as a downstream target or by modulating its anticancer efficacy. We observed a mutual synergism between 17-AAG and AhR in anticancer efficacy. Our data suggested that 17-AAG is useful for treating lung AD that expresses high levels of AhR.

Materials and Methods

Drugs

17-AAG was purchased from Enzo Life Sciences, Inc. (Farmingdale, New York, USA)

17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) was

purchased from Invivogen (San Diego, USA)

Cell lines and modulation of AhR expression

BEAS-2B cells are human immortalized bronchial epithelial cells. H1355, CL-5 and

CL1-5 are lung AD cell lines. H1355 and BEAS-2B cells were purchased from

American Type Culture Collection (Manassas, VA, USA). CL5 and CL1-5 cells were

the kind gift of Dr. Pan-Chyr Yang of National Taiwan University (Yang et al., 1992).

To perform RNA interference towards AhR (si-AhR), a short hairpin RNA was driven

by the human U6 promoter in an engineered pCDNA/HU6 vector (Chang, 2004). The

target site of si-AhR is GAATACTTCCACCTCAGTTGGC. The vector containing

si-AhR target site was transfected into H1355 cells for stable clone selection as

described previously (Chang et al., 2007). One stable clone of vector control (Si-NC)

and two stable si-clones (si-AhR-1 and si-AhR-2) were selected (Chang et al., 2007).

To overexpress AhR, the vector expressing AhR induced by tetracycline was

transfected into CL1-5 lung AD cells for selection of stable clone CL1-5 (TO-AhR)

(Cheng et al., 2012). Then, AhR expression was induced by the addition of 1 μg/ml

tetracycline analog doxycycline (Dox; Clontech, Mountain View, CA, USA). CL1-5

(TO-AhR) cells were cultured in 10% fetal calf serum/RPMI1640 (Gibco, Grand

Island, NY, USA) supplemented with penicillin/streptomycin (Gibco, Grand Island,

NY, USA). Wild type H1355 cells and stable clones derived from H1355 cells were

cultured in 5% fetal calf serum/RPMI1640 (Gibco, Grand Island, NY, USA)

supplemented with penicillin/streptomycin (Gibco, Grand Island, NY, USA).

Transient transfection

NFκB RelA siRNA (#6534, Cell Signaling, Danvers, MA, USA) or

NAD(P)H:quinone oxidoreductase (*NQO1)* siRNA

(5'-UUCUCCGAACGUGUCACGUTT-3') siRNA was transiently transfected into

H1355 cells with Lipofectamine^TM 2000 (Invitrogen, Grand Island, NY, USA) for 6 hr.

To perform RNA interference towards IL-6, a short hairpin RNA was driven by the

human U6 promoter in an engineered pCDNA/HU6 vector (Chang, 2004). The target

site of IL-6 is "GCCACTCACCTCTTCAGAA". Transient overexpression of AhR in

H1355 cells was previously described (Chen et al., 2012).After replacement with

fresh media, cells were incubated at 37°C for an additional 48 hours prior to testing of

gene expression.

Lung tumor growth in nude mice

Eight-week-old male BALB/C nude mice (BioLASCO, Taipei, Taiwan) were injected

subcutaneously with 5 x 10^6 H1355, si-NC or si-AhR stable clone (n = 6 mice/group).

Three weeks later, all animals had small palpable tumors. Cells were subcutaneously

injected once at day 1 and tumor volume was measured twice weekly for 43 days

using the formula: volume = a x b^2 x 0.4, where a and b are the larger and smaller

diameters, respectively. The animal protocol was approved by the Institutional Animal

Care and Use Committee of the National Health Research Institutes, Taiwan.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)
assay

Total RNA was extracted using the TRI reagent (Molecular Research Center,

Cincinnati, OH, USA). Three micrograms of total RNA were subjected to a reverse

transcription step using the ABI High-Capacity cDNA Archive Kit. Real-time PCR quantification was then performed using TaqMan universal PCR Master Mix (Roche, Branchburg, NJ, USA). *GAPDH* was used for normalization. The primers and probes for *AhR*, *CYP1B1*, *IL-6*, *IL-8*, *NQO1* and *GAPDH* were from the Assay-on-Demand Gene Expression Assay Mix (ABI, Foster City, NJ, USA).

