Resveratrol reduces drug resistance of SCLC cells by suppressing the inflammatory microenvironment and the STAT3/VEGF pathway

Cong Hou¹,², Lijun Lu², Zhanye Liu³, Yingjie Lian², Jianguang Xiao⁴

1. Department of Cardiothoracic Surgery, Zoucheng People's Hospital, 100 m East of Dart 342, Zoucheng, Jining City, Shandong, China, 273500.
2. Department of Thoracic Surgery, Caoxian People's Hospital, Qinghe East Road, Fumin Street South, Development Zone, Cao County, Heze City, Shandong, China, 274400.
3. Department of Thoracic Surgery, Caoxian People's Hospital, Caocheng Subdistrict, Cao County, Heze City, Shandong, China, 274400.
4. Department of Thoracic Surgery, Laizhou People’s Hospital, NO.1718, Wuli Street, Laizhou
City, Shandong, China, 261400.

# These authors contributed equally to this work.

*Corresponding author: Jianguang Xiao. Department of Thoracic Surgery, Laizhou People’s Hospital, NO.1718, Wuli Street, Laizhou City, Shandong, China, 261400. Tel: +86 13808919133. E-mail: xiaosaren88@sohu.com

**Running title:** Resveratrol reduces the drug-resistance of SCLC cells

**Keywords:** Small cell lung cancer, H69AR cells, Adriamycin, Resveratrol, IL-23, STAT3/VEGF signaling pathway.

**Abbreviations:**

SCLC, small cell lung cancer; Res, resveratrol; NSCLC, non-small cell lung carcinoma; STAT3, signal transducer and activator of transcription 3; MDR1, multidrug-resistance 1; si-STAT3, specific siRNA targeting STAT3; si-NC, negative control siRNA; qRT-PCR, quantitative real-time PCR; IC50, half-maximal inhibitory concentration

**Abstract**

DNA-damaging agents, such as adriamycin, are widely used for the treatment of small cell lung cancer (SCLC). However, drug resistance is one of the major challenges for treatment of SCLC. Herein, we investigated the mechanisms underlying drug resistance in SCLC cells and the effects of resveratrol (Res) on drug resistance. We report that adriamycin treatment of H69AR (multidrug-resistance phenotype) cells resulted in a lower rate of growth inhibition, up-regulation of MRP1 and P-gp and higher P-gp activity as compared with susceptible H69 cells treated with adriamycin. Moreover, the STAT3/VEGF pathway was overactivated in H69AR cells, especially after IL-23 treatment. The inflammatory microenvironment promoted the drug-resistance of...
H69AR cells by activating the STAT3/VEGF pathway. The addition of Res suppressed the expression levels of inflammatory mediators, inhibited the STAT3/VEGF pathway, impeded P-gp activity, and decreased the drug resistance of H69AR cells. H69AR cells exhibited adriamycin resistance through activation of the STAT3/VEGF pathway, and Res ameliorated the inflammatory microenvironment to suppress the STAT3/VEGF pathway to reduce drug resistance. Our results suggest that Res might have therapeutic potential for SCLC treatment.

Introduction

Lung cancer with the leading cancer-related mortality rate worldwide can be divided into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) [1]. SCLC is an aggressive disease of neuroendocrine origin which accounts for 15-20% of all lung carcinomas[2-5]. SCLC is considered different from the other lung cancer types for its early distant metastasis, aggressive growth, and the highest malignancy[2, 5, 6]. Clinically, it is proved that the poor prognosis of SCLC probably due to the rapid evolution from chemosensitivity to drug-resistance [7]. The widely used drugs for the treatment of SCLC are DNA-damaging agents[8], such as adriamycin, which show a potent effect on preventing cell division and enhancing cell death[9]. However, the cells often acquire drug-resistance, but the molecular mechanisms of drug-resistance are not fully elucidated in SCLC.

Inflammation can enhance cell proliferation and the production of survival signals to promote the development of tumors[10]. Not only does inflammation show a pro-cancer effect but also it induces angiogenesis by influencing immune regulation[10]. IL-23, which is overexpressed in lung cancer, is significantly associated between IL-23 and the recurrence and prognosis of SCLC[11]. It has been reported that IL-23 can mediate inflammatory processes providing a tumor microenvironment by activating the signal transducer and activator of transcription 3 (STAT3) signaling pathway in tumors[12, 13].

In several types of cancers including lung cancer, the malignant transformation is implicated with the STAT3 signaling pathway by constitutively activating the transcription factor signal transducer
and STAT3[14]. The STAT3 signaling pathway is activated by STAT phosphorylation, which increases the expression levels of target genes, such as VEGF in all tumor growth processes. In addition, recombinant human VEGF increases the expression of MRP1 or enhances the activity of MRP1 in K562 and BGC-823 cell lines [15]. MDR1, a multidrug-resistance gene in tumor cells, produces P-glycoprotein (P-gp) that alters intracellular drug distribution. Fabbro et al. have proved that VEGFR-2 and PKC412 inhibitors reverse the P-gp-mediated MDR role in cancer cells by blocking the VEGF cellular signaling pathway[16]. Hence, we conjecture that overactivation of the STAT3/VEGF signaling pathway was associated with drug-resistance of SCLC.

