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**Inhibition of stearyl-CoA desaturase 1 potentiates anti-tumor activity of
amodiaquine in non-small cell lung cancer**

Xiaolei Hu^{a, #}, Jiangning Xiang^{a, #}, Yibo Li^a, Yan Xia^a, Siyuan, Xu^a, Xiaoge Gao^{a, *}, and
Shuxi Qiao^{a, *}

a. Cancer Institute, Xuzhou Medical University, Xuzhou, Jiangsu Province 221004,
People's Republic of China.

#Xiaolei Hu and Jiangning Xiang contributed equally to this work.

*Correspondence to:

Dr. Shuxi Qiao, Email: qiaoshuxi1987@126.com, Dr. Xiaoge Gao E-mail:
gaoxiaoge@xzhmu.edu.cn.

Summary

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer related death with few therapeutic treatment options. Under adverse tumor microenvironment, autophagy is an important mechanism of metabolic adaptations to sustain the survival and proliferation of tumor cells. Therefore, targeting autophagic activity represents a promising opportunity for NSCLC treatment. Here, we found that amodiaquine (AQ) increased autophagosome numbers and LC3BII and p62 at protein levels in A549 lung cancer cells suggesting the blockade of autophagic flux by AQ. To identify the key metabolic vulnerability associated with autophagy inhibition by AQ treatment, we then performed transcriptomics analysis in the presence or absence of AQ in A549 lung cancer cells and found stearoyl-CoA desaturase 1 (SCD) 1 was one of the most highly upregulated with AQ exposure. The induction of SCD1 by AQ exposure at both protein and mRNA level suggests that SCD1 could represent a potential therapeutic target of AQ treatment. Treatment of AQ in combination with SCD1 inhibition by A939572 demonstrated robust synergistic anti-cancer efficacy in cell proliferation assay and a lung cancer mouse xenograft model. Taken together, our study identified SCD1 could be a new therapeutic target upon autophagy inhibition by AQ exposure. Combinational treatment of autophagy inhibition and SCD1 inhibition achieves synergistic anti-tumor effect both in vitro and in vivo. This combinational approach could be a promising strategy for NSCLC treatment.

Keywords: amodiaquine; stearoyl-CoA desaturase 1; autophagy; non-small cell lung cancer; combinational therapy

Introduction

Lung cancer is the most commonly diagnosed cancer in both men and women worldwide.¹⁾ Despite the advances of early screening and the application of therapeutic alternatives, lung cancer still remains one of the leading causes of cancer-related death, with very unoptimistic five-year survival rates.²⁾ Non-small cell lung cancer (NSCLC), the most common histological subtype, accounts for approximately 80-85% of lung cancers.³⁾ With the limited treatment options and the nature of highly refractory treatments of NSCLC, developing novel therapeutic strategies for NSCLC is in urgent need.

Amodiaquine (AQ), an analog of chloroquine (CQ) containing entity 4-amino-7-chloroquinoline, is an anti-malarial drug that is especially used to treat CQ resistant malaria strains.⁴⁾ Long time ago, AQ has been used to treat lupus erythematosus⁵⁾ and rheumatoid arthritis.⁶⁾ Recent evidences have demonstrated that AQ is a promising candidate for repurposing to treat cancers, such as NSCLC⁷⁾, malignant melanoma,⁸⁾ and multiple myeloma.⁹⁾ Autophagy is one of the major degradation pathways for cytoplasmic components in diverse physiological and pathological processes, such as adaption to stress conditions, clearing of damaged organelles and misfolded proteins.^{10,11)} Recently, our studies revealed that AQ could block autophagic-lysosomal to sensitize human melanoma cells to starvation- and chemotherapy-induced cell death.⁸⁾ However, AQ did not exhibit prospective *in vivo* efficiency as a single treatment. Therefore, seeking the therapeutic combinational opportunity with AQ is a primary goal of this study.

Stearoyl-CoA desaturase 1 (SCD1) is the limiting enzyme, which could catalyze the conversion of saturated fatty acids (SFAs) to Δ -9 monounsaturated fatty acids (MUFAs) that play critical roles for the synthesis of phospholipids, triglycerides, cholesteryl esters, and wax esters.¹²⁻¹⁴⁾ High expression and activity of SCD1 has been demonstrated in diverse tumors, including colon, esophageal, lung, liver, and breast cancer.¹⁵⁾ Correspondingly, SCD1 inhibition could block cell proliferation and induce cell apoptosis in cancers.¹²⁾ Therefore, SCD1 has become a promising target for anti-cancer therapy. In addition, SCD1 expression is tightly regulated by exogenous MUFAs availability and is a predictive biomarker for sensitivity to SCD1 inhibition.¹⁶⁾ It has been reported that activation of SCD1 is required for autophagy in *Drosophila*¹⁷⁾ and mammal cells via providing adequate unsaturated fatty acids.¹⁸⁾ Surprisingly, inhibition of SCD1 could induce autophagy by stimulating AMPK in hepatocellular carcinoma¹⁹⁾ and colorectal cancer cell.²⁰⁾ Thus, there is complicated relationship between autophagy and SCD1.