3-(4,5)-Dimethylthiahiazo(-z-yl)-3,5-di- phenytetrazoliumromide (MTT) assay

The cell lines were cultured onto 96-well microplates and treated with 17-AAG (BML-EI308, Enzo Life Sciences, NY, USA) for 48 hours. Then, MTT solution was added to each well, and the microplate was incubated at 37°C for 4 hours. Formazan crystals, produced by mitochondrial dehydrogenase activity in viable cells, were dissolved by the addition of dimethyl sulfoxide (DMSO) on a shaker at room temperature. The absorbance was read at 535 nm using a microplate reader.

Trypan blue exclusion assay for determination of viable cell numbers

Cells were cultured onto 12-well plates at a density of 1×10^{5} cells/well for 1 to 4 days in 2 ml of medium. Cells in each well were treated with 500 nM 17-AAG, harvested at the appropriate time, stained with trypan blue, and counted using a hemacytometer.

Colony formation assay

For the colony formation assay, 1000 transfected cells were plated onto a 6-well plate for two weeks. Colonies were fixed and stained with 1% crystal violet (with 30% ethanol).

Western immunoblotting

Cells were harvested for Western immunoblotting as previously described (Chang et

al., 2007) and stained with anti-AhR (BML-SA210, Enzo Life Sciences, Plymouth Meeting, PA, USA), anti-HSP90 (Assay Designs, Ann Arbor, MI, USA), anti-Arnt (H-172, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IκBα (C-21, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH (V-18, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CDK2 (D-12, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CDK4 (DCS-35, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin D1 (DCS-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Cyclin E (E-4, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rb (D20, Cell Signaling, Danvers, MA, USA), anti-phospho-Rb (ser807/811, Cell Signaling, Danvers, MA, USA), anti-p27 (F-8, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (clone CP74, Millipore, Temecula, CA, USA) and anti-NQO1 (C2C3, GeneTex, Hsinchu City, Taiwan) antibodies. The protein bands were detected by chemiluminescence.

Cell cycle analysis with flow cytometry

Cells were starved in serum-free medium for 24 hrs. Then, the medium was replaced with fresh medium containing 10% FBS and 17-AAG. After 24 hr incubation, cells were collected by trypsinization and gentle centrifugation. They were then fixed with 70% ethanol. The fixed cells were washed with phosphate buffered saline (PBS) and resuspended in propidium iodide (PI) solution (1% Triton X-100, 0.1 mg/ml RNase A, 4 ug/ml PI). DNA content in the cells was analyzed with FACSCalibur (BD Biosciences, San Diego, CA, USA).

Cell death as determined by the release of lactate dehydrogenase (LDH)

LDH activity was measured in collected medium following the manufacturer's instructions (CytoTox 96® Non-radioactive cytotoxicity assay, Promega, WI, USA).

The absorbance of the samples was measured at 490 nm using an ELISA reader (Molecular Devices SPECTRA max 190, Sunnyvale, CA, USA). The medium mixed with the reaction solution served as the background control.

Statistical analysis

Differences between groups in *in vitro* experiments were compared using ANOVA with Dunnett's test for multiple comparison. Differences were considered statistically significant at $p < 0.05$.

Results

Our previous report demonstrated that AhR positively regulates growth of lung cancer cells (Chang et al., 2007). Here, we showed that silencing of AhR expression reduces tumor growth of human lung AD H1355 cells *in vivo* (Fig. 1A). In the absence of exogenous ligand, AhR is associated with RelA and positively modulates NFκB activity, as well as up-regulates IL-6 expression, in H1355 cells (Chen et al., 2012). Here, we further demonstrated that silencing of RelA expression not only abolishes AhR-enhanced interleukin expression (Fig. 1B), but also inhibits colony formation of H1355 cells (Fig. 1C). Similarly, silencing of IL-6 expression inhibits AhR-enhanced colony formation of H1355 cells (Fig. 1D). It appears that AhR promotes growth of lung AD cells via RelA-dependent mechanisms. Moreover, 17-AAG has been reported to reduce AhR protein levels (Hughes et al., 2008). Treatment with 250 or 500 nM 17-AAG significantly reduced AhR protein levels and cytokines expression, but increased IkBα accumulation in H1355 cells (Fig. 2). Therefore, we speculated that AhR is partially involved in 17-AAG-mediated anticancer activity via modulation of NFκB activation.