The high expression level of MDR1 is ubiquitous in various cancers or diseases with drug-resistance. In pediatric soft tissue sarcoma, the MDR1 gene exerts a predominant role in innate drug resistance[17]. Meanwhile, the high expression level of MDR is partially responsible for drug-resistance of Burkitt lymphoma cells[18]. Iliodora et al. have revealed that overexpression of MDR1/P-gp is inhibited to overcome the vincristine-resistant effects in B lymphocytes thereby restoring the sensitivity of anticancer and antimicrobial drugs[19]. Besides, cisplatin-resistance in bladder cancer cells is reversed via reducing MDR1 expression[20]. Another example is that in the adriamycin-resistant human hepatic carcinoma mouse model, the anti-tumor efficacy of adriamycin is reversed by MDR1 siRNA[21].

Resveratrol (Res) has many therapeutic functions including anti-apoptosis, anti-oxidation, anti-tumor and anti-inflammation roles[22, 23]. Recent evidence has shown that Res plays an anti-tumor effect in SCLC[24]. A study has shown that Res increases the chemotherapy sensitivity of cholangiocarcinoma[25]. Besides, the negative regulation of the STAT3 pathway can overcome the drug-resistance in multiple myeloma cells[26] and prostate cancer cells[27]. However, whether Res can overcome drug resistance and increase drug sensitivity in SCLC cells under an inflammatory microenvironment has not been reported. This study aims to study the mechanism of the inflammatory microenvironment on drug resistance in SCLC cells and the effects of Res.

Materials and methods

FEBS Open Bio (2020) © 2020 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
Cell culture
Human SCLC cell lines H69 and H69AR (the multidrug-resistance phenotype) were obtained from the American Type Culture Collection (Manassas, VA, USA). Then the cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS, Thermo Fisher, Waltham, MA, USA) and 1% penicillin/streptomycin under 5% CO\textsubscript{2} at 37˚C.

MTT assay
To measure the viability of H69 and H69AR cells, the MTT Kit (Sigma, USA) was used. When cells at the logarithmic growth phase, they were plated (1.5×10\textsuperscript{4} cells/well) and treated with individual adriamycin (Houston, TX, USA) at different concentrations, or adriamycin combined with Res (Alexis Biochemicals, San Diego, CA) and/or IL-23, respectively. After treatment for 24 and 48 h, MTT was added and maintained for 4 h. Then we applied 200 µL DMSO to replace the culture medium and a microplate reader (Bio-Rad, Shanghai, China) was used to read the absorbance at 492 nm. The cell growth inhibition rate was calculated according to the formula (1 - the absorbance of reagent-treated cells/the absorbance of untreated control cells) × 100 %.

STAT3 knockdown
The si-STAT3 and the negative control siRNA (si-NC) were obtained from GenePharma (Shanghai, China). The H69AR cells were transfected with si-SIRT3 using lipofectamine 3000 (Life Technology, MD, USA Invitrogen). Then the cells were labeled as H69AR group, si-NC group, and si-STAT3 group for subsequent tests. 48 h after transfection, cells were collected for determination of siRNA transfection efficiency.

qRT-PCR
The relative expression level of MDR1 was measured by qRT-PCR. The TRIZOL Reagent Kit (Takara, Japan) was applied to isolate total RNA, and the miRNA First Strand cDNA synthesis Kit (Sangon Biotech, China) was applied to transcribed RNA into cDNA. qRT-PCR was performed with SYBR® Prime Script™ RT-PCR Kit (Invitrogen, USA). The primer sequences were listed below: MDR1 forward: 5’-CCCATCATGGCAATAGCAGG-3’, reverse: 5’-TGTTCAAACCTTTGCTCCTGA-3’; GAPDH forward: 
5ʹ-GTCTCCTCTGACTTCAACAGCG-3ʹ, reverse: 5ʹ-ACCACCCTGTTGCTGTAGCCAA-3ʹ. The Mx3000P real-time PCR system (Thermo Fisher, Waltham, MA, USA) was used. The PCR reaction conditions were described as follows: 95 °C for 15 min, and then 40 cycles of 94 °C for 15 s for, 60 °C for 1 min and 72 °C for 1 min. All procedures were conducted in triplicate. The $2^{-ΔΔCT}$ method was used to calculate the relative MDR1 expression.

