2-anilino-4-amino-5-aryltriazole-type compound AS7128 inhibits lung cancer growth through decreased iASPP and p53 interaction

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Lung cancer is the leading cause of cancer-related death worldwide. Thus, developing novel therapeutic agents has become critical for lung cancer treatment. In this study, compound AS7128 was selected from a 2-million entry chemical library screening and identified as a candidate drug against non-small cell lung cancer in vitro and in vivo. Further investigation indicated that AS7128 could induce cell apoptosis and cell cycle arrest, especially in the mitosis stage. In addition, we also found that iASPP, an oncogenic protein that functionally inhibits p53, might be associated with AS7128 through mass identification. Further exploration indicated that AS7128 treatment could restore the transactivation ability of p53 and, thus, increase the expressions of its downstream target genes, which are related to cell cycle arrest and apoptosis. This occurs through disruption of the interactions between p53 and iASPP in cells. Taken together, AS7128 could bind to iASPP, disrupt the interaction between iASPP and p53, and result in cell cycle arrest and apoptosis. These findings may provide new insight for using iASPP as a therapeutic target for non-small cell lung cancer treatment.

KEYWORDS
apoptosis, cell cycle, iASPP, lung cancer, p53
INTRODUCTION

Lung cancer remains the leading cause of cancer-related death worldwide. Although people are paying more attention to preventive medicine, an estimated 1.8 million new cases of lung cancer are diagnosed each year, and most of them are non-small cell lung cancer (NSCLC). Although target therapies are improving the clinical outcomes of lung cancer, the occurrence of drug resistance is a critical challenge. Thus, new therapeutics are needed.

In general, cancer occurs due to an alteration of cell division, which is an important process that lets cells grow, replicate their DNA and divide. The cell cycle process is usually controlled by complicated signaling pathways that include many regulators, such as p53. This regulator may protect the genome through its role in maintaining stability by ensuring that replication errors are corrected. As such, malfunctions of p53 may interfere with cell cycle arrest, apoptosis, senescence and autophagy.

Several studies indicate that the activity of p53 is suppressed through binding of murine double minute 2 (MDM2) and inducing p53 proteasome degradation. In addition, a high mutation rate of p53 has been detected in a majority of cancers. The mutant p53 may acquire oncogenic functions through alterations in its DNA binding ability, changes in the associated protein network or interference with its downstream gene expressions. In addition to cell proliferation, p53 is involved in the regulation of the epithelial-mesenchymal transition and cancer stem cell features. Hence, the restoration of wild-type p53 activity could be a path for developing novel anticancer therapeutics.

Emerging evidence shows that iASPP, which is encoded by the PPP1R13L gene, is overexpressed in various cancers. The family of apoptosis-stimulating proteins of p53 (ASPP) consists of ASPP1, ASPP2 and iASPP. The first 2 are considered as common activators of apoptosis-stimulating proteins of p53 (ASPP) consists of ASPP1, ASPP2 and iASPP. The first 2 are considered as common activators of p53, whereas iASPP acts as an inhibitor that can suppress the apoptotic transactivation potential of p53 by direct interaction. ASPP can also inhibit the apoptotic activity of p63 and p73 and promote p53-independent carcinogenesis.

In general, iASPP can be divided into 2 isoforms, which have 407 and 828 amino acids. The shorter one is a nuclear protein, and the longer one is located in both the cytosol and the nucleus. Nuclear iASPP may facilitate tumor progression and correlate with poor clinical outcome in prostate cancer and melanoma. All of the evidence suggests that targeting iASPP may be a feasible way to restore the function of p53. This idea was supported by 2 studies of p53-derived peptides, 37AA and A34, which can disrupt the interaction of iASPP with p73 and p53, respectively.

Recently, a 2-step high-throughput screening of several lung cancer cells with different EGFR status was performed in our laboratory. AS7128, a 2-anilino-4-amino-5-aroylthiazole compound, was one of the candidates selected for its activity against lung cancer cell growth with a reasonable therapeutic window compared with a control. In this study, we explored the potential mechanisms of AS7128 in vitro and in vivo. We present the interesting finding that AS7128 can inhibit lung cancer cell growth through restoring the activity of p53 by targeting iASPP. This may provide a new insight to develop novel therapeutics for NSCLC treatment in the future.

