SUPPLEMENTARY INFORMATION

Hydroxychloroquine based chemical drug for combination therapy with 5-Fu for inhibiting the pathway of Akt/mTOR in autophagy process on colon cancer

Zhi Liao1†, Yan Chen2†, Lizhu Han3,4, Dongke Yu3,4, Tingting Shi3,4, Zhixi Liu2, Hongtao Xiao2,[4*]

1 Department of Gynaecology and Obstetrics, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu 610072, Sichuan, China

2 Department of Clinical Pharmacy, Sichuan Cancer Hospital&Institute, Sichuan Cancer Center, School of Medicine, University of Electronic Science and Technology of China, Chengdu 610041, China

3 Department of Pharmacy, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu 610072, Sichuan, China

4 Personalized Drug Therapy Key Laboratory of Sichuan Province, Chengdu 610072, Sichuan, China

* Correspondence

Professor Hongtao Xiao,

Sichuan Cancer Hospital & Institute, Sichuan Cancer Centre, School of Medicine, University of Electronic Science and Technology of China, No. 55, Section 4, Renmin Nan Road, Chengdu, Sichuan 610041, PR China.

E-mail: xht927@163.com

Tel./ Fax: +86 028 85420338

† These authors made equal contributions to this work.

Supplementary S1 The antitumor activity of AHQ compared with HCQ *in vitro*

Cellular cytotoxicity of AHQ and HCQ respectively were measured on human colon cancer cell lines, human acute monocytic leukemia cell line, human mammary carcinoma cell line, human lung cancer cell lines, human neuroblastoma cell line, human ovarian cancer cell line and non-cancer cell lines by the standard CCK-8 assay. Cells were treated with various concentrations of AHQ or HCQ alone in 48 hours. The results of IC_{50} were calculated by graphphad prism 6.0. The data of IC_{50} were shown in table S1.

Supplementary S1 The IC_{50} of AHQ and HCQ respectively in ten cell lines

Type of cells	Cell Lines	IC_{50}(μg/ml)	
		AHQ	HCQ
Human colon cancer cells	HT29	0.69±0.03*	10.08±0.12
Human colon cancer cells	HCT116	3.02±0.11*	17.60±1.34
Human acute monocytic leukemia cells	THP-1	9.57±0.34	9.99±0.51
Human mammary carcinoma cells	MDA-MB-231	7.87±0.42	10.21±0.46
Human lung cancer cells	HCC827	10.82±0.72	9.52±0.99
Human lung cancer cells	A549	13.05±0.85	14.83±1.30
Human neuroblastoma cells	SH-SY5Y	8.49±0.88*	15.66±1.54
Human ovarian cancer cells	SKOV-3	11.25±1.56	14.74±1.36
Human embryonic kidney cells	293A	12.2±0.96	16.82±0.46
Human umbilical vein endothelial cells	HUVEC	18.35±1.10	19.80±1.73

Data obtained are presented as mean ± SD of three independent experiments triplicate experiments.*p<0.05, **p<0.01, versus HCQ.

Supplementary S2 The rightly specific process of AHQ synthesis

Hydroxychloroquine sulfate dissolved in water was mixed with 5 mol/L sodium hydroxide and stirred on the ice-bath. The formed white oil was heated up to room temperature and extracted with ethyl acetate three times. The ethyl acetate was combined and washed with saturated sodium chloride solution and water, dried with anhydrous sodium sulfate and evaporated in vacuum to obtain hydroxychloroquine. Then, the α-linolenic, N-Methylmorpholine, DMAP, and EDC were dissolved in methylene dichloride and agitated for 0.5 h on the ice-bath and under the protection of nitrogen. To the mixture, hydroxychloroquine were added and continuously stirred for 24 h at room temperature. The reaction mixture was added with methylene dichloride (100 mL), heated up to 50℃ and washed with hydrochloric acid solution (1 mol/L), saturated sodium chloride solution and water, successively. Then the reaction mixture was dried with anhydrous sodium sulfate and evaporated in vacuum to obtain brown oil. Then the brown oil was dissolved methyl tert-butyl ether and then transferred to a separatory funnel. The solution was washed with 1 mol/L HCl (200mL). The oil layer between methyl tert-butyl ether and aqueous layer was separated and redissolved in methanol. The solution was decolored by activated carbon and concentrated in vacuo to obtain the brown oil. The brown oil was purified by silica-gel column chromatography using 200-300 mesh silica gel. The eluent was methylene dichloride/methanol with volume ratio from 50:1 to 15:1. The α-linolenic acid conjugated hydroxychloroquine was obtained by evaporating methylene dichloride/methanol. The route of AHQ synthesis was supplied in Figure 2.