AhR expression was variable in human lung cells: AhR expression was high in H1355 and CL5 cells, but was relative low in BEAS-2B and CL1-5 (Fig 3A). Interestingly, high-AhR expressing cells (H1355 and CL5) were more sensitive to 17-AAG than low-AhR expressing cells (BEAS-2B and CL1-5) (Fig 3A). To understand the function of AhR expression in anticancer activity of 17-AAG, we established one stable clone of vector control (si-NC) from H1355 cells, two stable AhR interference clones from H1355 cells (si-AhR-1 and -2) and one inducible AhR clone from CL1-5 cells (TO-AhR). When we modulated AhR expression in lung AD H1355 and CL1-5 cells, surprisingly AhR expression was positively correlated with anticancer activity

of 17-AAG. While silencing of AhR expression in H1355 cells reduced activity of 17-AAG, induction of AhR expression in CL1-5 cells enhanced activity of 17-AAG (Figs. 3B and 3C). The detailed effects of 17-AAG on cell growth and death were further investigated. Treatment of si-NC cells with 100 or 500 nM 17-AAG for 96 hrs markedly reduced viable cell numbers to 11% or 4% of vehicle-treated cells (Fig 3D). In AhR silenced clones AhRi-2, treatment with 100 or 500 nM 17-AAG only reduced viable cell numbers to 21% or 13% of vehicle-treated cells (Fig 3D). Furthermore, high concentration of 17-AAG caused cell death in si-NC cells, but not in AhR-silenced cells (Figure 3E). It appears that silencing of AhR expression reduces growth inhibitory effect and cytotoxicity of 17-AAG. We further investigated the interaction between AhR expression and 17-AAG on cell cycle progression and its regulators. After cells were synchronized at G0/G1 phase via starvation for 24 hrs, 17-AAG prevented si-NC cells from entering the cell cycle and caused G1 arrest 24 hrs later (Fig 3F). No increase in apoptotic cells was observed after treatment with 17-AAG for 24 hrs (data not shown). Silencing of AhR expression allowed cells to enter the cell cycle, but some cells were blocked in the G2 phase. Silencing of AhR expression also significantly reduced 17-AAG-induced G1 arrest, which was accompanied by increased G2 arrest (Fig. 3F). Regardless of AhR expression, treatment with 500 nM 17-AAG significantly reduced S phase cells (Fig. 3F).

We further investigated the expressions of cell cycle regulators. Treatment with 250 or 500 nM 17-AAG markedly reduced protein levels of CDK2, CDK4, cyclin D1, cyclin E, and phosphorylated Rb (pRb) in si-NC cells (Fig. 4A). Silencing of AhR expression prevented reduction of these proteins by 17-AAG. Consistently, induction of AhR expression accelerated reduction of these proteins by 17-AAG in CL1-5 (TO-AHR) cells (Fig. 4B). It has been reported that NQO1 converts 17-AAG into a

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metabolite with high growth inhibitory activity (Siegel et al., 2011). Interestingly, AhR expression positively modulated *NQO1* expression in H1355 and CL1-5 cells (Fig 4C). Thus, it is likely that increased *NQO1* expression participates in the AhR-mediated enhancement of 17-AAG anticancer activity. Indeed, reduction of *NQO1* expression prevented cyclins and CDKs degradation in 17-AAG-treated CL1-5 (TO-AhR) cells following induction of AhR (Fig .4D). Furthermore, reduction of *NQO1* expression reduced growth inhibitory effect of 17-AAG in H1355 cells. (Fig 4E). These results suggested that high level of AhR expression enhances 17-AAG-mediated cyclins and CDKs reduction via up-regulation of *NQO1* expression.