MATERIALS AND METHODS

Cell lines and culture conditions

The human lung cancer cell lines A549 (adenocarcinoma, ATCC CCL-185), H1975 (adenocarcinoma, ATCC CRL-5908), H460 (large cell carcinoma, ATCC HTB-177), H838 (adenocarcinoma, ATCC CRL-5844) and H1299 (adenocarcinoma, ATCC CRL-5803), and the foreskin fibroblast cell line Hs68 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). A549 and Hs68 were grown in DMEM medium (Thermo Fisher Scientific, Rockford, IL, USA), and the other cell lines were grown in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (v/v), penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were grown at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Chemical library

The 2-million screening compound library contains commercial synthetic molecules, known bioactive inhibitors and approved drugs, pure natural products, and proprietary collections. The synthetic molecules in the 2-million library can be clustered into approximately 5300 groups, based on 85% structure similarity. The diversity is approximately 0.87, calculated using a modified centroid-diversity sorting algorithm. Compounds were prepared at 1 mmol/L in 100% DMSO in 1536-well propylene plates.

Xenograft tumor growth in vivo

Experiments were carried out using BALB/c-nu mice, and all of the animal procedures were in accordance with the procedures and...
of guidelines of the Institutional Animal Care and Use Committee. A total of \(3 \times 10^6\) H1975 human lung cancer cells were subcutaneously injected into the flank of 6-week-old BALB/c-nu mice (purchased from BioLABSCO, Yilan, Taiwan). Three days after the inoculation with cancer cells, the animals were divided into 4 groups. Seven days after tumor implantation, both vehicle and 3 doses of AS7128 were suspended in 50% PEG400 (in PBS) and given intraperitoneally to the animals twice a week for 18 days. Body weight and tumor size were measured each time before drug delivery.

The tumor volume was calculated using the largest diameters (a) and smallest diameters (b) as \(V = 0.5 \times ab^2\). In addition, mice sera were collected to examine the levels of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), blood urine nitrogen (BUN) and creatinine (CREA) after the mice were sacrificed. HE stain was purchased from the National Taiwan University College of Medicine Laboratory Animal Center (Taipei, Taiwan). A POD In Situ Cell Death Detection Kit was used to determine the cell apoptosis in the tumor section according to the manufacturer’s protocol (Roche Diagnostics, Indianapolis, IN, USA).

### 2.4 Immunoprecipitation and protein identification

H1975 cells were lysed on ice in 20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 100 mmol/L Na3VO4, 50 mmol/L NaF, 30 mmol/L sodium pyrophosphate and 0.5% NP-40. A 25-fold dilution of a stock solution was treated with a Mini Protease Inhibitor Cocktail Tablet (Roche Diagnostics) dissolved in 2 mL of distilled water, and clarified by centrifugation. The supernatants were taken as the total cell lysates. The compound-associated proteins were immunoprecipitated using AS7128 conjugated magnetic beads (which were synthesized as in Figure S1 and Data S1). The immunoprecipitated proteins were separated by SDS-PAGE, and the proteins were identified in a selected region by mass spectrometry.

The isolated gels were subjected to trypsin digestion and analyzed by mass spectrometry (LTQ-orbitrap XL; Institute of Biological Chemistry, Academia Sinica, Taiwan). The protein identification was processed by Proteome Discoverer software workflow (Thermo Fisher Scientific) using the Mascot search engine against the Swiss-Prot Homo sapiens protein database. Non-specific binding protein were eliminated from control group first. The remaining interactors were mapped using the CRApome database\(^{27}\) and a recent study\(^{28}\) to determine the contaminant frequency of observations across AP-MS; and those frequency more than 15% were also be eliminated as the non-specific binders in this filter step. Then, the confidential interacting proteins were used to enrich their biological process annotations by Gene Ontology (GO) analysis; and we finally selected the potential targets more focusing on those related to apoptosis- and cell-cycle-related proteins (detailed proteins are listed in Tables S1 and S2).

