

Profile of Apoptotic Proteins after Curcumin Treatment by Antibody Array in H69AR Lung Cancer Cells

Süleyman İlhan¹ 

¹Manisa Celal Bayar University, Faculty of Science and Letters, Department of Biology, Manisa, Turkey

ORCID IDs of the authors: S.I. 0000-0002-6584-3979

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ABSTRACT

Objective: The aim of the study was to investigate the changes in the expression levels of apoptosis-related proteins after treatment with curcumin (Cur) on multiple drug-resistant H69AR non-small cell lung cancer cells.

Materials and Methods: Viability of H69AR cells after Cur exposure (5-100 µg/mL) was evaluated via MTT assay at 24, 48 and 72 h. Apoptosis was assessed via ELISA assay. Apoptosis related proteins of breast cancer cell lines were analyzed by a Human Apoptosis Antibody Array. Protein-protein interactions were analyzed and visualized by using the STRING database.

Results: Cur inhibited cell viability and induced apoptosis in H69AR cells. The IC50 value of Cur in H69AR cells was 8.75 µg/mL. The array results showed that the protein levels of pro-apoptotic proteins such as Bad, Bax, Caspase-3, TRAIL R1, TRAIL R2, FADD, Fas, SMAC/DIABLO, HMOX2 were significantly increased by 2.4-, 3.1-, 2.6-, 3.1-, 3.4-, 2.4-, 2.1-, 4.1- and 5.5-fold in H69AR cells ($p < 0.05$). Moreover, the protein levels of the anti-apoptotic proteins such as Bcl-2, cIAP-1, CLU and HIF1A were significantly decreased by 4.1-, 3.2-, 2.2- and 2.0-fold, respectively in H69AR cells by Cur exposure ($p < 0.05$).

Conclusion: Findings of this study suggested that Cur induced apoptosis of human H69AR cells via mediating several proteins involved in both extrinsic and intrinsic apoptotic pathways.

Keywords: Curcumin, apoptosis, protein array, protein-protein interactions

INTRODUCTION

Cancer is one of the major public health problems globally. Lung cancer is the most common cancer type and a leading cause of cancer-related deaths. Despite advances in lung cancer therapy, the mortality rate of the disease is extremely high and the 5-year survival rate is approximately 16%. (1). Non-small cell lung cancer (NSCLC) is the most common type, which composes of nearly 85% of all cases. Toxic effects and various side effects limit the efficiency of standard therapy for lung cancer (2). Moreover, drug resistance that leads to tumor recurrence and disease progression is the major problem for therapeutic failure (3). Therefore, there is an urgent need for novel alternative agents to inhibit proliferation and to induce apoptosis of multiple drug-resistant (MDR) lung cancer cells.

Natural plant products are a valuable source of novel bio-active compounds having potential cytotoxic activities. Curcumin (Cur) is a polyphenolic compound originated from the plant *Curcuma longa* L. called turmeric (4) and was extracted from the rhizomes of this plant in a pure crystalline form in 1870 (5,6). Extensive research in the past 50 years has revealed several biological functions of Cur such as anti-proliferative, anti-inflammatory, and antioxidant properties. Recently, Cur has attracted great attention for its anticancer properties on breast, lung, prostate, and brain tumors (7). It is also known that Cur is a potent inducer of apoptotic cell death in several cancer types (8-11).

There are many studies investigating the possible apoptotic effects of Cur on various human cancer cells (7-11), but there is no study on MDR H69AR small cell lung cancer cells. In the current study, potential cyto-



Corresponding Author: Süleyman İlhan

E-mail: suleyman.ilhan@cbu.edu.tr

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toxic and apoptotic effects of Cur were investigated on MDR H69AR human lung cancer cells. Moreover, the dominant signaling cascades and apoptotic players by Cur-induced cytotoxicity were screened by antibody array analysis.

MATERIALS AND METHODS

Cells and Culture

The human H69AR lung cancer cells utilized in the experiments were obtained from the American Type Culture Collection (ATCC, CRL-11351). H69AR cells were maintained in RPMI 1640 medium (Sigma-Aldrich, UK) with stable L-glutamine (1%) (Sigma-Aldrich, UK), fetal bovine serum (20%) (Sigma-Aldrich, UK) and, streptomycin (1%) (Sigma-Aldrich, UK) in 75 cm² flasks (Cellstar, UK). Cells were incubated in a 37 °C incubator during the experiments.