Supplementary S3 The process of flow cytometric apoptosis analysis

HT-29 and HCT116 cells were seeded in a 6-well plate at a density of 1×10^6 cells per well for 24h. HT-29 cells were separately treated with 10μmol/L of 5-Fu, 10μmol/L of AHQ and 5-Fu+AHQ (with ratio 1:1) in DMEM medium, while HCT116 cells with 10μmol/L of 5-Fu, 80μmol/L of AHQ and 5-Fu+AHQ (with ratio 1:8)

respectively. After 48-72 h, cells were trypsinized, washed, and centrifuged. The collected cells were suspended. 5 μL of annexin-V FITC and 5 μL of PI were added. The mixture was incubated for 15 min in the dark and then was analyzed by flow cytometer (BD FACS Canto II).

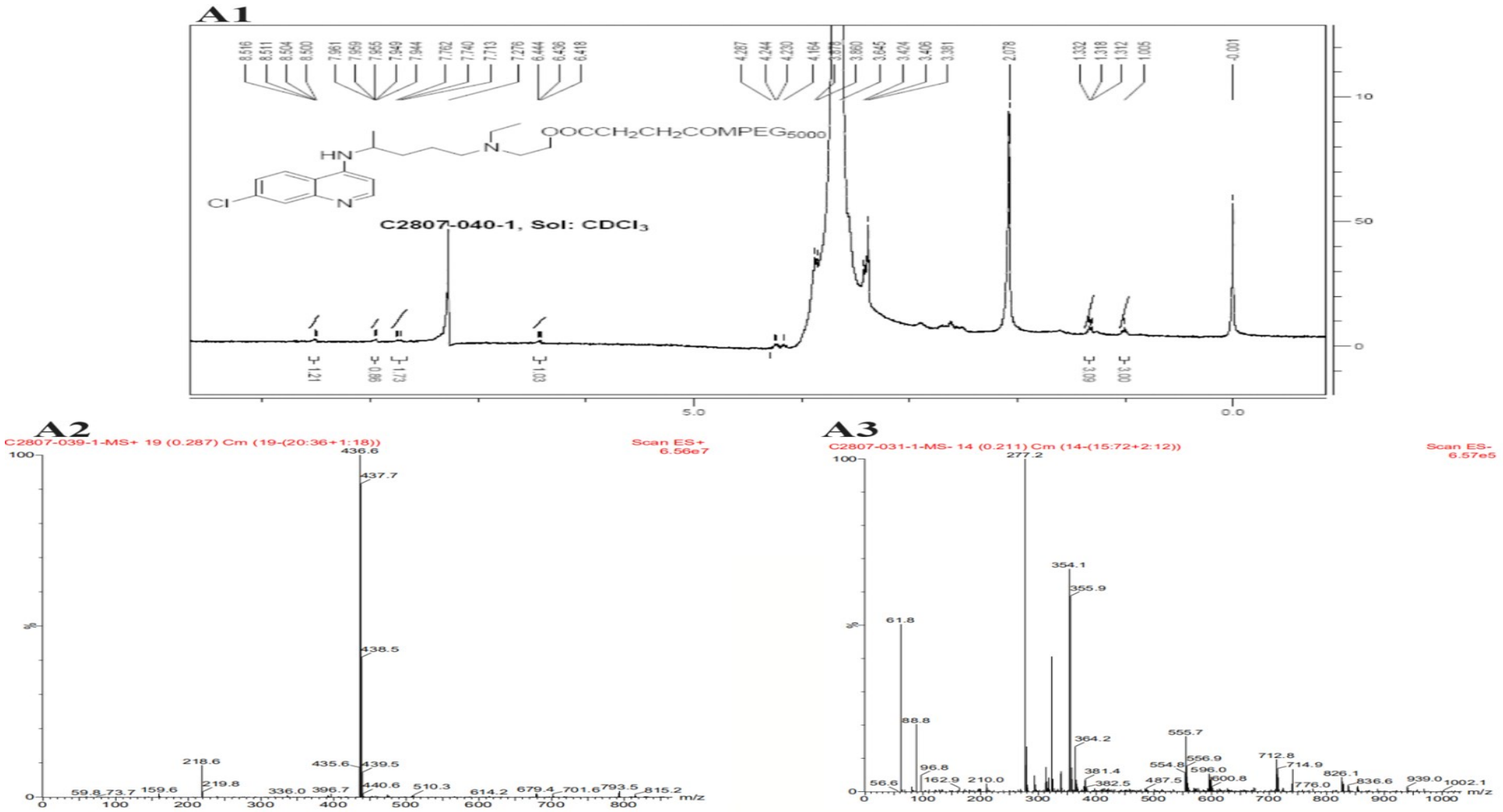
Supplementary S4 The process of flow cytometric cell cycle analysis