**Western blot**

The protein expression levels were measured by western blot. In brief, total protein was obtained using the specific protein extraction Kit (BestBio Institute of Biotechnology, Wuhan, China). The amounts of total protein were quantified by the BCA assay (Keygen Institute of Biotechnology, Nanjing, China). Protein was resolved by 6-15% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA), then blocked with 5% non-fat milk. Primary antibodies including anti-P-gp (1:1000; ab170904; Abcam, Cambridge, UK), anti-STAT3 (1:1000; ab76315; Abcam Cambridge, UK), anti-IL-8 (1:1000; ab18672; Abcam, Cambridge, UK), anti-IL-23 (1:1000; ab45420; Abcam, Cambridge, UK) and anti-VEGF (1:1000; ab46154; Abcam, Cambridge, UK), anti-IL-1β (1:1000; 12703; Cell Signaling Technologies, MA, USA), anti-p-STAT3 (1:1500; 9145; Cell Signaling Technologies, MA, USA), anti-MRP1 (1:1000; 72202; Cell Signaling Technologies, MA, USA), anti-IL-1β (1:1000; 12703; Cell Signaling Technologies, MA, USA), and anti-p-NF-κB (1:800; sc-136548; Santa Cruz, CA, USA) were added and incubated overnight at 4°C. The secondary antibodies (ab205718, ab190475; Abcam, Cambridge, UK) were applied to the membranes and incubated for 2 h, subsequently. Immunoreactive signals were revealed by the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Munchen, Germany). Image J software was applied to analyze protein expressions.

**Flow cytometry**

The apoptosis of H69 and H69AR cells was measured by flow cytometry. Briefly, the cells were collected after treatment with adriamycin, or its combination with Res or/and IL-23 and Res, and incubated with ECD (5 μl) and Annexin V-FITC (5 μl) solution (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min. The cell apoptosis rates were analyzed under a flow cytometer (BD...
Rhodamine 123 accumulation assay

The accumulation of rhodamine 123 which reflecting P-gp activity was detected in H69 and H69AR cells. Briefly, after treatment, the H69 and H69AR cells were treated with rhodamine 123 (5 μM) (Sigma Chemical Company, USA) and incubated at 37 °C for 2 h. Then cold PBS and 1% Triton X-100 were used to wash H69 and H69AR cells. The accumulation of rhodamine 123 was measured using a confocal laser scanning microscope (OLYMPUS, Japan). Image J software was applied to analyze the above data.

Statistical analysis

All the experiments were performed in triplicate and repeated at least 3 times. The data were presented as mean ± standard deviation (SD). Student unpaired t-test and one-way analysis of variance (ANOVA) were used for statistical analysis. \( p < 0.05 \) was considered statistically significant.

Results

The adriamycin resistance of H69AR cells

To assess the drug-resistance of SCLC cells treated with adriamycin, the following experiments were conducted. MTT assay was executed for detecting the growth inhibition effect of adriamycin (2, 5, 10, 20, 50 μM) treatment for 48 h in H69 and H69AR cells. The results showed that adriamycin inhibited the growth of both H69 cells and H69AR cells in a dose-dependent manner, and a lower growth inhibition rate was detected in H69AR cells than H69 cells (\( p < 0.01 \), Fig. 1A).

What is more, to evaluate the adriamycin resistance of H69AR cells, we calculated and obtained the ratio of the half-maximal inhibitory concentration (IC50) of adriamycin of H69AR cells which was 10 μM. And the flow cytometry for the detection of cell apoptosis showed consistent results (Fig. 1B). Besides, the results of qRT-PCR indicated that the expression level of MDR1 in H69 cells was lower than H69AR cells (\( p < 0.01 \), Fig. 1C). The results in Fig.1D showed that the MRP1 and P-gp expressions were elevated in H69AR cells (\( p < 0.01 \)). The results of the rhodamine
accumulation assay indicated that P-gp activity in H69AR cells was higher than H69 cells (Fig. 1E). These findings confirmed that H69AR cells showed superior resistance to adriamycin in comparison to H69 cells.

**The inflammatory microenvironment promoted drug-resistance of SCLC cells by activating the STAT3/VEGF signaling pathway**

To investigate the mechanism of the inflammatory microenvironment on the promotion of drug-resistance of H69AR cells, we conducted the following experiments. Western blot was used to detect the expression levels of STAT3, p-STAT3 and VEGF and the results reported that the levels of p-STAT3 and VEGF were up-regulated in H69AR cells compared with H69 cells \((p<0.01)\), which indicated an overactivation of STAT3/VEGF pathway in drug-resistant H69AR cells (Fig. 2A). IL-23 promoted the inflammatory processes in the tumor microenvironment. To detect the effect of the inflammatory microenvironment on drug-resistance, the H69AR cells were treated by IL-23 \((0, 5, 10, 20 \text{ ng/ml})\) and adriamycin \((10 \mu\text{M})\) for 24 h and 48 h. The MTT assay was performed to test the growth inhibition rate. As shown in Fig. 2B, the growth inhibition induced by adriamycin in H69AR cells was significantly relieved under treatment of 10 ng/ml of IL-23, and it remained stable under 10-20 ng/ml of IL-23 treatments. The results indicated that the inflammatory microenvironment mediated the drug-resistance of H69AR, and 10 ng/ml IL-23 was chosen as the appropriate concentration for further analysis. To study the effect of the inflammatory microenvironment on STAT3/VEGF signaling pathway, the siRNA of STAT3 was transfected into H69AR to knock down endogenous STAT3. The results of qRT-PCR analysis showed that the STAT3 expression was dramatically reduced in H69AR+si-STAT3 group compared with H69AR group and H69AR+si-NC group \((p<0.01, \text{ Fig. 2C})\). The results of qRT-PCR showed that the MDR1 expression level in the H69AR+IL-23 group was significantly up-regulated, while that in H69AR+si-STAT3 group was down-regulated. Meanwhile, the decline of the MDR1 level was reversed by co-treatment with IL-23 \((p<0.01, \text{ Fig. 2D})\). The results of western blot and Rhodamine 123 accumulation assay suggested that the changes of the expression levels of STAT3/VEGF pathway-related proteins (p-STAT3, VEGF) and drug resistance-related...
proteins (MRP1, and P-gp), as well as P-gp activity, were consistent with the trend above (Fig. 2E, F). To study the changes of adriamycin resistance of H69AR cells after treatment by si-STAT3 and IL-23, MTT assay and flow cytometry were conducted. After treatment of 10 µM adriamycin, the growth inhibition rate in H69AR group was close to that in the H69AR+IL-23 group and the growth inhibition rate of H69AR cells was significantly increased by si-STAT3 transfection, which could be abolished by co-treatment with IL-23 (Fig. 2G). Similar results were found in cell apoptosis tested by flow cytometry (Fig. 2H). The above data showed that the inflammatory microenvironment promoted the drug-resistance of H69AR cells through activating the STAT3/VEGF signaling pathway.