Curcumin Treatment

Cur (Sigma-Aldrich, UK) was prepared as 1 mg/mL ethanol (EtOH) stock solution and stored for experiments at 4 °C. The EtOH concentration in all experimental wells was lower than 0.1% and was not toxic to the cells (Figure 1A-C).

MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, UK) was utilized to calculate the viability of cells after Cur exposure. Cells (10⁴/well) were propagated onto 96-well plates in a 200 µL medium. After 24, 48 and 72 h of exposure with increasing concentrations of Cur (5-100 µg/mL), 20 µL MTT was added to each well and incubated for an additional 4 h at 37°C. After the incubation period, wells were drained and existent crystals were dissolved by DMSO (Sigma-Aldrich, UK). Conversion of MTT into formazan by mitochondrial succinate dehydrogenase and other oxidoreductases was detected via spectrophotometer (570 nm, Tecan Infinite 200 PRO, Switzerland) (12). The half-maximal inhibitory concentration (IC₅₀) was evaluated via Graphpad 5.0 software.

Detection of Apoptosis via ELISA Assay

Apoptosis was determined through the Cell Death Detection kit (Sigma Aldrich, UK). This ELISA kit detects DNA fragments in the cytoplasm of apoptotic cells. For this experiment, cells were exposed to Cur (5-100 µg/mL) for 24, 48 and 72 h. After each incubation period, cells were lysed and cytoplasmic fractions were

obtained. Then, 20 µL of the cytoplasmic fractions were put into a streptavidin-coated 96-well. The mixture of “anti-DNA” and “anti-histone” was added to all wells and additionally incubated for 2 h. After washing the plates, diammonium salt was added and optical densities were measured at 405 nm and 490 nm (Tecan Infinite 200 PRO, Switzerland). The fold changes in each treated well were determined as compared to untreated controls.

Protein Array

Apoptosis related proteins of H69AR cells were analyzed by an Apoptosis Antibody Array (R&D Systems, USA). The principle of the array method involves a sandwich immunoassay, which is membrane-based (13). To optimize the exposure time, which allows us the detection of all protein spots, different exposure times were tested for each membrane ranging from 15 s to 15 min. Finally, it was optimized to use for 5 min. The proteins were detected by chemiluminescence methods involving an incubation step with an antibody (biotinylated) for 1 h and, with streptavidin (HP-conjugated) for 1 h. Chemiluminescence imaging was conducted via UVP Biolmaging Systems. Kodaarray® 2.6 software was used to quantitate the pixel intensity in each spot (14).

Analysis of Protein-Protein Interaction

Protein-protein interactions were analyzed and visualized by using the STRING database. (version 11.0; <http://string.embl.de>) (15).

Statistical Analysis

All statistical analyses were done by using Graphpad Prism 5.0 software (USA). For significant values, a one-way analysis of variance test (ANOVA) was utilized (16). Statistically significant data were those with a p value ≤0.05.

RESULTS

Cur Inhibits the Viability of H69AR Human Lung Cancer Cells

The effect of Cur on the viability of H69AR cells was assessed in a concentration- and time-dependent manner. As shown in Figure 1(A-C), the viability of H69AR cells was reduced at 24, 48 and 72 h. Reduction in the viability was also concentration-dependent at all tested time points. There were 1%, 73%, and 96% reductions in the viability of H69AR cells exposed to 5, 25, and 100 µg/mL Cur respectively, as compared to control cells at 24 h (p<0.05). There were 2%, 88%, and 97% reductions in the viability of H69AR cells exposed to 5, 25 and 100 µg/mL Cur respectively, as compared to control cells at