17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) is the other derivative of geldanamycin. 17-DMAG is water soluble and less dependent on NQO1 (Gaspar et al., 2009). As shown in Figure 5A, 17-DMAG was more effective than 17-AAG in H1355 cells. Modulation of AhR expression in H1355 and CL1-5 cells still positively correlated with anticancer activity of 17-DMAG (Fig. 5B and 5C, although the dependence on AhR expression was less for 17-DMAG activity than for 17-AAG activity. Similar to 17-AAG, 17-DMAG significantly reduced proteins levels of AhR and cyclin D1, but increased the accumulation of IkBα in H1355 cells (Fig. 5D). Cytokine expression was also reduced by 17-DMAG (Fig. 5E). Therefore, AhR is also a target for 17-DMAG and might partially involve in 17-DMAG-mediated anticancer activity.

Discussion

The incidence of lung ADs has been increasing worldwide. However, the etiology of lung ADs is not well understood, and more efficient or targeted therapies are in urgent need. AhR expression is elevated in human lung ADs and promotes tumor growth. Here, we presented evidence of novel synergistic effects between AhR and 17-AAG in the inhibition of lung AD cell growth. For example, 17-AAG reduced AhR protein levels and its regulated genes, suggesting that AhR is an anticancer target of 17-AAG. On the other hand, AhR increased *NQO1* expression and, in turn, sensitized lung AD cells to 17-AAG. AhR expression enhanced the growth inhibitory effect of 17-AAG by decreasing protein levels of cyclins and CDKs in lung AD cells. These results suggested that 17-AAG is an effective anticancer agent for the treatment of cancers that express high levels of AhR, such as lung AD.

Recently, we demonstrated that AhR increases NF-kB activity by direct association with RelA, and thus upregulates IL-6 expression in lung AD cells (Chen et al., 2012). IL-6 has been shown to play a role in tumor progression (Smith et al., 2001). Here, we further demonstrated that knockdown of RelA or IL-6 expression by siRNA interference reduces AhR-facilitated colony formation of lung AD cells, confirming the involvement of RelA/IL-6 in AhR-promoted tumor growth. Our previous report demonstrated that up-regulation of AhR reduces IκBα level (Chen et al., 2012). Treatment with 17-AAG not only increased the accumulation of IkBα protein, but also reduced IL-6/Il-8 expression and AhR protein, suggesting that 17-AAG inhibits RelA-dependent AhR-promoted tumor growth.

As the first hsp90 inhibitor to undergo clinical trials, 17-AAG has shown promising results in the treatment of HER2-positive breast cancer and multiple myeloma (Modi

et al., 2011; Richardson et al., 2011). However, the requirement of metabolic activation of 17-AAG by NQO1 may limit the usage of 17-AAG in cancer therapy (Gaspar et al., 2009; Kelland et al., 1999). The hydroquinone 17AAGH2, converted from 17-AAG by NQO1, exhibits a greater affinity to Hsp90 than 17AAG (Guo et al., 2005). Hence, 17-AAG causes greater growth inhibition in NQO1-expressing cells than in NQO1-null cells (Guo et al., 2005). AhR is one of the transcription factors that regulates NQO1 expression (Yeager et al., 2009). Our present study demonstrated that *AhR* expression positively modulates *NQO1* expression in lung AD cells. Knockdown of *NQO1* expression abolished 17-AAG-induced cyclins and CDKs reduction in CL1-5 cells in which there was overexpression of AhR. It is quite conceivable that high level of AhR expression results in up-regulation of *NQO1*, which in turn increases the metabolism of 17-AAG to 17AAGH2 for stronger inhibitory effect on Hsp90. This leads to enhanced anticancer effect of 17-AAG in cells with high AhR expression levels (Figure 6).

17-DMAG is a potent and water soluble hsp90 inhibitor, but less dependent on NQO1(Gaspar et al., 2009). However, AhR expression still moderately modulated anticancer activity of 17-DMAG. Similar to 17-AAG, 17-DMAG effectively reduced AhR protein levels, accompanied with reduced mRNA levels of IL-6 and IL-8. AhR not only promoted tumor growth and mediated IL-6 expression in lung cancer cells, but also has been implicated to associate with multidrug resistance phenotype via up-regulating ATP-binding cassette transporter ABCG2 protein in esophageal carcinoma cells (To et al., 2012). Thus, we anticipate that application of 17-AAG and DMAG after chemotherapy may be beneficial to eliminate those AhR up-regulated drug resistant cells.