### 2.5 Real-time quantitative RT-PCR

Total RNA was extracted from cells and reverse transcribed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and Random Hexamer primers (Thermo Fisher Scientific) in the presence of an RNase inhibitor according to the manufacturer’s instructions. The detection primers of each gene are shown in Table S3. The reaction signals were detected by SYBR Green reagent (Thermo Fisher Scientific), and TATA-Box Binding Protein (TBP) was used as an internal control (GenBank X54993). The expression level of the detection gene relative to that of TBP was defined as \(-\Delta\Delta C_t = -[C_t\text{ of Gene} - C_t\text{ of TBP}]\), and the ratio was calculated as \(2^{-\Delta\Delta C_t}\). Experiments were performed in duplicate, and no-template controls were included in each assay.

### 2.6 Statistical analysis

The data are presented as the means ± SD or SEM, and the significance of differences was analyzed using Student’s t test. All experiments were performed in triplicate, the statistical testing was 2-tailed, and \(P < .05\) was considered statistically significant.

The details of other methods are listed in Data S1.

### 3 RESULTS

#### 3.1 Identification of AS7128 that possesses non-small cell lung cancer inhibitory activities

Through high-throughput screening, we identified the 2-anilino-4-amino-5-arylthiazole-type compound AS7128, which has the chemical structure shown in Figure 1A. AS7128 could inhibit the viabilities of several lung cancer cells with IC\(_{50}\) values of 0.1-0.3 μmol/L. Furthermore, it has 10 times higher potency for cancer cells than normal cells (Figure 1B). This suggests that AS7128 has potential for lung cancer treatment. As such, we further investigated its antitumor efficacy in vivo.

Athymic nude mice bearing subcutaneous H1975 tumors were intraperitoneally treated with DMSO (as a control) or 0.5, 1 and 3 mg/kg of AS7128 twice a week for 18 days. The body weight and tumor volume were monitored for each treatment period. The results showed that treatment with AS7128 significantly inhibited H1975 xenograft tumor growth compared with the control without altering the body weight between the 4 groups (\(n = 4\) for each group; average tumor size, 1625.4 mm\(^3\) for 0.5 mg/kg, 288.9 mm\(^3\) for 3 mg/kg on day 24; \(P = .014\) for 1 mg/kg and 0.018 for 3 mg/kg, both compared with the DMSO control; Figure 1C, D).

HE staining and TUNEL assay analysis revealed that AS7128 inhibits tumor growth and induces apoptosis in the tumor region (Figure 1E,F). In addition, mice sera were collected to perform biochemical analyses of GOT, GPT, BUN and CREA to evaluate cytotoxicity of AS7128 (with focus on the effects on the liver and kidney). The results showed that there was no statistical difference between the control and the 3 treated groups (Figure S2). These results suggest that AS7128 could inhibit tumor growth in vitro and in vivo.
FIGURE 1  Tumor growth inhibition by AS7128 in vitro and in vivo. A, Chemical structure of AS7128. B, The cell viability of different lung cancer cell lines against AS7128 was determined by SRB assay after 72 h of treatment. Hs68: normal fibroblast. Experiments were performed in triplicate. C, D, Nude mice were subcutaneously injected with $3 \times 10^6$ H1975 cells. Mice were treated with DMSO, 0.5, 1 or 3 mg/kg of AS7128 intraperitoneally twice a week for 18 d after 7 d of tumor implantation. Mice tumor volume (C) and body weight (D) were monitored twice a week. The data are presented as the mean ± SEM and were analyzed using Student’s t-test. Asterisks represent statistically significant differences (*P < .05). E, Tumor photographs after sacrifice (upper panel). Scale: 1 cm. Tissue morphology was examined by HE staining (lower panels). Scale: 50 μm. F, Cell apoptosis status was examined by TUNEL staining. Scale: 50 μm

A549
H460
H838
H1975
H1299
Hs68
FIGURE 2  Induction of apoptosis and cell cycle arrest by AS7128 in H1975 cells. H1975 cells were treated with 250 nmol/L of AS7128 for the indicated time. Apoptosis and cell cycle status were determined by the following experiments. A, Cleaved Caspase 3 and PARP were detected by immunoblotting. β-actin was used as the loading control. B, Cells were harvested for annexin V-PI double staining and evaluated for cell apoptosis by flow cytometry. The bar graphs reveal the apoptotic percentage of H1975 cells with AS7128 treatment. C, AS7128-treated cells were stained with PI to analyze the DNA content by flow cytometry. D, Expressions of indicated proteins of H1975 cells were detected by immunoblotting. GAPDH was used as the loading control. The densitometry value was measured by ImageJ. The data are presented as the mean ± SD. Experiments were performed in triplicate.
3.2 | AS7128 induced cell apoptosis and cell cycle arrest in H1975 cells