**Res played an anti-inflammatory role to inhibit drug-resistance**

The H69AR cells were treated by Res (0, 50, 100, 150, 200 µM) and adriamycin (10 µM) for 24 h and 48 h respectively, then the adriamycin resistance of H69AR cells was detected by MTT assay. Fig. 3A showed that the growth inhibition rate of H69AR cells induced by adriamycin was significantly increased under the treatment of Res (concentration from 0 to 200 µM), and the rate flattened out by Res concentration above 100 µM. As shown in Fig. 3B, the H69AR cells were treated by Res (0, 50, 100, 150 and 200 µM), IL-23 (10 ng/ml) and adriamycin (10 µM) for 24 h and 48 h respectively. The results suggested that Res decreased drug-resistance of H69AR cells in the inflammatory microenvironments. Besides, 100 µM was selected as the appropriate dose of Res used in the subsequent experiments. To detect the anti-inflammation role of Res, the expression levels of inflammation-related factors were assessed. Compared with H69AR group, the expression levels of p-NF-κB, IL-1β, IL-8 and IL-23 were remarkably promoted in the H69AR+IL-23 group ($p < 0.01$), while these increased expression levels were reduced by the addition of Res (Fig. 3C). Therefore, the above results suggested that Res played an anti-inflammatory role and reversed adriamycin resistance of H69AR cells.

**Res reversed STAT3/VEGF-mediated drug-resistance by ameliorating the inflammatory microenvironment**

To investigate whether Res can reverse drug-resistance, the following experiments were
performed. MTT assay was conducted for detecting the effects of Res (100 µM) on the growth inhibition rate of H69AR cells under adriamycin (10 µM) treatment. We found that Res reversed adriamycin resistance in H69AR+Res group compared with H69AR group, and the depressed growth inhibition rate under IL-23 treatment was elevated in H69AR+IL-23+Res group (Fig. 4A). The above results suggested that Res reversed the adriamycin resistance of H69AR cells. The changes in cell apoptosis revealed by flow cytometry were consistent with the above results (Fig. 4B). As shown in Fig. 4C, the MDR1 expression level was significantly decreased in H69AR+Res group and increased in H69AR+IL-23 group, while the elevated MDR1 expression level in H69AR cells under IL-23 environment was further declined by Res treatment ($p < 0.01$). The changes in the expression levels of p-STAT3, VEGF, MRP1 and P-gp, as well as the P-gp activity, were consistent with the above trend in these groups ($p < 0.01$, Fig. 4D, E). All the findings indicated that Res inhibited the expression levels of inflammatory mediators in the tumor microenvironment and then reduced STAT3/VEGF-mediated adriamycin resistance of H69AR cells.

Discussion

SCLC, as an aggressive disease, is highlighted by high morbidity and mortality[3] and the major obstacle is Multidrug-resistance (MDR) in SCLC treatment. H69 cell line is a drug-sensitive SCLC cell line, while the H69AR cell line was a multidrug-resistance phenotype. Our results showed that in H69AR cells, the drug-resistant proteins and transporters were overexpressed compared with H69 cells, after treatment with adriamycin, H69AR cells developed adriamycin resistance.