Knockdown of or interference with AhR expression has been shown to reduce cell

proliferation and arrest cell cycle progression in several kinds of cells. G1 arrest has been observed in AhR-silenced HepG2 and medulloblastoma cells (Abdelrahim et al., 2003), whereas G2 arrest has been observed in AhR-null fibroblasts (Elizondo et al., 2000). In our present study, reduction of AhR expression in lung AD H1355 cells moderately increased the distribution of cells in the G2 phase. Treatment with 250-500 nM 17-AAG further increased the percentage of G2 cells in AhR silenced clones of H1355 cells, but increased the percentage of G1 cells in si-NC H1355 clone. Furthermore, the significant reduction in S cells by 17-AAG was not affected by AhR expression. It appears that 17-AAG has multiple effects on cell cycle progression, and AhR expression mainly modulates the effects of 17-AAG on G1 phase.

AhR is associated with Hsp90, co-chaperone 23 and immunophilin-like protein XAP2 in the cytoplasm (Meyer and Perdew, 1999; Meyer et al., 1998). Hsp90 is important for maintenance of ligand binding conformation, as well as cytosolic localization of AhR (Song and Pollenz, 2002). Geldanamycin specifically binds to the ATP binding pocket located in the N terminus of Hsp90 (Stebbins et al., 1997), which causes dissociation of XAP2 from the mature AhR-Hsp90-p23 complex (Kazlauskas et al., 2001). As XAP2 is critical for maintaining a stable AhR-chaperone complex in the cytoplasm (Kazlauskas et al., 2001; Meyer and Perdew, 1999; Meyer et al., 1998), geldanamycin-induced dissociation of XAP2 protein may result in AhR degradation. Geldanamycin also causes nuclear translocation of AhR without triggering AhR signaling in the nuclei (Kazlauskas et al., 2001). As a derivative of geldanamycin, 17-AAG has similar effects on Hsp90 binding and AhR protein degradation. However, the actual mechanisms of 17-AAG-induced AhR degradation remain to be clarified.

Targeting of Hps90 for cancer therapy is still under investigation. Neckers and Workman (Neckers and Workman, 2012) noted that success will likely lie in treating

cancers that are addicted to particular driver oncogene products that are sensitive

Hsp90 clients. Here we provide evidence that AhR not only serves as a sensitive

Hsp90 client protein, but also enhances the anticancer efficacy of 17-AAG via

up-regulation of *NQO1* in lung AD cells (Figure 6). This novel mechanism provides

information for selection of subtype of patients that be may effectively treated with

17-AAG and will be investigated with the new generation of Hsp90 inhibitors in the

future.

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Authorship Contributions

Participated in research design: Lin, Chang, Chen

Conducted experiments: Chen, Tsai, Shen

Contributed new reagents or analytic tools: Li

Performed data analysis: Lin, Chang, Tsai, Shen, Chen

Wrote or contributed to the writing of the manuscript: Lin, Chang, Chen

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Footnote

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Figure Legends

Figure 1. AhR promotes growth of lung AD H1355 cells via RelA dependent mechanisms. (A) Growth of subcutaneously implanted wild type, si-NC clone and siAhR clones of H1355 cells in null mice. Data are presented as the mean +/- standard deviation (S.D.) of six mice in each group. The insert presents the results of Western immunoblotting. (B) H1355 cells were transiently transfected with AhR expression vector and/or siRelA. The mRNA levels were quantified with real-time RT-PCR at 48 h after transfection. Data are presented as the mean +/- S.D. of three replicates in each group. (C) H1355 cells were transiently transfected with AhR expression vector and/or siRelA. (D) H1355 cells were transiently transfected with AhR expression vector and/or si-IL-6. Cell growth was determined with colony formation assay for 14 days. Data are presented as the mean +/- S.D. of three replicates in each group. *, p < 0.05, compared with si-NC cells.