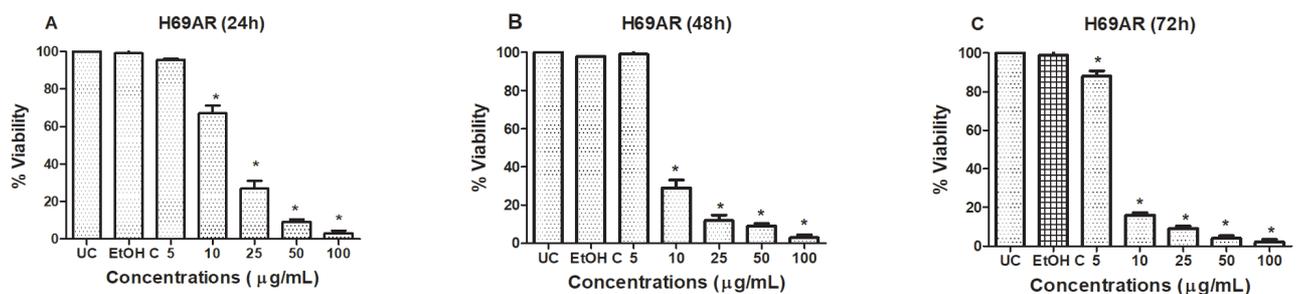


Figure 1. Concentration- and time-dependent inhibition of the viability of H69AR lung cancer cells by Cur. Cell viability was determined by the MTT assay. The results are the mean of 3 different experiments (±SD) (*p<0.05 compared to untreated cells) (UC: Untreated control, EtOH C: Ethanol control).

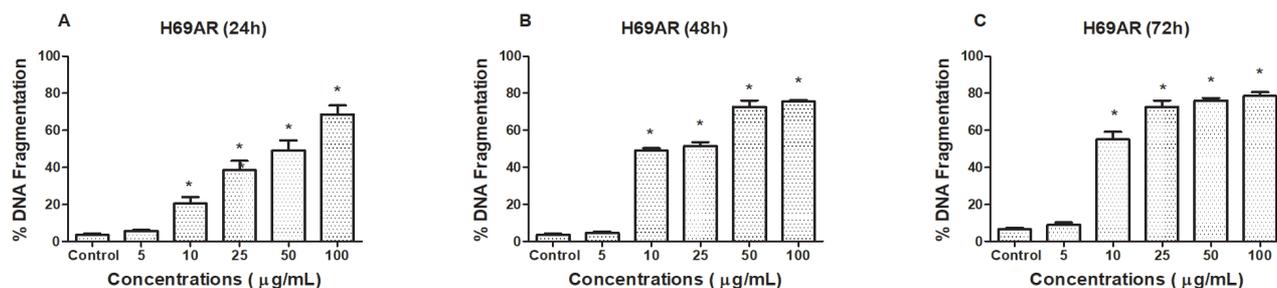


Figure 2. Induction of % of DNA fragmentation in H69AR cells in response to Cur treatment at different time points. (* $p < 0.05$ compared to untreated cells).

48 h ($p < 0.05$). The most effective cytotoxic time point was accepted as 48 h and IC_{50} value of Cur in H69AR cells was 8.75 µg/mL.

Induction of Apoptotic Cell Death by Cur in H69AR Cells

To assess the induction of apoptosis after exposure to Cur in H69AR cells, increasing concentrations of Cur were applied for 24h, 48 h and 72 h and then apoptotic cell death was evaluated. As shown in Figure 2, Cur exposure increased the DNA fragments concentration dependently in H69AR cells ($p < 0.05$).

Changes in Apoptotic Proteins by Cur in H69AR Cells

Differences in the expression of apoptosis-related proteins were profiled by using apoptosis array in H69AR cells. Table 1 shows the changes in the expression of apoptotic proteins in H69AR cells after Cur exposure. The results revealed that pro-apoptotic proteins such as Bad, Bax, Caspase-3, TRAIL R1, TRAIL R2, FADD, Fas, SMAC/DIABLO, HMOX2 were significantly increased by 2.4-, 3.1-, 2.6-, 3.1-, 3.4-, 2.4-, 2.1-, 4.1- and 5.5-fold in H69AR cells, respectively, as compared to control cells at 48 h ($p < 0.05$). The anti-apoptotic proteins such as Bcl-2, cIAP-1, CLU and HIF1A were significantly decreased by 4.1-, 3.2-, 2.2- and 2.0-fold in H69AR cells, respectively, at 48 h ($p < 0.05$).

Protein-Protein Interaction

To analyze protein-protein interactions, the STRING database was utilized. Figure 3 indicates the protein-protein interaction generated by STRING with an average local clustering coefficient of 0.491.