STAT3 targets Bcl-XL, Cyclin D1, C-MYC, MCL1 and VEGF, which is involved in various cellular processes[28]. Compared with matched primary tumors, the STAT3 phosphorylation level is increased in drug-resistant recurrent tumors [29, 30]. Inactivation of STAT3 can overcome drug-resistance in lung cancer[31, 32]. In osteosarcoma cells, inhibition of STAT3 increases the sensitivity of chemotherapy-resistant cells and eliminates drug efflux[33]. Our results also
indicated that the drug-resistance of lung cancer cells was achieved through activating the STAT3/VEGF signaling pathway, which was consistent with previous research [33]. What is more, the inflammatory microenvironment is involved in the drug resistance of cancer cells. Peng et al. have provided evidences that Res regulates drug-resistance of SCLC via inhibition of PI3K/BMX/STAT3 signaling pathway[34]. The activation of the STAT3 pathway plays a crucial role in the transcriptional regulation of MDR1 and MRPs gene expressions[35]. The activation of STAT3 can raise the expression level of MRP1, and therefore, MRP1 overexpression is often detected in various types of cancers. In leukemia cells, the inhibition of the STAT3 pathway can promote the intracellular accumulation of adriamycin, and down-regulate the expression levels of MDR1 and MRP1, thus increasing drug sensitivity[36]. Also, the STAT3 signaling pathway is the main intrinsic tumor inflammation pathway, because it is frequently activated in the malignant cells, and regulates many tumor inflammatory genes in the tumor microenvironment[37]. Our results indicated that the expression levels of STAT3, P-gp, and MRP1 were reduced in STAT3 knockdown H69AR cells, which was consistent with the above results. At the same time, we found that the p-STAT3, VEGF, P-gp, and MRP1 expressions in the inflammatory microenvironment were elevated, indicating that the inflammatory microenvironment enhanced the drug-resistance of SCLC by activating the STAT3/VEGF signaling pathway.

Chronic resveratrol treatment appears to have neuroprotective potential through performing its anti-oxidative and anti-apoptotic functions[38]. The anti-oxidative and anti-inflammatory effects of Res were demonstrated in various tumors. In melanoma cells, Res chronic pretreatment was confirmed to inhibit the inflammation and EMT to induce an anti-tumor hosting microenvironment for cancer therapies[39]. Furthermore, Res has an anti-tumor effect in SCLC cells[24]. Bhardwaj and his colleagues found that Res inhibited the IL-6-induced activation of STAT3[26]. We found that Res played an anti-inflammatory role by decreasing the expressions of p-NF-κB, IL-1β, IL-8, and IL-23, then inactivated the STAT3/VEGF signaling pathway, thus reducing drug-resistance of H69AR cells.

In summary, Res overcame STAT3/VEGF-mediated multidrug-resistance by suppressing the

FEBS Open Bio (2020) © 2020 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
inflammatory microenvironment, which provided a sound basis for SCLC treatment using Res alone or in combination with other agents in clinical trials. However, the clinical application of Res needs to be further clarified.

Authors' contributions
Cong Hou and Lijun Lu designed and wrote the paper, Zhanye Liu and Yingjie Lian analyzed data and performed the experiments. Jianguang Xiao conceived the experiments and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Acknowledgment
The authors wish to thank the contractor (Shandong Excalibur Medical Research Ltd.) for providing the results of the western blot.

Availability of data and materials
The datasets used during the present study are available from the corresponding author upon reasonable request.

Funding
The authors declare that there are no sources of funding to be acknowledged.

Competing interests
The authors declare no conflict of interest.

References
1. Lv, C., Hong, Y., Miao, L., Li, C., Xu, G., Wei, S., Wang, B., Huang, C. & Jiao, B. (2013) Wentilactone A as a novel

potential antitumor agent induces apoptosis and G2/M arrest of human lung carcinoma cells, and is mediated by HRas-GTP accumulation to excessively activate the Ras/Raf/ERK/pS3-p21 pathway, Cell Death Dis. 4, e952.

2. Chen, Y. T., Feng, B. & Chen, L. B. (2012) Update of research on drug resistance in small cell lung cancer chemotherapy, Asian Pac J Cancer Prev. 13, 3577-81.

3. Rodriguez, E. & Lilenbaum, R. C. (2010) Small cell lung cancer: past, present, and future, Curr Oncol Rep. 12, 327-34.

4. Yu, J., Wang, S., Zhao, W., Duan, J., Wang, Z., Chen, H., Tian, Y., Wang, D., Zhao, J., An, T., Bai, H., Wu, M. & Wang, J. (2018) Mechanistic Exploration of Cancer Stem Cell Marker Voltage-Dependent Calcium Channel alpha2delta1 Subunit-mediated Chemotherapy Resistance in Small-Cell Lung Cancer, Clin Cancer Res. 24, 2148-2158.

5. Shepherd, F. A., Crowley, J., Van Houtte, P., Postmus, P. E., Carney, D., Chansky, K., Shaikh, Z., Goldstraw, P., International Association for the Study of Lung Cancer International Staging, C. & Participating, I. (2007) The International Association for the Study of Lung Cancer lung cancer staging project: proposals regarding the clinical staging of small cell lung cancer in the forthcoming (seventh) edition of the tumor, node, metastasis classification for lung cancer, J Thorac Oncol. 2, 1067-77.

6. Mirski, S. E., Gerlach, J. H. & Cole, S. P. (1987) Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin, Cancer Res. 47, 2594-8.

7. Stewart, C. A., Gay, C. M., Xi, Y., Sivajothi, S., Sivakamasundari, V., Fujimoto, J., Bolisetty, M., Hartsfield, P. M., Balasubramaniyan, V., Chalishazar, M. D., Moran, C., Kalhor, N., Stewart, J., Tran, H., Swisher, S. G., Roth, J. A., Zhang, J., de Groot, J., Gilsson, B., Oliver, T. G., Heymach, J. V., Wistuba, I., Robson, P., Wang, J. & Byers, L. A. (2020) Single-cell analyses reveal increased intratumoral heterogeneity after the onset of therapy resistance in small-cell lung cancer, Nature cancer. 1, 423-436.