Figure 2. In H1255 cells, 17-AAG reduces AhR protein levels and AhR-regulated gene expression. H1355 cells were treated with 100, 250 or 500 nM 17-AAG for 24 h. The protein (A) and mRNA (B) levels were determined on Western immunoblotting and real-time RT-PCR, respectively. Data are presented as the mean +/- S.D. of three replicates in each group. *, P < 0.05, compared with DMSO-treated cells.

Figure 3. AhR enhances anticancer activity of 17-AAG in lung AD cells. (A) BEAS-2B, H1355, CL5 and CL1-5 cells were treated with different concentrations of 17-AAG for 48 h. (B) Wild type (WT), si-NC and si-AhR clones of H1355 cells were treated with different concentrations of 17-AAG for 48 h. (C) CL1-5 (TO-AhR) with or without doxycycline treatment was treated with different concentrations of 17-AAG for 48 h. Cell viability was determined on MTT assay at 48 h and AhR

protein was detected on Western immunoblotting at 24 hr. (D) si-NC and siAhR clones (si-AhR-1 and si-AhR-2) were treated with 100 or 500 nM 17-AAG for 24 to 96 h. Cell growth was determined by trypan blue exclusion method. (E) si-NC and si-AhR clones (si-AhR-1 and si-AhR-2) were treated with 500 nM 17-AAG for 96 h. Cell death was detected by measuring LDH activity in conditioned media at 96 h. (F) si-NC and si-AhR clones (si-AhR-1 and si-AhR-2) were treated with 500 nM 17-AAG for 24 h. The cell cycle distribution was determined by flow cytometry. Data are presented as the mean +/- S.D. of three replicates in each group. *, P < 0.05, compared with si-NC cells.

Figure 4. AhR is required for 17-AAG-induced reduction of cell cycle regulators in lung AD cells. (A) si-NC and si-AhR clones cells were treated with 0.01% DMSO, or 250 or 500 nM 17-AAG for 24h. (B) CL1-5 (TO-AhR) cells with or without doxcycline (Dox) pretreatment were treated with 0.01% DMSO, or 0.5 or 1 μM 17-AAG for 24 h. (C) The relative mRNA levels of *NQO1* were quantified in si-NC and si-AhR clones of H1355 and CL1-5 (TO-AhR) cells with or without doxcycline treatment on real-time RT-PCR. (D) CL1-5 (TO-AhR) cells with or without doxcycline (Dox) pretreatment and with or without silencing of *NQO1* were treated with 0.01% DMSO or 0.5 μM 17-AAG for 24 h. Cell cycle regulators, NQO1 and AhR were detected on Western immunoblotting. Each immunoblot was replicated for three different treatments. (E) H1355 cells were transfected with si-NC or si-NQO1 for treated with 0.01% DMSO, 100 nM or 500 nM 17-AAG for 48 h. Cell viability was determined with trypan blue exclusion method. Data are presented as percentage of control from three replicates in each group. *P<0.05, compared with cells transfected with si-NC and treated with the same dose of 17-AAG.

Figure 5. Mutual interaction between AhR expression and 17-DMAG activity. (A)

H1355 cells were treated with different concentrations of 17-AAG and 17-DMAG for 48 h. (B) si-NC and si-AhR clones of H1355 cells were treated with different concentrations of 17-DMAG for 48 h. (C) CL1-5 (TO-AhR) cells with or without doxycycline (Dox) pretreatment were treated with 17-DMAG for 48 h. Cell viability was determined with MTT assay. H1355 cells were treated with 0.01% double distilled water (d_2H_2O), 100 or 500 nM 17-DMAG for 24 hr. (D) Protein and (E) mRNA levels were detected with Western immunoblotting and real-time RT-PCR, respectively. Each assay was replicated for three different treatments. *, $P < 0.05$, compared with 0.01% distilled water-treated cells.

Figure 6. The proposed synergistic mechanisms between AhR expression and 17-AAG on tumor growth inhibition.

Figure 1

Figure 2

(A)

(B)

Figure 3

Figure 4

Figure 5

Figure 6