To understand the action mechanism of AS7128, we investigated whether the cytotoxic effect was due to cell apoptosis. The immunoblotting showed that cleavage caspase-3 and PARP, 2 important apoptotic markers, appeared after H1975 cells were treated with AS7128 (Figure 2A). In addition, the flow cytometry examination indicated that the positive staining population of both annexin V and PI increased in AS7128-treated H1975 cells (29.55% of treated cells vs 2.35% of the control group and 15.80% of treated cells vs 2.94% of the control group in early and late apoptosis, respectively, at 72 hour; Figure 2B). The data suggest that AS7128 treatment causes apoptosis in lung cancer cells.

We next explored whether the apoptotic effect of AS7128 treatment was derived from unregulated cell proliferation. The results indicated that the cell population of AS7128-treated H1975 cells was significantly decreased in the G1 stage and increased in the G2/M stages in asynchronous conditions (Figure 2C). The compound would, thus, induce a sub-G1 population of H1975 cells.

We also examined the expression levels of several cell cycle checkpoints, including cyclin D1, E, A and B1 and phospho-histone H3. As shown in Figure 2D, the expressions of cyclin B1 and serine 10 in phospho-histone H3 were increased after H1975 cells were treated with AS7128. Collectively, the data suggest that treatment with AS7128 might induce cell apoptosis through cell cycle G2/M arrest in H1975 cells.

3.3 | AS7128 arrested H1975 cell in M phase

To examine the events of AS7128-induced cell cycle arrest, H1975 cells were synchronized at the G1/S transition by double thymidine block and subsequently released into fresh medium containing DMSO or 250 nmol/L of AS7128 for 12 hour. The cell cycle statuses were monitored each hour by measuring the DNA contents with PI staining. As expected, cells treated with AS7128 were arrested in the G2/M phases, but most of the control cells re-entered the G1 phase at 12 hour after release (Figure 3A).

We reperformed the same experiment and extended the releasing time to 18 hour to clarify whether the arrested H1975 cells could pass the G2/M phases after releasing for 12 hour under AS7128 treatment. The results showed that most of the H1975 cells were still arrested in the G2/M phases after 18 hour of release with AS7128 treatment (Figure S3). This result was further confirmed by examining the protein expression levels of different cell cycle checkpoints. As shown in Figure 3B, the expressions of cyclin B1 and serine 10 in p-histone H3 (2 specific G2 and M phase markers) were significantly increased in treated cell lysates compared with the control.

Next, we examined how AS7128 treatment induced G2/M arrest in cells. The cyclin B-cdc2 complex is required for the G2/M transition. Activation of the cyclin B-cdc2 complex may trigger cells to enter the M phase. This activation comprises several steps, including cdc2 dephosphorylation at Thr14 and Tyr15 and phosphorylation at Thr161.29 To investigate whether the AS7128 treatment could interfere with the steps of G2/M transition, H1975 cells were synchronized at the G1/S stage by double thymidine block and released into fresh medium with or without AS7128. Protein extracts were collected to analyze the phosphorylation levels of cdc2 at Tyr15 and Thr161at each indicated time point.

Surprisingly, both the AS7128-treated and DMSO control groups presented similar patterns of these 2 phosphorylation statuses (Figure 3C). This implied that AS7128 treatment may cause incomplete M phase progression in H1975 cells. To test our hypothesis, we focused on the effect of AS7128 treatment during the mitosis processes. Similarly, H1975 cells were synchronized and released with or without AS7128 treatment. Cells were fixed and stained with DAPI and then cells with different chromosome arrangements were counted in at least 12 fields to analyze the percentage of each mitotic stage, including interphase, prophase, and after prometaphase.