8. Cheung-Ong, K., Giaever, G. & Nislow, C. (2013) DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology, Chem Biol. 20, 648-59.

9. Moyal, L., Goldfeiz, N., Gorovitz, B., Rephaeli, A., Tal, E., Tarasenko, N., Nudelman, A., Ziv, Y. & Hodak, E. (2018) AN-7, a butyric acid prodrug, sensitizes cutaneous T-cell lymphoma cell lines to doxorubicin via inhibition of
10. Coussens, L. M. & Werb, Z. (2002) Inflammation and cancer, *Nature*. **420**, 860-7.

11. Cam, C., Karagoz, B., Muftuoglu, T., Bigi, O., Emirzeoglu, L., Celik, S., Ozgun, A., Tuncel, T. & Top, C. (2016) The inflammatory cytokine interleukin-23 is elevated in lung cancer, particularly small cell type, *Contemp Oncol (Pozn)*. **20**, 215-9.

12. Langowski, J. L., Zhang, X., Wu, L., Mattson, J. D., Chen, T., Smith, K., Basham, B., McClanahan, T., Kastelein, R. A. & Oft, M. (2006) IL-23 promotes tumour incidence and growth, *Nature*. **442**, 461-5.

13. Watford, W. T., Hissong, B. D., Bream, J. H., Kanno, Y., Muul, L. & O'Shea, J. J. (2004) Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4, *Immunol Rev*. **202**, 139-56.

14. 6;98(3):295-303Bromberg JF, W. M., Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr (1999) Stat3 as an oncogene, *Cell*. 6, 295-303.

15. Li, J., Wu, X., Gong, J., Yang, J., Leng, J., Chen, Q. & Xu, W. (2013) Vascular endothelial growth factor induces multidrug resistance-associated protein 1 overexpression through phosphatidylinositol-3-kinase /protein kinase B signaling pathway and transcription factor specificity protein 1 in BGC823 cell line, *Acta Biochim Biophys Sin (Shanghai)*. **45**, 656-63.

16. Fabbro, D., Ruetz, S., Bodis, S., Pruschy, M., Csermak, K., Man, A., Campochiaro, P., Wood, J., O'Reilly, T. & Meyer, T. (2000) PKC412--a protein kinase inhibitor with a broad therapeutic potential, *Anticancer Drug Des*. **15**, 17-28.

17. Molina-Ortiz, D., Torres-Zarate, C., Cardenas-Cardos, R., Palacios-Acosta, J. M., Hernandez-Arrazola, D., Shalkow-Klincovstein, J., Diaz-Diaz, E. & Vences-Mejia, A. (2018) MDR1 not CYP3A4 gene expression is the predominant mechanism of innate drug resistance in pediatric soft tissue sarcoma patients, *Cancer biomarkers : section A of Disease markers*. **22**, 317-324.

18. Tabata, M., Tsubaki, M., Takeda, T., Tateishi, K., Tsurushima, K., Imano, M., Satou, T., Ishizaka, T. & Nishida, S. (2020) Dasatinib reverses drug resistance by downregulating MDR1 and Survivin in Burkitt lymphoma cells, *BMC complemenary medicine and therapies*. **20**, 84.

19. Pop, I. V., Pop, L. M., Ghetie, M. A. & Vitetta, E. S. (2009) Targeting mammalian target of rapamycin to both downregulate and disable the P-glycoprotein pump in multidrug-resistant B-cell lymphoma cell lines,
20. Oh, S. S., Lee, K. W., Madhi, H., Jeong, J. W., Park, S., Kim, M., Lee, Y., Han, H. T., Hwangbo, C., Yoo, J. & Kim, K. D. (2020) Cordycepin Resensitizes T24R2 Cisplatin-Resistant Human Bladder Cancer Cells to Cisplatin by Inactivating Ets-1 Dependent MDR1 Transcription, *International journal of molecular sciences*. 21.

21. Yang, H., Ding, R., Tong, Z., Huang, J., Shen, L., Sun, Y. U., Liao, J., Yang, Z., Hoffman, R. M., Wang, C. & Meng, X. (2016) siRNA Targeting of MDR1 Reverses Multidrug Resistance in a Nude Mouse Model of Doxorubicin-resistant Human Hepatocellular Carcinoma, *Anticancer research*. 36, 2675-82.

22. Hsieh, T. C. & Wu, J. M. (2010) Resveratrol: Biological and pharmaceutical properties as anticancer molecule, *Biofactors*. 36, 360-9.

23. Blanquer-Rossello, M. D., Hernandez-Lopez, R., Roca, P., Oliver, J. & Valle, A. (2017) Resveratrol induces mitochondrial respiration and apoptosis in SW620 colon cancer cells, *Biochim Biophys Acta Gen Subj*. 1861, 431-440.