In the present study, we identified that AQ treatment blocks autophagic-lysosomal flux and increased the number of autophagosome in A549 lung cancer cells. Transcriptomics analysis revealed that SCD1 is highly upregulated upon AQ treatment in lung cancer cells and elevated SCD1 protein level is accompanied by LC-3BII accumulation. Consequently, a new hypothesis was proposed that elevated SCD1 expression might be a molecular vulnerability of AQ treatment and inhibition of SCD1 could enhance the anti-tumor activity of AQ. Our subsequent results showed that combinational treatment of AQ and SCD1 inhibitor indeed exhibited synergistic

anti-tumor activity *in vitro* and *in vivo*. Interestingly, AQ treatment induces expression of Sterol regulatory element binding proteins 2 (SREBP2) together with elevated expression of SCD1. In summary, our findings indicate that up-regulation of SCD1 induced by AQ could promote tumor sensitivity to SCD1 inhibitors, which provides a combinational opportunity of AQ and SCD1 inhibitors as novel strategies for the clinical application of AQ.

Materials and methods

Cell lines and reagents

Human lung cancer cell line A549 was cultured in F12 medium and H460 was cultured in DMEM medium supplemented with heat-activated fetal bovine serum and GlutaMAX. All the cultures were maintained in humidified incubator with 5% CO₂ at 37 °C. Both A549 and H460 cells were purchased from the national collection of authenticated cell cultures (Shanghai, China) and have been authenticated using STR (or SNP) profiling within the last three years. The antibodies against GAPDH (#5174), SCD1 (#2794), p62 (#88588), were all obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody against LC3B (NB100-2220) was purchased from Novus Biologicals (Shanghai, China). The antibodies against SREBP2 were purchased from Abcam group (ab30682, Shanghai, China) and Proteintech Group (28212-1-AP, Wuhan, China). All the secondary antibodies labeled with HRP were obtained from Beyotime (Nanjing, China). AQ, 3-Methyladenine (3-MA), Pepstatin Ammonium (Pepstatin A), A939572 (SCD1 inhibitor), Fatostatin, and Oleic acid (OA) were all purchased from MCE (Shanghai, China).

mRFP-GFP-LC3 puncta formation assay

mRFP-GFP-LC3 puncta formation assay was performed following our previous descriptions.²¹⁾ Briefly, lung cancer cells were transfected with adenovirus mRFP-GFP-LC3 (Hanbio Biotech, Shanghai, China), and then treated with 10 μmol/l AQ for 24 h. Finally, the RFP and GFP fluorescence was captured by confocal laser scanning microscopy (LSM880, Carl Zeiss, Germany).

SDS-PAGE and Western-blotting assay

Lung cancer cells were treated under the indicated conditions, then lysed in RIPA buffer with protease inhibitor. Subsequently, 20 µg of protein samples were separated with SDS-PAGE and transferred to PVDF membrane. After blocking and incubation successively with primary and secondary antibodies, the chemical signals were visualized with Tanon imaging system (Tanon Science & Technology Co., Ltd., China).

RNA-seq and differentially expressed gene analysis

RNA-sequencing for AQ treated and control lung cancer cells (triplicates) was performed using illumina Novaseq 6000 system from CapitalBio Technology Co., Ltd (Beijing, China). Transcripts with fold change (FC) > 1.0 and a *p*-value < 0.05 were considered as significantly differentially expressed, which were utilized to investigate the function and mechanisms of AQ in lung cancer.

Real-time quantitative PCR (Q-PCR)

After treatment under indicated conditions, total RNA was isolated from lung cancer cells using Trizol reagent (Invitrogen, CA, USA). The cDNA was generated by reverse transcription using purified RNA and a PrimeScript RT reagent kit (TaKaRa, Dalian, China). Amplification reaction was performed using Roche Lightcycler[®] 96 system and SYBR Green master mix (Vazyme, Nanjing, China) according to manufacturer's instructions. Primers used for QPCR amplification were shown as following:

SCD1	(F-CCTGGTTTCACTTGGAGCTGTG;
R-TGTGGTGAAGTTGATGTGCCAGC),	SREBP1

(F-ACTTCTGGAGGCATCGCAAGCA; R-AGGTTCCAGAGGAGGCTACAAG),
SREBP2 (F-CTCCATTGACTCTGAGCCAGGA;
R-GAATCCGTGAGCGGTCTACCAT), PPAR- α
(F-TCGGCGAGGATAGTTCTGGAAG; R-GACCACAGGATAAGTCACCGAG),
PPAR- γ (F-AGCCTGCGAAAGCCTTTTGGTG;
R-GGCTTCACATTCAGCAAACCTGG), and GAPDH
(F-GTCTCCTCTGACTTCAACAGCG; R-ACCACCCTGTTGCTGTAGCCAA).

Construct of shRNA and establishment of stable cell lines

Firstly, SREBP2 shRNAs or scrambled sequences were cloned to the lentivirus expression vector pLKO.1 to obtain shSREBP2#1, shSREBP2#1, and shCon plasmids. These plasmids were co-transfected with packing plasmids (pSPAX and pMD2G) into 293T cells to generate lentivirus, which was used to infect lung cancer cells, and construct stable cell lines under puromycin selection. The target sequences of SREBP2 shRNA were described as following: shSREBP2#1: GGGACCATTCTGACCACAATG, sh SREBP2#2: GCTGCCAAGGAGAGTCTATAC.