The criteria for the 3 phases are shown below the fluorescence image, and the results are presented as histograms (Figure 3D). Compared with the DMSO group, the percentage of prophase cells was significantly higher in the AS7128-treated group in a time-dependent manner. Taken together, the data suggest that treatment with AS7128 may lead to the arrest of H1975 cells in the prophase mitotic stage.

3.4 | iASPP might be the action target of AS7128

Because AS7128 treatment could cause cell cycle arrest in the prophase and induce cell apoptosis, we investigated its mechanism of action. AS7128 was conjugated into magnetic beads (Figure 4A) and used to identify potential targets in H1975 lung cancer cells. The pull-down samples were separated by 2 SDS-PAGEs with different concentrations of acrylamide. Seven regions with significantly different protein expressions were selected for protein identification by LC/MS-MS (Figure S4A).

A total of 527 proteins were identified, of which 390 showed significantly differential expressions in the AS7128-treated group after filtering out non-specific binding proteins with over 15%
frequency according to Marcon and Mellacheruvu’s research.²⁷,²⁸ In addition, GO enrichment analysis revealed that 36 of these proteins were involved in cell apoptosis or cell cycle regulation (Figure S4B and Table S2). Among them, we chose the 2 proteins iASPP and CSE1L for further validation, which are related to p53-induced cell apoptosis. The results showed that iASPP but not CSE1L could be co-immunoprecipitated with AS7128 conjugated magnetic beads (Figure 4B and Figure S4C; NIH-3T3 was loaded as an antibody positive control). This implied that iASPP might be a potential target for AS7128 treatment in lung cancer.

3.5 AS7128 could restore p53 function through disruption of the interaction between p53 and iASPP

A previous study indicated that the expression of iASPP could inhibit cell apoptosis through the binding of p53.³⁰ We wondered whether AS7128-induced cell apoptosis would result in interruption of the interaction between iASPP and p53. To test this hypothesis, the interaction between iASPP and p53 was detected by co-immunoprecipitation assays with or without AS7128 treated with H460 cells. The results showed that the binding of iASPP and p53 was inhibited under AS7128 treatment (Figure 4C) and also in p53 mutated H1975 cells (Figure S5).

We also examined whether the transactivation activity of p53 could be restored when AS7128 disrupted the iASPP-p53 interaction. As expected, the transactivation activity of p53 could be restored and presented nearly a 1.6-fold increase in the reporter assay (Figure 4D). In addition, the mRNA and protein expression levels of p53 downstream genes were significantly upregulated after treatment with AS7128 for 24 and 48 hour (Figures 4E,F and Figure S6). This treatment also induced cell apoptosis via PARP cleavage (Figure 4G) in both A549 and H460 cells. The data suggest that AS7128 could bind to iASPP, disrupt the interaction between iASPP and p53, and result in cell cycle arrest and apoptosis through restoring the transactivation activity of p53 and turning on the expressions of its downstream target genes in lung cancer cells (Figure 5).

4 DISCUSSION

Despite treatment advances made over the past 20 years, the prognosis of lung cancer remains poor.³¹,³² Thus, new anticancer drugs are being developed to target certain molecular pathways, such as epidermal growth factor receptor (EGFR) or p53 signaling. We identified a small molecule, AS7128, that could inhibit lung cancer cell growth in vitro and in vivo. Treatment with AS7128 led to cell cycle arrest in the mitosis stage and induced cell apoptosis. AS7128 could also be associated with the oncogenic protein iASPP and restore the transactivation ability of p53. This increases the expressions of downstream target genes, which are related to cell cycle arrest and apoptosis, through disruption of the interaction between p53 and iASPP in cells. AS7128 may, therefore, serve as a novel strategy for lung cancer treatment.

AS7128 is a type of 2-acrylamino-4-amino-5-aroylthiazole compound, and has been reported as a new class of inhibitors of tubulin polymerization.³³ Romagnoli et al. report that the inhibition of tubulin polymerization of 2-anilino-4-amino-5-aroylthiazoles might occur through binding to the colchicine site. For this reason, we tested whether AS7128 treatment could interfere with the dynamics of microtubules in our studies. As expected, AS7128 could cause tubulin depolymerization and delay microtubule regrowth after cold treatment in lung cancer cells (Figure S7). However, it is very interesting that our protein identification indicated that candidates other than tubulin could be potential targets for AS7128. Using AS7128-conjugated beads and mass spectrometry identification, we found that iASPP was presented in the pull-down complex of AS7128. Previous studies showed that iASPP is overexpressed in NSCLC, gastric cancer and cervical cancer, and it is correlated with pathological stages. Downregulation of iASPP could inhibit cell proliferation, invasion and migration, and it induced cell apoptosis.¹⁴,³⁴⁻³⁶ Moreover, iASPP could suppress both p53-dependent and independent apoptotic activity through interaction with p53, p63 or p73.¹⁸⁻²⁰ Thus, iASPP could be a suitable drug target for lung cancer therapy.