HT-29 and HCT116 cells (1×10^6 cells per well) were treated with 5-Fu alone, AHQ or their combination for 48h and 72h respectively. After the treatment, cells were converted into single cell suspension and fixed with 75% ethanol overnight at -20 °C. Cells were then centrifuged and resuspended in PBS for 2h followed by RNase A (20μm) treatment for 2h at 37°C. PI was added and incubated for another 20min at room temperature. Flow cytometric analysis was performed immediately using a BD FACS Verse flow cytometer using FlowJo software (FlowJo, OR, USA) .

Supplementary S5 The process of *in vivo* antitumor analysis

The mice were housed under SPF conditions in facilities. When the tumour reached approximate 100 mm^3, mice were randomly divided into four groups (5 mice per group) and were respectively treated with negative group (Vehicle), 5-Fu(25mg/kg), AHQ(50mg/kg) and 5-Fu+AHQ (5-Fu(25mg/kg), AHQ (50mg/kg)). Tumour size was measured by a slide calliper, and tumour volume was also calculated every three days. The tumour volumes were calculated. After mice were scarified, tumors were weighted and the tumour growth inhibition rate was calculated. The experiments were performed according to institutional guidelines and approved by the Institution Animal Care and Use Committee of Sichuan Provincial People's Hospital. Disposal of animal carcasses was done by a professional company (Dashuo Biotechnology Company, Ltd., Chengdu, Sichuan, China).

Supplementary S6 **Figure S6. The H-NMR of chemical conpound A1, the mass-spectropy of A2 and A3, respectively.**