Cell proliferation assay

The effects of AQ and A939572 on lung cancer cell proliferation were measured with a MTT assay kit (KeyGEN Biotech, Nanjing, China) according to manufacturer's instructions. Briefly, the cells were seeded on 96-well plates overnight, and then further treated with AQ and/or A939572 under indicated concentrations for 48h. After incubated with MTT for 4h, the absorbance was read at 570 nm with a full

wavelength microplate reader (BioTek Instruments, VT, USA).

Xenograft tumor model

Twenty female Balb/c nude mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained at Xuzhou Medical University Animal Center (Xuzhou, China). The mice were subcutaneously inoculated in the flank with human lung cancer cell H460 (6×10^6 cells per mouse). When tumor volume reached 100 mm^3 in all mice, they were randomly divided into four groups (five mice per group), which were respectively treated with or without 40mg/kg AQ and/or A939572 by daily oral gavage. For the control group, the mice received equal volume of vehicle by the same way. The length and width of tumors were measured with a caliper, and tumor volumes were calculated as $\text{length} \times \text{width}^2 / 2$. After treatment for 17 days, all tumor-bearing mice were sacrificed. The tumors were surgically isolated and photographed. All Animal studies were conducted in strict accordance with the recommendations of the Experimental Animal Care and Use Guidelines of the Experimental Animal Ethics Committee of Xuzhou Medical University, and were approved by the Experimental Animal Ethics Committee of Xuzhou Medical University (Permit Number: 201547).

Statistical analysis

All *in vitro* experiments were repeated at least three times and the data was presented as mean $\pm$ standard deviation (SD). One-way analysis of variance ANOVA was used to analyze multiple group comparisons following normality. Graphpad Prism 8.0.1 (GraphPad Software, Inc. CA, USA) was used to analyze all data, and a

value of $p < 0.05$ was considered to be a significant difference.

Results

AQ inhibits autophagic-lysosomal function in lung cancer cells

Previously, AQ has been reported as a promising candidate for cancer therapy by inhibiting autophagic-lysosomal function in malignant melanoma cells.⁸⁾ Herein, mRFP-GFP-LC3 puncta formation assay was performed to analyze the effect of AQ on autophagic flux in lung cancer cells. As shown in Figure 1A, significant red and green puncta formation could be induced in AQ treated cells compared to control, which indicated the increased number of autophagosomes. Meanwhile, yellow puncta presented in the merged image indicated either autophagosomal or autolysosomal localization. Usually, acidic environments of lysosome could quench the GFP-fluorescence.⁸⁾ Therefore, autolysosomal co-localization of red and green fluorescence suggested the impaired acidification of autolysosomes. In order to further explore whether AQ treatment could induce disrupted acidification of lysosomes in lung cancer cells, p62, an autophagic cargo receptor and substrate, was analyzed under the indicated conditions. The accumulation of p62 after treatment with AQ implied the blockade of autolysosomal flux (Figure 1B). As known, accumulation of LC3BII is involved in autophagosomes formation presenting on their membrane.²²⁾ AQ treatment induced massive turnover of LC3BI to LC3BII in the dose- and time-dependent manners (Figure 1B). The increase of p62 and LC3BII is consistent with the blockade of autophagic flux. Taken together, our results demonstrated that AQ treatment inhibited autophagic-lysosomal function leading to the increased number of autophagosomes in lung cancer cells.

AQ up-regulates SCD1 associated with the inhibition of autophagy in lung cancer cells

In order to identify the key metabolic pathway altered by autophagy inhibition by AQ treatment in lung cancer cells, RNA-Seq analysis of A549 lung cancer cells with or without AQ treatment was performed. As shown in Table 1, 52 genes associated with cholesterol biosynthesis and fatty acid *de novo* biosynthesis were significantly changed. Among them, SCD1 which was one of the most highly up-regulated in response to AQ treatment, aroused our great interest. Furthermore, the expression of SCD1 was confirmed by Q-PCR and Western-blotting after treatment with AQ. Our results showed that AQ could remarkably increase the expression of SCD1 in the dose- and time-dependent manners in A549 lung cancer cells (Figure 2A and 2B).

SCD1 can catalyze the conversion of SFAs (saturated fatty acids) to MUFAs (monounsaturated fatty acids), which is involved in the formation of initial autophagosomes membrane.¹⁸⁾ Moreover, SCD1 expression is tightly regulated in a context-dependent manner, including hormones and dietary elements, especially the intracellular levels of MUFAs.²³⁾ Accordingly, we proposed a hypothesis that AQ-induced the increase of autophagosomes could affect intracellular levels of MUFAs, and then regulate the expression of SCD1. In order to explore the relationship between autophagosomes and AQ induced up-regulation of SCD1, 3-MA (3-Methyladenine) that is a well-known inhibitor of autophagy via its inhibitory effect on class III PI3K and can suppress autophagosomes formation,²⁴⁾ was introduced to our further investigations. As shown in Figure 2C and 2D, 3-MA could remarkably

suppress the elevation of SCD1 by AQ. Simultaneously, 3-MA could inhibit the conversion of LC3BI to LC3BII induced by AQ. Interestingly, OA (oleic acid), as the major catalyzed product of SCD1 in cells, was used as the supplement of intracellular MUFAs in this study. Our result showed that OA could significantly promote AQ-induced conversion of LC3BI to LC3BII and reverse the up-regulation of SCD1 induced by AQ (Figure 2E). In summary, our results suggested that inhibition of autophagy by AQ might induce the decrease of cytoplasmic MUFAs, and then up-regulate the expression of SCD1 in lung cancer cells.