P53 is critical for regulating the cell cycle and apoptosis, and it is also known as a classical tumor suppressor, with over 50% human cancers carrying loss-of-function mutations.²⁷ As such, reactivation of p53 is one direction for the development of new therapeutic strategies for cancer treatment. Some approaches have been used to reactivate wild-type (WT) or mutant (Mt) p53. Virus-based WT p53 gene therapy provides functional p53 and induces cell apoptosis to enhance anticancer activity.²⁸,²⁹ Moreover, some small molecules have been reported to restore the function of WT p53 by blocking the interaction between MDM2/MDM4 and p53, inhibiting the E3 ligase activity of MDM2, blocking SIRT to increase the stability of p53, or inhibiting the nuclear export of p53.³⁰⁻³¹ Some of these molecules are being clinically evaluated.

We demonstrated that AS7128 could restore the transactivity of p53 through disrupting the interaction between p53 and its inhibitor,
In comparison with virus-based p53 injection, using small compounds would be more convenient and economical. Although the activation of p53 is required in anticancer treatment, p53-mediated apoptosis may be harmful in normal tissues. For example, hypoxia could induce ischemia. Moreover, most p53 inhibitors like MDM2 or iASPP are usually overexpressed and act as an oncogene in cancers. Therefore, regulating p53 activity by targeting its inhibitors may be safer than using p53 itself. Thus, developing novel drugs that target iASPP might be a good option for restoring the function of p53 in cancer treatment.

The small peptides 37AA and A34 derived from p53 can increase the expression of p53 downstream genes, dissociate p73 or p53 from iASPP, and suppress tumor growth in vitro and in vivo. Silencing the expressions of iASPP could not only inhibit cell proliferation in vitro and tumor growth in vivo, but also upregulate the mRNA expression levels of p21 and PUMA. When we used siRNA to mimic the effect of AS7128 on targeting iASPP, we found that the mRNA and protein levels of BAX and 14-3-3 sigma were also upregulated in iASPP-silenced A549 and H460 cells (Figure S8). This suggests that iASPP is, indeed, a potential target for inhibiting lung cancer cell growth. Furthermore, miR-124 and miR-140 could regulate the expression levels of iASPP, as well as inhibit tumor growth in cervical cancer and glioma, respectively. We showed that treatment with AS7128 inhibited tumor growth in vitro and in vivo, as well as upregulated the expression levels of p53 downstream genes by disrupting the interaction between p53 and iASPP. Using a small molecule is a better way to reduce the costs of synthesis and storage and increase the delivery efficiency in clinical applications.

In conclusion, we identified the compound AS7128, which could bind to the oncoprotein iASPP, disrupt the interaction between iASPP and p53, and restore the function of p53 by activating the expression of p53 downstream genes and inducing cell apoptosis. Because p53 is an important tumor suppressor gene, reactivation of p53 function is proposed as an ideal approach for lung cancer therapy. The identification of AS7128 as an anti-lung cancer small molecule through reactivating p53 by targeting iASPP may provide new insight for further development of lung cancer treatments.

**ACKNOWLEDGMENTS**

The authors thank the following individuals for providing technical support: Dr Yih-Leong Chang and Dr Chen-Tu Wu (Department of Pathology and Graduate Institute of Pathology, College of Medicine, National Taiwan University).

**CONFLICT OF INTEREST**

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Cheng H-W, Chein R-J, Cheng T-J, et al. 2-anilino-4-amino-5-aryltiazole-type compound AS7128 inhibits lung cancer growth through decreased iASPP and p53 interaction. Cancer Sci. 2018;109:832–842. https://doi.org/10.1111/cas.13489