DISCUSSION

Curcumin, the active component of *C. longa*, has been used traditionally for centuries to treat a variety of impairments and studied on a large scale for its anti-proliferative, anti-inflammatory, and anti-oxidant effects throughout the past few decades (17-19). It has been shown in many studies that Cur has potent antiproliferative effects and exerts activity through multiple signaling mechanisms (20-22). However, in the literature, there is no study investigating the effect of Cur on H69AR small cell lung cancer cells. The H69AR cells are resistant to many chemotherapeutic drugs such as adriamycin, epirubicin, daunomycin, mitoxantrone, etoposide, vincristine and vinblastine. Therefore, H69AR is known as a multi-drug resistant lung cancer cell line (23). The

results revealed that Cur inhibited the viability of H69AR cells in a concentration-dependent manner. In a study by Li et al., similar results were obtained by human lung carcinoma A549 cells (24). Cur exposure significantly inhibited A549 cell proliferation and also induced apoptosis concentration dependently. Yang et

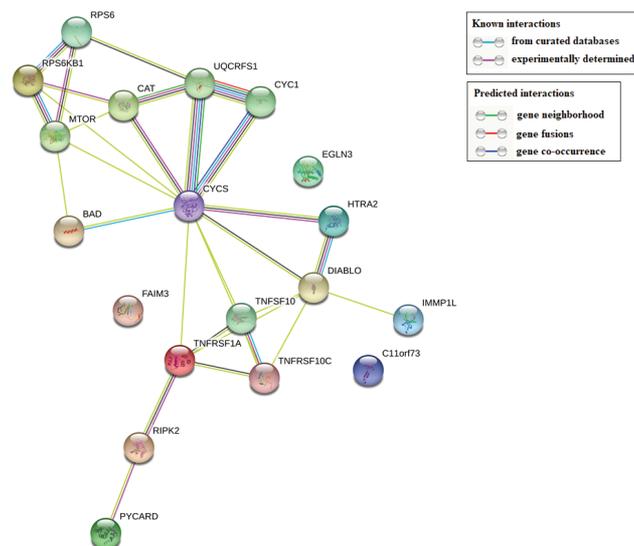


Figure 3. The protein-protein interactions map of the identified proteins. STRING database, version 11 (<http://string.embl.de>) was used to determine the protein-protein interactions of the proteins identified by antibody array analysis (RPS6: 40S Ribosomal Protein S6; RPS6KB1: Ribosomal Protein S6 Kinase B1; MTOR: Mechanistic Target of Rapamycin Kinase; UQCRCF1: Ubiquinol-Cytochrome C Reductase, Rieske Iron-Sulfur Polypeptide 1, CYC1: Cytochrome C1, CYCS: Cytochrome C, EGN3: Egl-9 Family Hypoxia Inducible Factor 3, FAIM3: Fas Apoptotic Inhibitory Molecule 3, HTRA2: HtrA Serine Peptidase 2, DIABLO: Diablo IAP-Binding Mitochondrial Protein, TNFSF10: TNF Superfamily Member 10, TNFRSF1A: TNF Receptor Superfamily Member 1A, TNFRSF10C: TNF Receptor Superfamily Member 10C, RIPK2: Receptor Interacting Serine/Threonine Kinase 2, PYCARD: Caspase Recruitment Domain-Containing Protein 5, IMMP1L: Inner Mitochondrial Membrane Peptidase Subunit 1, C11orf73: Heat Shock Protein Nuclear Import Factor Hikeshi, CLU1: Clusterin Like 1, RFW2: COP1 E3 Ubiquitin Ligase).

Table 1. Changes in apoptosis related proteins in H69AR cells after exposure to curcumin (8.75 µg/mL) for 48 h. The results are the mean of two independent experiments (±SD) (ND: not detectable, Ns: non-significant changes).