It has been demonstrated that lysosome could mediate the degradation of membrane-rich organelles and cell debris delivered by autophagy, and release amino acids and cholesterol.²⁵⁾ Our previous studies have revealed that AQ could inhibit autophagy by increasing lysosomal pH via 4-aminoquinolines.⁸⁾ Pepstatin ammonium (Pepstatin A), a specific inhibitor of aspartic protease produced by actinomycetes, could prevent autophagy through suppressing protein cleavage inside lysosomes.²⁶⁾ To investigate if inhibition of lysosomal function could impact SCD1 level, Pepstatin A was used to treat A549 lung cancer cells in the presence or absence of AQ. Our results showed that Pepstatin A could up-regulate the expression of SCD1 and obviously promote the up-regulation of SCD1 induced by AQ (Figure 2F), which implied that lysosome function inhibited by AQ could affect the expression of SCD1.

AQ potentiates anti-tumor activity of SCD1 inhibitor *in vitro* and *in vivo*

As known, SCD1 plays key roles in cancer cell proliferation via providing MUFAs to sustain the increasing demand for membrane phospholipids.²⁷⁾ Usually,

SCD1 expression serves as a biomarker for its inhibitor sensitivity in cancer.²⁸⁾ Our above results have demonstrated that AQ was able to up-regulate the expression of SCD1. Therefore, we hypothesized that elevated SCD1 expression might be a molecular vulnerability of AQ treatment and inhibition of SCD1 could enhance the anti-tumor activity of AQ. Here The *in vitro* analysis of the anti-proliferative effect of AQ and A939572 (an orally available a small molecule inhibitor of SCD1) on lung cancer cells was measured by MTT assay. As shown in Figure 3A and 3B, AQ could inhibit A549 and H460 cell proliferation with IC₅₀ value of 40 and 11.2 $\mu\text{mol/l}$, respectively. A939572 inhibited A549 and H460 cell proliferation with IC₅₀ value of 10 $\mu\text{mol/l}$ and 5.5 $\mu\text{mol/l}$ (Figure 3A and 3B). Importantly, combination treatment of AQ and A939572 under indicated concentration exhibited robust synergistic inhibition on cell proliferation compared to single treatment of AQ (Figure 3C and 3D). Furthermore, the *in vivo* analysis of the synergistic effect about AQ and A939572 was confirmed by A549 lung cancer xenograft mouse models (Figure 3E). Our results showed that co-administration of AQ and A939572 significantly attenuated tumor growth as compared to single treatment of AQ and A939572 (Figure 3F). At the end of treatment, combination of AQ and A939572 achieved robust inhibition of A549 lung tumor growth as evidenced by tumor size and weight (Figure 3G and 3H). Taken together, our results demonstrated that inhibition of SCD1 absolutely enhanced anti-tumor activity of AQ on lung cancer *in vivo* and *in vitro*.

SREBP2 mediates up-regulation of SCD1 induced by AQ

SREBP2 (Sterol regulatory element binding protein 2) could specifically bind to

the sterol regulatory element-1 (SRE1) to promote the expression of cholesterol biosynthesis associated genes.²⁹⁾ Interestingly, our RNA-Seq results showed that AQ could up-regulate the expression of SREBP2 (Table 1). Our following Q-PCR results also confirmed that AQ could significantly up-regulate SREBP-2 in a concentration dependent manner but not SREBP1, PPAR α , or PPAR γ (Figure 4A), which are all classical transcriptional factors of SCD1.³⁰⁾ Fatostatin, a specific inhibitor of SREBP, can impair the activation of SREBP2.³¹⁾ To investigate the relationship of SCD1 and SREBP2 expression in AQ-treated cells, Fatostatin was tested in this study. Our results showed that fatostatin could significantly reverse the up-regulation SCD1 induced by AQ (Figure 4B). In order to further confirm SREBP2 to be involved in up-regulation of SCD1 induced by AQ, two specific shRNAs of SREBP2 were synthesized. Our results showed that both shSREBP2#1 and shSREBP2#2 could effectively knockdown the expression of SREBP2 at mRNA and protein levels (Figure 4C and 4D). Furthermore, SREBP2 knockdown could obviously AQ mediated induction of (Figure 4E). In addition, 3-MA could inhibit the up-regulation of SREBP2 induced by AQ, which implied that increase of autophagosomes could up-regulate the expression of SREBP2 (Figure 4F). Taken together, SREBP2 played key roles in AQ induced up-regulation of SCD1.

Discussion

NSCLC with the most frequent Ras mutation is aggressive and refractory to treatment.²⁾ To date, direct inhibition of the Ras protein still remains challenging³²⁾ that leads to the research for alternative approach for treating Ras-driven cancers. Autophagy, as a highly conserved degradation process, plays a complex role in tumor formation and progression.³³⁾ Recent studies showed that oncogenic Ras mutation tumors require autophagy for their metabolic adaptations and survival in unfavorable tumor microenvironments.³⁴⁾ Therefore, autophagy is a promising opportunity for NSCLC treatment. Indeed, many clinical trials are evaluating chloroquine (CQ) and hydroxychloroquine (HCQ) for cancer treatment as single or in combination with chemotherapeutic agents.³⁵⁾

For now, a number of clinical trials have revealed the promising roles of CQ, an autophagy inhibitor, as a novel anti-tumor drug.³⁶⁾ AQ, as another autophagy inhibitor from the same category has caught lots of attention and demonstrated anti-cancer activity in different tumor cells.^{7-9,37,38)} New modality of AQ using nanoparticles approach have demonstrated superior promise *in vitro* systems when compared with plain AQ.⁷⁾ However, the *in vivo* anti-tumor efficacy of AQ has not been evaluated. Furthermore, AQ or CQ as a single treatment is not sufficient to induce tumor cell death and combination with chemotherapeutic agents could be a viable approach.^{8,36)} Therefore, its limited therapeutic efficacy renders us to further identify the metabolic vulnerabilities and can be potentially used as combinational therapies.