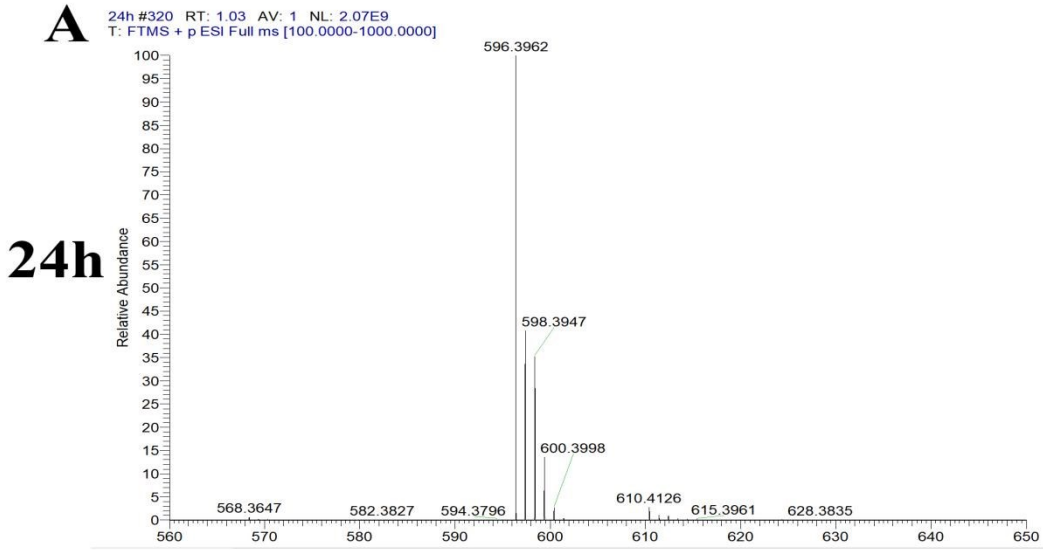


Supplementary S7 The ^{1}H-NMR spectral characterization of AHQ

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.53 (d, *J* = 5.6 Hz, 1H), 7.99 (d, *J* = 2.2 Hz, 1H), 7.76 (d, *J* = 9.0 Hz, 1H), 7.36 (dd, *J* = 9.0, 2.2 Hz, 1H), 6.43 (d, *J* = 5.7 Hz, 1H), 5. 43-5.30(m, 6H), 4.17 (t, *J* = 6.1 Hz, 2H), 3.74 (dd, *J* = 12.1, 6.2 Hz, 1H), 2.84 - 2.79 (m, 5H), 2.74 (t, *J* = 6.2 Hz, 2H), 2.53-2.53 (m, 3H), 2.35 (t, *J* = 7.6 Hz, 1H), 2.27 (t, *J* = 7.6 Hz, 2H), 2.06 (dq, *J* = 12.9, 7.1 Hz, 6H), 1.38-1.27 (m, 17H), 1.00 (dt, *J* = 18.8, 7.3 Hz, 5H), 0.92- 0.84 (m, 1H).

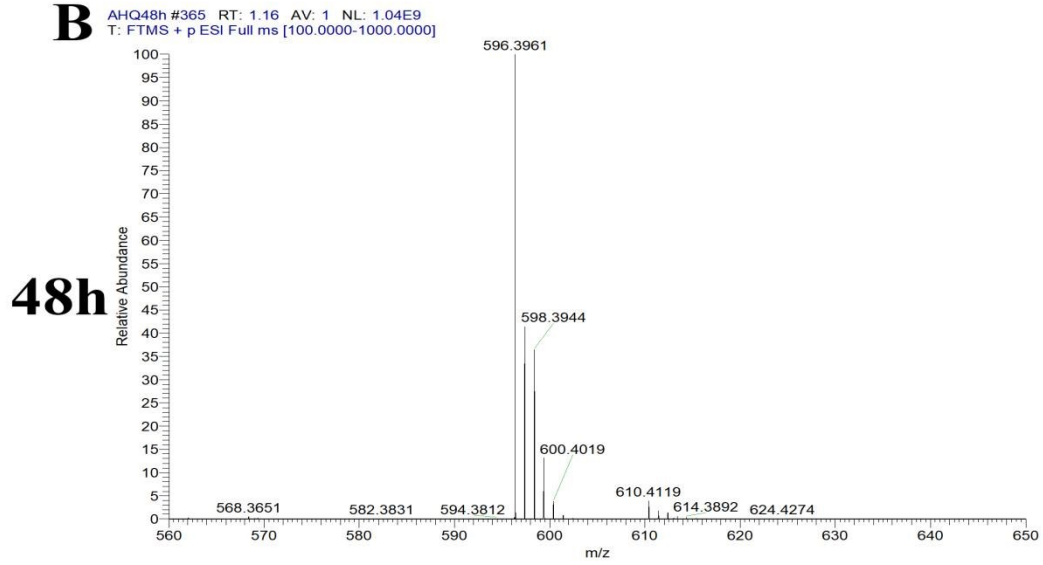
Supplementary S8 The stability of ester bond in AHQ of 24h, 48h and 72h *in vitro*