H69AR			
Symbol	Protein name	Up/down-regulation	Fold change
Bad	BCL2 Associated Agonist Of Cell Death	↑	2.4±0.2
Bax	BCL2 Associated X Protein	↑	3.1±2.8
Bcl-2	BCL2 Apoptosis Regulator	↓	4.1±2.4
Bcl-x	Bcl-2-Like Protein 1	↔	Ns
Pro-Caspase-3	Pro- apoptosis-Related Cysteine Peptidase	↓	2.0±0.8
Cleaved Caspase-3	Cleaved apoptosis-Related Cysteine Peptidase	↑	2.6±1.2
Cat	Catalase	↑	2.1±2.3
cIAP-1	Apoptosis Inhibitor 1	↓	3.2±0.2
cIAP-2	Apoptosis Inhibitor 2	↔	Ns
CLSPN	Claspin	↔	Ns
CLU	Clusterin	↓	2.2±3.0
CYCS	Cytochrome c	↑	4.2±2.8
TRAIL R1/DR4	Death receptor 4	↑	3.1±2.6
TRAIL R2/DR5	Death receptor 5	↑	3.4±0.8
FADD	Fas Associated via Death Domain	↑	2.4±2.2
Fas/TNFRSF6/CD95	Fas Cell Surface Death Receptor	↑	2.1±0.4
HIF-1A	Hypoxia Inducible Factor 1 Subunit Alpha	↓	2.0±0.2
HO-1/HMOX1/HSP32	Heme Oxygenase 1	↑	2.5±0.8
HO-2/HMOX2	Heme Oxygenase 2	↑	5.2±3.8
HSP27	Heat Shock 27 KDa Protein 1	↑	2.8±0.4
HSP60	Heat Shock 60kDa Protein 1	↔	Ns
HSP70	Heat Shock 70 KDa Protein 4	↑	2.2±0.4
HTRA2/Omi	HtrA Serine Peptidase 2	↑	3.6±2.6
Livin	Baculoviral IAP Repeat Containing 7	↔	Ns
PON2	Paraoxonase 2	↔	Ns
p21/CIP1/CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	↔	ND
p27/Kip1	Cyclin Dependent Kinase Inhibitor 1B	↔	ND
Phospho-p53 (S15)	Tumor Protein P53 (Phospho- Ser15)	↑	3.2±1.2
Phospho-p53 (S46)	Tumor Protein P53 (Phospho- Ser46)	↑	3.6±2.4
Phospho-p53 (S392)	Tumor Protein P53 (Phospho- Ser392)	↑	2.8±1.2
Phospho-Rad17 (S635)	Cell Cycle Checkpoint Protein RAD17 (Phospho-Ser635)	↔	ND
SMAC/Diablo	Diablo IAP-Binding Mitochondrial Protein	↑	4.1±0.8
Survivin	Baculoviral IAP Repeat Containing 5	↔	ND
TNF RI/TNFRSF1A	TNF Receptor Superfamily Member 1A	↔	Ns
XIAP	X-Linked Inhibitor of Apoptosis	↔	Ns

al. also investigated the cytotoxic effect of curcumin on NCI-H446 cells and determined the IC₅₀ value as 15 µM (25). In a study by Jin et al., the apoptotic effect of Cur was investigated on A549 cells at 10-40 µM concentrations at 24 h and showed the apoptotic cell death ascended by the increasing concentrations, as compared to the control cells (26). Further, in the current study, apoptotic effect of Cur was also evaluated in H69AR cells by flow cytometer and found that Cur induced apoptosis at 48 h.

To explore the potential molecular mechanisms responsible for the apoptotic activity, an antibody protein array that provides

the simultaneous evaluation of 35 apoptosis-associated proteins was used. Apoptosis is a programmed cell death characterized by chromatin condensation, phosphatidylserine externalization, DNA fragmentation and apoptotic bodies which can be activated via internal or external signals (27). Apoptosis that has been initiated by external signals such as tumor necrosis factor receptor (TNF), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL) and Fas cell surface death receptor (Fas) is called the extrinsic apoptotic pathway. However, the intrinsic (mitochondrial) apoptotic pathway is initiated internally and includes Bcl-2 family proteins (27-29). The Bcl-2 family proteins containing both