Here using a transcriptomics approach, we for the first time identified SCD1 is highly expressed in A549 lung cancer cell lines upon AQ treatment (Table 1, Figure 2A and 2B). SCD1 plays a key role in tumor lipid metabolism and membrane architecture via providing substrates for the synthesis of phospholipids, triglycerides, cholesteryl esters, and wax esters.³⁹⁾ SCD1 has become an important therapeutic target for cancer treatment.¹⁵⁾ In the present study, our results showed that inhibition of SCD1 indeed enhanced anti-tumor activity of AQ in lung cancer *in vitro* and *in vivo* (Figure 3). It has been reported that activation of autophagy serves as a survival signal when SCD1 is inhibited in colorectal cancer cells, suggesting that combining SCD1 inhibitor with autophagy inhibitors could be a promising anticancer therapy.²⁰⁾ It should be noted that 28c (a small molecule inhibitor of SCD1) inhibits starvation-induced autophagy and SCD1 activity is required for the earliest step of autophagosome formation by providing MUFAs.¹⁸⁾ It's tempting to speculate that SCD1 inhibition could further abolish autophagy machinery in addition to AQ treatment. However, these proposed mechanisms underlying the synergistic effect of AQ and SCD1 inhibition remain to be elucidated.

We further revealed that AQ-induced high expression of SCD1 is elevated through SREBP2 in a dose-dependent manner (Figure 4). Previous investigations have demonstrated that inhibition of lysosomal function could affect endoplasmic reticulum (ER) cholesterol level leading to SREBP2 activation.²⁵⁾ Therefore, AQ-induced autolysosomal-function blockade might also activate SREBP2 via down-regulating cholesterol. Interestingly, 3-MA, a widely used inhibitor of

autophagy via suppressing the formation of autophagosome, could reverse the up-regulation of SREBP2 induced by AQ (Figure 2D and 4F), which implied that there is complicated relationship between autophagy and SREBP2 activation. And the exact mechanisms should be explored in further investigations.

It has been shown that overexpress SREBP-1a could up-regulate SCD1 expression in transgenic mice,⁴⁰⁾ suggesting that SCD1 could be a downstream target of SREBP family members. Moreover, the expression of SREBP2 was positively correlated with that of SCD1 in human colon cancer, and SREBP signaling was also under the control of SCD1 in colon cancer cells.⁴¹⁾ In our present study, genetic knockdown of SREBP2 could substantially suppress SCD1 expression and reverse the up-regulation of SCD1 induced by AQ (Figure 4E). All of which suggested that SREBP2 signaling participated in the regulation of SCD1 induced by AQ, but the mechanisms need be further elucidated in the future.

Conclusion

Repurposing of FDA-approved drugs increasingly attracted more attention in the developing of anti-cancer drugs because of the clinical safety. Our present study revealed that inhibition of SCD1 could be a novel and effective approach for promoting the application of AQ in cancer therapy. However, only one pharmacological inhibitor of SCD1, A939572 was used that limit current study to a certain degree. In the future, we will further evaluate the effects of genetic knockdown of SCD1 in combination with AQ treatment and whether addition of oleic acid. This combinational treatment of inhibition of autophagy and SCD1 should also be

evaluated in other types of NSCLC tumor models.

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Conflict of Interest

The authors declare no conflict of interest.