24. Li, W., Shi, Y., Wang, R., Pan, L., Ma, L. & Jin, F. (2018) Resveratrol promotes the sensitivity of small-cell lung cancer H446 cells to cisplatin by regulating intrinsic apoptosis, *Int J Oncol*. 53, 2123-2130.

25. Frampton, G. A., Lazcano, E. A., Li, H., Mohamad, A. & DeMorrow, S. (2010) Resveratrol enhances the sensitivity of cholangiocarcinoma to chemotherapeutic agents, *Lab Invest*. 90, 1325-38.

26. Bhardwaj, A., Sethi, G., Vadhan-Raj, S., Bueso-Ramos, C., Takada, Y., Gaur, U., Nair, A. S., Shishodia, S. & Aggarwal, B. B. (2007) Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells, *Blood*. 109, 2293-302.

27. Kotha, A., Sekharam, M., Cilenti, L., Siddiquée, K., Khaled, A., Zervos, A. S., Carter, B., Turkson, J. & Jove, R. (2006) Resveratrol inhibits Src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein, *Mol Cancer Ther*. 5, 621-9.

28. Guo, S., Zhi, Y., Yang, H., Yu, Y., Wang, Y., Zhang, J., Wang, G., Zhang, L., Sun, B. & Zhang, Y. (2014) Bcl-2 expression is associated with poor prognosis of solitary plasmacytoma of bone, *Ann Hematol*. 93, 471-7.

29. Lee, E. S., Ko, K. K., Joe, Y. A., Kang, S. G. & Hong, Y. K. (2011) Inhibition of STAT3 reverses drug resistance acquired in temozolomide-resistant human glioma cells, *Oncol Lett*. 2, 115-121.
30. Lee, H. J., Zhuang, G., Cao, Y., Du, P., Kim, H. J. & Settleman, J. (2014) Drug resistance via feedback activation of Stat3 in oncogene-addicted cancer cells, *Cancer Cell.* **26**, 207-21.

31. Shien, K., Papadimitrakopoulou, V. A., Ruder, D., Behrens, C., Shen, L., Kallhor, N., Song, J., Lee, J. J., Wang, J., Tang, X., Herbst, R. S., Toyooka, S., Girard, L., Minna, J. D., Kurie, J. M., Wistuba, II & Izzo, J. G. (2017) JAK1/STAT3 Activation through a Proinflammatory Cytokine Pathway Leads to Resistance to Moleculary Targeted Therapy in Non-Small Cell Lung Cancer, *Mol Cancer Ther.* **16**, 2234-2245.

32. Kotmakci, M., Cetintas, V. B. & Kantarci, A. G. (2017) Preparation and characterization of lipid nanoparticle/pDNA complexes for STAT3 downregulation and overcoming chemotherapy resistance in lung cancer cells, *Int J Pharm.* **525**, 101-111.

33. Wang, Z., Wang, C., Zuo, D., Zhang, T., Yin, F., Zhou, Z., Wang, H., Xu, J., Mao, M., Wang, G., Hua, Y., Sun, W. & Cai, Z. (2019) Attenuation of STAT3 Phosphorylation Promotes Apoptosis and Chemosensitivity in Human Osteosarcoma Induced by Raddeanin A, *Int J Biol Sci.* **15**, 668-679.

34. Peng, J., Wang, Q., Liu, H., Ye, M., Wu, X. & Guo, L. (2016) EPHA3 regulates the multidrug resistance of small cell lung cancer via the PI3K/BMX/STAT3 signaling pathway, *Tumour Biol.* **37**, 11959-11971.

35. Yun, M., Lee, D., Park, M. N., Kim, E. O., Sohn, E. J., Kwon, B. M. & Kim, S. H. (2015) Cinnamaldehyde derivative (CB-PIC) sensitizes chemo-resistant cancer cells to drug-induced apoptosis via suppression of MDR1 and its upstream STAT3 and AKT signalling, *Cell Physiol Biochem.* **35**, 1821-30.

36. Zhang, X., Xiao, W., Wang, L., Tian, Z. & Zhang, J. (2011) Deactivation of signal transducer and activator of transcription 3 reverses chemotherapeutics resistance of leukemia cells via down-regulating P-gp, *PLoS One.* **6**, e20965.

37. Yang, C. L., Liu, Y. Y., Ma, Y. G., Xue, Y. X., Liu, D. G., Ren, Y., Liu, X. B., Li, Y. & Li, Z. (2012) Curcumin blocks small cell lung cancer cells migration, invasion, angiogenesis, cell cycle and neoplasia through Janus kinase-STAT3 signalling pathway, *PLoS One.* **7**, e37960.

38. Ozacmak, V. H., Sayan-Ozacmak, H. & Barut, F. (2016) Chronic treatment with resveratrol, a natural polyphenol found in grapes, alleviates oxidative stress and apoptotic cell death in ovariectomized female rats subjected to chronic cerebral hypoperfusion, *Nutritional neuroscience.* **19**, 176-86.

39. Menicacci, B., Laurenzana, A., Chilla, A., Margheri, F., Peppicelli, S., Tanganelli, E., Fibbi, G., Giovannelli, L., Del
Rosso, M. & Mocali, A. (2017) Chronic Resveratrol Treatment Inhibits MRC5 Fibroblast SASP-Related Protumoral Effects on Melanoma Cells, The journals of gerontology Series A, Biological sciences and medical sciences. 72, 1187-1195.