pro-apoptotic and anti-apoptotic members are the main regulators of intrinsic apoptosis. Pro-apoptotic proteins such as Bad and Bak, which are essential for mitochondrial permeabilization, are activators of apoptosis whereas anti-apoptotic members such as Bcl-2, Bcl-xL and Mcl-1 inhibit apoptotic cell death (28). Results revealed an increase in the pro-apoptotic proteins belonging to the intrinsic apoptotic pathways such as Bad, Bax and a decrease in Bcl-2. Zhu et al. showed that protein expression of Bax was induced, while Bcl-2 was reduced indicating the induction of apoptosis by the high dosing groups of Cur in pancreatic cancer cells (30). Yang et al., also demonstrated changes in Bax, Bcl-2 and Bcl-xL proteins in NCI-H446 cells by 15 μ M Cur for 48 h (25). Cytochrome c release from the mitochondria is essential for the activation of caspase-9 and as a result, activation of apoptosis (27). In the same study, authors also stated that Cur induced cytochrome c, caspase-9 and caspase-3 levels. In the current study, array results support the findings of Yang et al. in the case of induction in cytochrome c release and increase in caspase-3 levels (25). However, the array results of the current study do not support some of the findings of Yang et al. They reported that Cur did not cause any change in the expression of FAS and TRAIL apoptotic receptors and stated that Cur did not induce death receptor-mediated pathways (25). In the current study, Cur induced the main death receptors (TRAIL R1, TRAIL R2, FADD and Fas) indicating the induction of death receptor-mediated apoptotic pathway in H69AR cells. Reactive oxygen species (ROS) and mitochondria both play a vital role in the activation of apoptosis. Cytochrome c release is known to be mainly regulated via ROS (29). In many studies, the induction of the ROS-mediated mitochondrial pathway in Cur-treated cells has been demonstrated (31,32). Results of Kuttikrishnan et al. revealed the activation of the intrinsic apoptotic pathway via the generation of ROS in acute lymphoblastic leukemia cells by Cur treatment (33). Similarly, according to array results, Cur induced the proteins involved in ROS-mediated apoptotic pathways such as HIF-1A, HSP27 and HSP70. Here, changes in apoptotic proteins by Cur treatment were investigated by using a protein array method, but this needs to be verified by other methods such as western blotting or qPCR.

To investigate the possible protein-protein interactions of the proteins obtained from array data, bioinformatics STRING analysis was carried out. Combining the protein array data with protein interactome data allows us to investigate the network of proteins that Cur is probably interacting with. The network interaction map revealed complex protein-protein interactions that include direct and indirect functional protein connections. Of these proteins, Ribosomal Protein S6 (RPS6) is a cytoplasmic ribosomal protein and one of the components of the 40S ribosomal subunit. It is the downstream substrate of PI3K/Akt/mTOR/p70S6 kinase pathways and is phosphorylated by several kinases such as Ribosomal Protein S6 Kinase B1 (RPS6KB1) (34). In the literature, it has been shown that Cur inhibits the mTOR pathway via decreasing the phosphorylation of RPS6 leading to blocking the proliferation of human colorectal cancer cells, malignant glioma cells and intestinal epithelial cells (35-37). According to the map, Cur could induce apoptosis in H69AR cells by inhibiting mTOR pathway and phosphorylation of RPS6.

The interaction map also revealed the interaction of TNFSF10 (TRAIL) with Receptor-interacting serine threonine kinase 2 (RIPK2) which is one of the members of the serine/threonine protein kinase family. It acts by binding to the inhibitor of apoptosis proteins (AIPs) such as XIAP, cIAP-1 and cIAP-2. It was shown by the array data that Cur induced both TRAIL R1 and TRAIL R2 and subsequently inhibited cIAP-1, which leads to enhancement of the apoptotic pathway. The STRING data implies the possible interaction of TNFSF10 with RIPK2 and Caspase Recruitment Domain-Containing Protein 5 (PYCARD) after Cur treatment in H69AR cells. PYCARD functions as a key mediator in the activation of the mitochondrial apoptotic pathway and also regulates mitochondrial translocation of BAX and activates initiator caspases, which were found to be increased after Cur treatment in H69AR cells (38).

Although the present findings are notable, there are limitations to the study. The effects of Cur on human MDR small cell lung cancer cells were only detected on H69AR cells. The study should be expanded by using other NSCLC cell lines.

CONCLUSION

Collectively, the novel findings of this study from array data and STRING database suggested that Cur inhibited cell viability and induced apoptotic cell death in human H69AR cells via mediating several proteins involved in both extrinsic and intrinsic apoptotic pathways. By expanding our knowledge of the heterogeneous, biological behavior of the MDR H69AR cells, novel treatment approaches can be developed for the treatment of MDR small cell lung cancer.

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