Reference

1. Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJ, Jr., Wu YL, Paz-Ares L. Lung cancer: current therapies and new targeted treatments. *Lancet*, 389, 299-311 (2017).
2. Bade BC, Dela Cruz CS. Lung Cancer 2020: Epidemiology, Etiology, and Prevention. *Clin. Chest Med.*, 41, 1-24 (2020).
3. Roman M, Baraibar I, Lopez I, Nadal E, Rolfo C, Vicent S, Gil-Bazo I. KRAS oncogene in non-small cell lung cancer: clinical perspectives on the treatment of an old target. *Mol. Cancer*, 17, 33 (2018).
4. Parhizgar AR, Tahghighi A. Introducing New Antimalarial Analogues of Chloroquine and Amodiaquine: A Narrative Review. *Iran J. Med. Sci.*, 42, 115-128 (2017).
5. Maguire A. Amodiaquine hydrochloride in the treatment of chronic discoid lupus erythematosus. *Lancet*, 1, 665-667 (1962).
6. Kersley GD, Palin AG. Amodiaquine and hydroxychloroquine in rheumatoid arthritis. *Lancet*, 2, 886-888 (1959).
7. Parvathaneni V, Kulkarni NS, Chauhan G, Shukla SK, Elbatanony R, Patel B, Kunda NK, Muth A, Gupta V. Development of pharmaceutically scalable inhaled anti-cancer nanotherapy - Repurposing amodiaquine for non-small cell lung cancer (NSCLC). *Mater. Sci. Eng. C Mater. Biol. Appl.*, 115, 111139 (2020).
8. Qiao S, Tao S, Rojo de la Vega M, Park SL, Vonderfecht AA, Jacobs SL, Zhang DD, Wondrak GT. The antimalarial amodiaquine causes autophagic-lysosomal and proliferative blockade sensitizing human melanoma cells to starvation- and chemotherapy-induced cell death. *Autophagy*, 9, 2087-2102 (2013).
9. Salentin S, Adasme MF, Heinrich JC, Haupt VJ, Daminelli S, Zhang Y, Schroeder M. From malaria to cancer: Computational drug repositioning of amodiaquine using PLIP interaction patterns. *Sci. Rep.*, 7, 11401 (2017).
10. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. *Mol. Cancer*, 19, 12 (2020).
11. Fan D, Yu S, Yang Y, Qu S. Pyoluteorin Induces Apoptosis and Autophagy in NSCLC Cells. *Biol. Pharm. Bull.*, 44, 976-983 (2021).
12. Igal RA. Stearoyl-CoA desaturase-1: a novel key player in the mechanisms of cell proliferation, programmed cell death and transformation to cancer. *Carcinogenesis*, 31, 1509-1515 (2010).
13. Wu X, Zou X, Chang Q, Zhang Y, Li Y, Zhang L, Huang J, Liang B. The evolutionary pattern and the regulation of stearyl-CoA desaturase genes. *Biomed. Res. Int.*, 2013, 856521 (2013).
14. Thon M, Hosoi T, Chea C, Ozawa K. Loss of Stearoyl-CoA Desaturase-1 Activity Induced Leptin Resistance in Neuronal Cells. *Biol. Pharm. Bull.*, 40, 1161-1164 (2017).
15. Tracz-Gaszewska Z, Dobrzyn P. Stearoyl-CoA Desaturase 1 as a Therapeutic Target for the Treatment of Cancer. *Cancers (Basel)*, 11, 948 (2019).
16. Roongta UV, Pabalan JG, Wang X, Ryseck RP, Fargnoli J, Henley BJ, Yang WP, Zhu J, Madireddi MT, Lawrence RM, Wong TW, Rupnow BA. Cancer cell dependence on unsaturated fatty acids implicates stearyl-CoA desaturase as a target for cancer therapy. *Mol. Cancer Res.*, 9, 1551-1561 (2011).
17. Kohler K, Brunner E, Guan XL, Boucke K, Greber UF, Mohanty S, Barth JM, Wenk MR, Hafen E. A combined proteomic and genetic analysis identifies a role for the lipid desaturase Desat1 in starvation-induced autophagy in Drosophila. *Autophagy*, 5, 980-990 (2009).
18. Ogasawara Y, Itakura E, Kono N, Mizushima N, Arai H, Nara A, Mizukami T, Yamamoto A. Stearoyl-CoA desaturase 1 activity is required for autophagosome formation. *J Biol Chem*, 289, 23938-23950 (2014).
19. Huang GM, Jiang QH, Cai C, Qu M, Shen W. SCD1 negatively regulates autophagy-induced cell

death in human hepatocellular carcinoma through inactivation of the AMPK signaling pathway. *Cancer Lett.*, 358, 180-190 (2015).

20. Ono A, Sano O, Kazetani KI, Muraki T, Imamura K, Sumi H, Matsui J, Iwata H. Feedback activation of AMPK-mediated autophagy acceleration is a key resistance mechanism against SCD1 inhibitor-induced cell growth inhibition. *PLoS One*, 12, e0181243 (2017).

21. Gao X, Jiang P, Zhang Q, Liu Q, Jiang S, Liu L, Guo M, Cheng Q, Zheng J, Yao H. Peglated-H1/pHGFK1 nanoparticles enhance anti-tumor effects of sorafenib by inhibition of drug-induced autophagy and stemness in renal cell carcinoma. *J. Exp. Clin. Cancer Res.*, 38, 362 (2019).

22. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy*, 3, 542-545 (2007).

23. Liu X, Strable MS, Ntambi JM. Stearoyl CoA desaturase 1: role in cellular inflammation and stress. *Adv. Nutr.*, 2, 15-22 (2011).

24. Miller S, Oleksy A, Perisic O, Williams RL. Finding a fitting shoe for Cinderella: searching for an autophagy inhibitor. *Autophagy*, 6, 805-807 (2010).

25. Eid W, Dauner K, Courtney KC, Gagnon A, Parks RJ, Sorisky A, Zha X. mTORC1 activates SREBP-2 by suppressing cholesterol trafficking to lysosomes in mammalian cells. *Proc. Natl. Acad. Sci. U S A*, 114, 7999-8004 (2017).

26. Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M. Pepstatin, a new pepsin inhibitor produced by Actinomycetes. *J. Antibiot. (Tokyo)*, 23, 259-262 (1970).

27. Igal RA. Roles of StearoylCoA Desaturase-1 in the Regulation of Cancer Cell Growth, Survival and Tumorigenesis. *Cancers (Basel)*, 3, 2462-2477 (2011).