Figure Legends

Fig.1. Adriamycin resistant profiles of SCLC cells. (A) The growth inhibition rate of H69 and H69AR cells was measured by MTT assay after adriamycin treatment. ** p<0.01, vs. control. (B) Cell apoptosis was determined by flow cytometry after adriamycin treatment. (C) qRT-PCR analysis of MDR1 expression levels. (D) Western blot analysis of the expression levels of MRP1 and P-gp, GAPDH was used as a loading control. This part of the results was provided by the contractor. (E) The activity of P-gp was detected by rhodamine 123 accumulation assay. Student unpaired t-test and one-way analysis of variance (ANOVA) were used for statistical analysis. ** p<0.01, vs. H69 group. All the results were shown as mean ± SD, data were obtained from at least three independent experiments.

Fig.2. The inflammatory microenvironment plays a role in drug resistance in cancer cells by activating STAT3/VEGF pathway. (A) Western blot analysis of the expression levels of STAT3, p-STAT3, and VEGF in H69 and H69AR cells, GAPDH was used as a loading control. This part of the results was provided by the contractor. (B) The cell growth inhibition rate was measured by MTT assay in H69AR cells treated with adriamycin and IL-23 for 24 h or 48 h. (C) Knockdown of STAT3, the relative mRNA expression level of STAT3 in H69AR cell was detected by qRT-PCR. Then cells were divided into 4 groups (H69AR, H69AR+IL-23, H69AR+si-STAT3, H69AR+IL-23+STAT3). (D) Relative mRNA expression levels of MDR1 were detected by qRT-PCR. (E) The expression levels of STAT3, p-STAT3, VEGF, MRP1 and P-gp were detected by western blot. This part of the results was provided by the contractor. (F) The activity of P-gp was detected by rhodamine 123 accumulation assay, scale bar: 50 μm. (G) The cell inhibition rate was measured by MTT assay and (H) cell apoptosis was determined by flow cytometry. Student unpaired t-test and one-way analysis of variance (ANOVA) were used for statistical analysis. *
Fig. 3. Res plays an anti-inflammatory role and increases drug sensitivity of H69AR cells. The cell growth inhibition rate was measured by MTT assay in H69AR cells (A) treated with adriamycin and Res, (B) treated with adriamycin, Res and IL-23 for 24 h, 48 h, respectively. (C) Western blot analysis of the expression levels of p-NF-κB, IL-1β, IL-8, and IL-23 in H69AR cells, GAPDH was used as a loading control. This part of the results was provided by the contractor. Student unpaired t-test and one-way analysis of variance (ANOVA) were used for statistical analysis. ** p<0.01, vs. H69AR group. All the results were shown as mean ± SD, data were obtained from at least three independent experiments.

Fig. 4. Res reverses STAT3/VEGF-mediated drug-resistance. Cells were divided into 4 groups (H69AR, H69AR+Res, H69AR+IL-23, H69AR+Res+IL-23). (A) The cell growth inhibition rate was measured by MTT assay in H69AR cell. (B) Cell apoptosis was determined by flow cytometry. (C) The relative mRNA expression level of MDR1 in H69AR cells was detected by qRT-PCR. (D) The expression levels of STAT3, p-STAT3, VEGF, MRP1 and P-gp were detected by western blot. This part of the results was provided by the contractor. (E) The activity of P-gp was detected by rhodamine 123 accumulation assay, scale bar: 50 μm. Student unpaired t-test and one-way analysis of variance (ANOVA) were used for statistical analysis. * p<0.05, ** p<0.01, vs. H69AR group; ## p<0.01, vs. H69AR+IL-23 group. All the results were shown as mean ± SD, data were obtained from at least three independent experiments.
**Figure 1**

(A) Inhibition rate of growth (%) of H69 and H69AR cells with increasing concentrations of Adriamycin. 

(B) Flow cytometry analysis showing the percentage of cells in the sub-G1 phase at different Adriamycin concentrations for H69 and H69AR cells. 

(C) Western blot analysis showing the relative mRNA levels of MRP1 in H69 and H69AR cells. 

(D) Western blot analysis showing the protein expression levels of MRP1, P-gp, and GAPDH in H69 and H69AR cells.

(E) Confocal microscopy images of Rhodamine 123 and DAPI staining in H69 and H69AR cells, with Merge showing the combined images.

feb4_13230_f1.tif
Figure 3: Effects of IL-23 on the growth inhibition of H9AR cells.

A) Inhibition rate of growth (%) over time (24h and 48h) with different concentrations of Res (μM) and Adriamycin (10 μM).

B) Inhibition rate of growth (%) at 48h and 24h with different concentrations of IL-23 (ng/ml) and Adriamycin (10 μM).

C) Western blot analysis showing the expression levels of p-NF-κB, IL-1β, IL-8, and IL-23 with different treatments: H9AR, H9AR+IL-23, and H9AR+Res+IL-23. The molecular weights (kDa) for each protein are indicated.