A



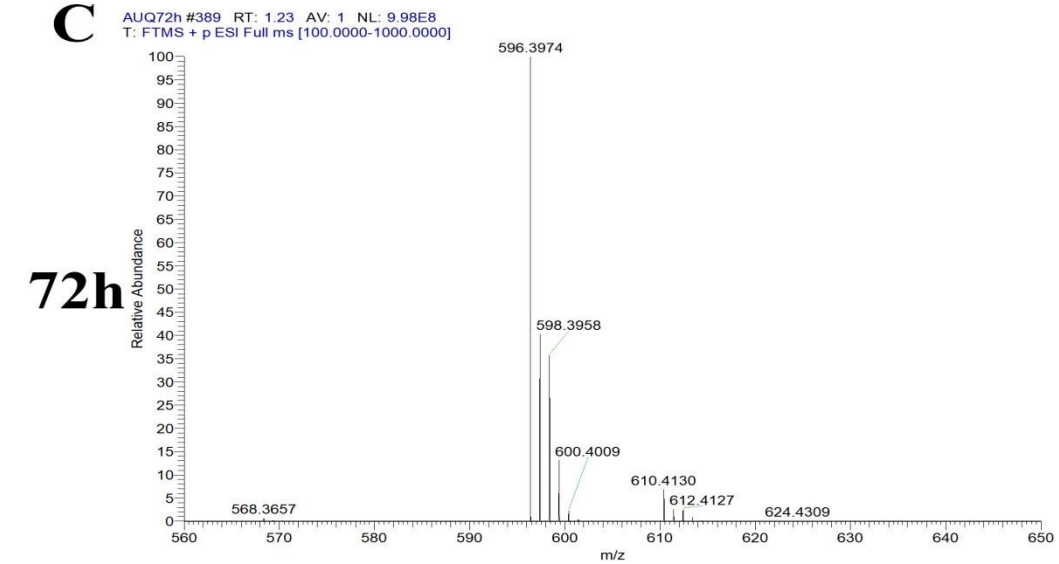
24h

B



48h

C



72h

Supplementary S9 Combination index of AHQ and 5-Fu with ratio 1:1 at different therapeutic time with different concentration on HT-29 cell line.

Time/Total Dose (μmoL)		5	10	20	40	80
24h	Effect(%)	19.20±16.12	30.40±17.53	41.46±14.35	51.54±11.83	60.62±10.90
	CI	1.06	0.65	0.63	0.76	0.99
48h	Effect(%)	42.77±7.34	55.87±7.53	65.18±11.56	73.46±9.72	82.18±7.75
	CI	1.03	0.64	0.64	0.70	0.69
72h	Effect(%)	58.59±6.63	67.23±1.23	73.66±9.27	80.46±9.55	88.42±5.79
	CI	1.06	0.99	0.99	1.03	1.01

Data obtained are presented as mean ± SD of three independent experiments triplicate experiments.

Supplementary S10 Combination index of AHQ and 5-Fu with ratio 8:1 at different therapeutic time with different concentration on HCT116 cell line.

Time/Total Dose (μmoL)		11.25	22.5	45	90	180
24h	Effect(%)	25.76±6.41	32.81±6.48	42.71±4.88	58.39±3.38	96.29±1.10
	CI	1.07	0.64	0.91	1.24	0.43
48h	Effect(%)	40.34±6.61	44.26±5.16	52.33±5.84	83.64±1.50	95.46±2.15
	CI	1.50	1.68	1.46	0.77	0.63
72h	Effect(%)	57.43±13.20	64.41±8.45	76.24±7.77	89.66±4.78	96.76±1.25
	CI	0.69	0.94	0.99	0.80	0.59

Data obtained are presented as mean ± SD of three independent experiments triplicate experiments.

Supplementary S11 The tumor volume of mice in each group during therapeutic days

Days	Control	5-Fu	AHQ	5-Fu+AHQ
0	107.48±48.94	93.06±80.46	90.15±57.25	85.66±70.17
3	153.07±77.99	128.09±103.44	139.08±101.61	94.69±90.00
6	175.34±97.10	166.88±124.12	169.72±109.36	106.87±103.31
9	237.17±103.09	286.10±172.71	206.19±131.01	135.49±111.70
12	330.93±134.90	365.79±201.13	268.42±201.59	138.48±96.29
15	443.39±158.21	390.31±294.64	355.50±225.90	164.33±101.65*
18	636.21±188.70	468.93±289.78	525.58±245.86	166.65±150.20**#$
21	771.43±298.61	467.76±318.97	604.60±405.16	197.83±165.50**$
24	842.97±286.29	668.14±444.87	585.51±252.46	225.12±198.70**#
27	1154.56±229.68	786.89±408.45	758.82±359.18	297.42±252.99**#$
30	1361.58±298.89	916.34±374.56*	1007.23±413.85	362.21±241.45**#$$
33	1838.82±330.16	1181.98±365.86**	1145.13±310.63**	473.43±275.09**##$$

*P＜0.05, **P＜0.01, versus control； #P < 0.05, ##P < 0.01, versus 5-Fu; $P < 0.05, $$P < 0.01, versus AHQ; &P < 0.05, &&P < 0.01, versus 5-Fu+AHQ.