28. Oatman N, Dasgupta N, Arora P, Choi K, Gawali MV, Gupta N, Parameswaran S, Salomone J, Reisz JA, Lawler S, Furnari F, Brennan C, Wu J, Sallans L, Gudelsky G, Desai P, Gebelein B, Weirauch MT, D'Alessandro A, Komurov K, Dasgupta B. Mechanisms of stearyl CoA desaturase inhibitor sensitivity and acquired resistance in cancer. *Sci. Adv.*, 7, eabd7459 (2021).

29. Hua X, Yokoyama C, Wu J, Briggs MR, Brown MS, Goldstein JL, Wang X. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc. Natl. Acad. Sci. U S A*, 90, 11603-11607 (1993).

30. Mauvoisin D, Mounier C. Hormonal and nutritional regulation of SCD1 gene expression. *Biochimie*, 93, 78-86 (2011).

31. Shao W, Machamer CE, Espenshade PJ. Fatostatin blocks ER exit of SCAP but inhibits cell growth in a SCAP-independent manner. *J. Lipid Res.*, 57, 1564-1573 (2016).

32. Vasan N, Boyer JL, Herbst RS. A RAS renaissance: emerging targeted therapies for KRAS-mutated non-small cell lung cancer. *Clin. Cancer Res.*, 20, 3921-3930 (2014).

33. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat. Rev. Cancer*, 17, 528-542 (2017).

34. Kimmelman AC. Metabolic Dependencies in RAS-Driven Cancers. *Clin. Cancer Res.*, 21, 1828-1834 (2015).

35. Verbaanderd C, Meheus L, Huys I, Pantziarka P. Repurposing Drugs in Oncology: Next Steps. *Trends Cancer*, 3, 543-546 (2017).

36. Kimura T, Takabatake Y, Takahashi A, Isaka Y. Chloroquine in cancer therapy: a double-edged sword of autophagy. *Cancer Res.*, 73, 3-7 (2013).

37. Kambey PA, Chengcheng M, Xiaoxiao G, Abdulrahman AA, Kanwore K, Nadeem I, Jiao W, Gao D. The orphan nuclear receptor Nurr1 agonist amodiaquine mediates neuroprotective effects in 6-OHDA Parkinson's disease animal model by enhancing the phosphorylation of P38 mitogen-activated kinase

but not PI3K/AKT signaling pathway. *Metab. Brain Dis.*, 36, 609-625 (2021).

38. Espinoza JA, Zisi A, Kanellis DC, Carreras-Puigvert J, Henriksson M, Huhn D, Watanabe K, Helleday T, Lindstrom MS, Bartek J. The antimalarial drug amodiaquine stabilizes p53 through ribosome biogenesis stress, independently of its autophagy-inhibitory activity. *Cell Death Differ.*, 27, 773-789 (2020).

39. Luo X, Cheng C, Tan Z, Li N, Tang M, Yang L, Cao Y. Emerging roles of lipid metabolism in cancer metastasis. *Mol. Cancer*, 16, 76 (2017).

40. Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J. Clin. Invest.*, 98, 1575-1584 (1996).

41. Qin XY, Kojima S. Inhibition of Stearoyl-CoA Desaturase-1 Activity Suppressed SREBP Signaling in Colon Cancer Cells and Their Spheroid Growth. *Gastrointest. Disord.*, 1, 191-200 (2019).

Figure 1. AQ inhibited autophagic-lysosomal function in A549 lung cancer cell.

(A) mRFP-GFP-LC3 puncta formation assay was used to analyze the autophagic flux in the presence or absence of AQ in lung cancer cells. AQ treatment increased the number of yellow puncta indicating autophagosomal or autophagic-lysosomal localization. (B) Western-blotting assay results showed that AQ treatment induced the increase of LC3BII and p62 in the dose- and time-dependent manners.

Figure 2. AQ induces up-regulation of SCD1 in A549 lung cancer cells.

(A) Q-PCR results showed that AQ induced SCD1 expression in a dose-dependent manner. (B) Western-blotting results showed that AQ upregulates the protein level of SCD1 in the dose- and time-dependent manners. (C) Q-PCR results showed that 3-MA partially reversed the up-regulation of SCD1 induced by AQ. (D) Western-blotting results showed that 3-MA partially reversed the enrichment of LC3BII and the up-regulation of SCD1 induced by AQ. (E) Western-blotting results showed that OA promoted the accumulation of LC3BII and partially reversed AQ induced SCD1 elevation. (F) Western-blotting results showed that Pepstatin A enhanced AQ-induced SCD1 upregulation. Significant differences were denoted by ** for $p < 0.01$ and *** for $p < 0.001$.

Figure 3. SCD1 inhibition potentiates the anti-tumor activity of AQ *in vitro* and *in vivo*.

MTT assay was performed to measure the inhibitory activity of AQ and A939572 (SCD1 inhibitor) on A549 and H460 lung cancer cells *in vitro*. (A and B) AQ and A939572 inhibited A549 and H460 cell proliferation. (C and D) A939572 enhanced the inhibitory effect of AQ on both A549 and H460 cell proliferation. Lung cancer xenograft mouse model was successfully established to investigate the anti-tumor activity of AQ and A939572 *in vivo*. (E) Schematic diagram illustrated the therapeutic schedule. (F, G and H) A939572 enhanced the inhibitory effect of AQ on tumor growth (size and weight) *in vivo*. Significant differences were denoted by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Figure 4. SREBP2 mediated up-regulation of SCD1 induced by AQ. (A) Q-PCR results showed that AQ induced up-regulation of SREBP2, but not SREBP1, PPARα, or PPARγ. (B) Western-blotting results showed that Fatostatin (a specific inhibitor of SREBP activation) block the up-regulation of SCD1 induced by AQ. (C and D) Q-PCR and Western-blotting results showed that shSREBP2#1 and shSREBP2#2 could significantly knock-down the expression of SREBP2. (E) Western-blotting results showed that genetic knockdown of SREBP2 could abolish the up-regulation of SCD1 induced by AQ. (F) Q-PCR result showed that 3-MA could inhibit the up-regulation of SREBP2 induced by AQ. Significant differences were denoted by * for $p < 0.05$ and *** for $p < 0.001$.

Table 1. Gene expression profiles associated with cholesterol biosynthesis and fatty acid biosynthesis after AQ treatment in lung cancer cell

Gene name	Entrez ID	Fold Change	p-value
ACAA1 (acetyl-CoA acyltransferase 1)	30	-2.2	0.012
ACACA (acetyl-CoA carboxylase alpha)	31	-1.9	0.015
ACAT2 (acetyl-CoA acetyltransferase 2)	39	6.6	0.0001
ACOT7 (acyl-CoA thioesterase 7)	11332	-9	0.003
ACSL3 (acyl-CoA synthetase long chain family member 3)	2181	1.9	7.10E-05
CYP2D6 (cytochrome P450 family 2 subfamily D member 6)	1565	4.6	0.039
CYP4F12 (cytochrome P450 family 4 subfamily F member 12)	66002	5.5	0.04
CYP51A1 (cytochrome P450 family 51 subfamily A member 1)	1595	2.3	1.40E-07
DHCR7 (7-dehydrocholesterol reductase)	1717	9.2	0.0001
DHCR24 (24-dehydrocholesterol reductase)	1718	2.8	0.005
EBP (emopamil binding protein (sterol isomerase)	10682	2.4	0.022
ELOVL1 (ELOVL fatty acid elongase 1)	64834	-5.4	0.009
ELOVL5 (ELOVL fatty acid elongase 5)	60481	1.2	0.001
ELOVL6 (ELOVL fatty acid elongase 6)	79071	2.1	0.0002
ELOVL7 (ELOVL fatty acid elongase 7)	79993	2.5	0.049
FADS1 (fatty acid desaturase 1)	3992	4.5	0.001
FADS2 (fatty acid desaturase 2)	9415	4.4	3.70E-05
FASN (fatty acid synthase)	2194	3.1	0.004
FDFT1 (farnesyl-diphosphate farnesyltransferase 1)	2222	11.3	0.0001
FDPS (farnesyl diphosphate synthase)	2224	5.8	0.005
HADH (hydroxyacyl-CoA dehydrogenase)	3033	5.6	0.023
HADHA (hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha)	3030	7.5	0.038
HELZ2 (helicase with zinc finger 2)	85441	-1.7	0.002
HES1 (hes family bHLH transcription factor 1)	3280	-2	0.013
HHEX (hematopoietically expressed homeobox)	3087	1.6	0.004
HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase)	3156	1.5	5.70E-05
HMGCS1 (3-hydroxy-3-methylglutaryl-CoA synthase 1)	3157	4.7	0.009
HNF1A (HNF1 homeobox A)	6927	2.1	0.03
HSD17B7 (hydroxysteroid 17-beta dehydrogenase 7)	51478	2.8	1.50E-05
HSD17B12 (hydroxysteroid 17-beta dehydrogenase 12)	51144	-2.05	0.037
IDI1 (isopentenyl-diphosphate delta isomerase 1)	3422	3.5	0.007
INSIG1 (insulin induced gene 1)	3638	7.2	0.004
LBR (lamin B receptor)	3930	1.1	0.037
LSS (lanosterol synthase)	4047	7.9	0.017
MNX1 (motor neuron and pancreas homeobox 1)	3110	1.4	0.028
MSMO1 (methylsterol monooxygenase 1)	6307	11.2	3.30E-06
MVD (mevalonate diphosphate decarboxylase)	4597	4.8	0.044

MVK (mevalonate kinase)	4598	7.2	0.005
NFYC (nuclear transcription factor Y subunit gamma)	4802	-1.1	0.016
NSDHL (NAD(P) dependent steroid dehydrogenase-like)	50814	3.3	0.009
NR5A2 (nuclear receptor subfamily 5 group A member 2)	2494	1.4	0.015
PPT1 (HBS1 like translational GTPase)	5538	-4.3	0.0004
SAR1B (secretion associated Ras related GTPase 1B)	51128	-1.2	0.005
SCD1 (stearoyl-CoA desaturase 1)	6319	3.9	0.003
SEC24A (SEC24 homolog A, COPII coat complex component)	10802	-1.5	0.007
SEC24C (SEC24 homolog C, COPII coat complex component)	9632	1.7	0.021
SP1 (Sp1 transcription factor)	6667	-5.4	0.009
SREBP2 (sterol regulatory element binding transcription factor 2)	6721	5.1	0.008
SQLE (squalene epoxidase)	6713	2.9	0.04
TGS1 (trimethylguanosine synthase 1)	96764	-2.3	0.029
TM7SF2 (transmembrane 7 superfamily member 2)	7108	5.8	0.02
UCP2 (uncoupling protein 2)	7351	-11.4	4.00E-05