

Cite this: *RSC Adv.*, 2018, 8, 40974

Synthesis and anti-glioblastoma effects of artemisinin-isothiocyanate derivatives†

Chan Myae Nyein,^{‡abc} Xiaolin Zhong,^{‡a} Junfeng Lu,^b Huijuan Luo,^a Jiamin Wang,^a Simona Rapposelli,^{id ef} Mingtao Li,^d Ying Ou-yang,^g Rongbiao Pi^{*bd} and Xixin He^{id *a}

A series of novel artemisinin (ART) derivatives containing an isothiocyanate (ITC) group were synthesized. All the compounds showed more potent anti-tumor effects than those of parent dihydroartemisinin (DHA) towards glioblastoma multiforme U87 *in vitro*. Among them, **5b** had the strongest cytotoxic activity which exerted its effects in a concentration-dependent but not time-dependent manner (IC₅₀ 7.41 μM for 24 h, 7.35 μM for 72 h). Pyknosis was observed in **5b**-treated U87 cells. Multiple intrinsic apoptotic pathways were induced by **5b** including the upregulation of caspase 9, the release of cytochrome c, an increase of the proapoptotic protein Bax, a decrease of the anti-apoptotic protein Bcl 2, and the activation of execution pathways by the upregulation of caspase 3. In addition to apoptosis, an autophagic mechanism was also involved in **5b**-induced cytotoxicity to human GBM U87 cells by upregulating the expression of LC3-II and downregulating p62. Furthermore, **5b** also significantly attenuated the migration of U87 cells. Therefore, our results suggest that **5b** may be a promising molecule for the further development of a novel drug for the treatment of glioblastoma.

Received 2nd October 2018
Accepted 19th November 2018

DOI: 10.1039/c8ra08162j

rsc.li/rsc-advances

Introduction

Glioblastoma multiforme (GBM) is the most prevalent and severe primary central nervous system tumor. Even when using conventional treatments such as surgery, radiation therapy and chemical treatment, the average survival time is less than 15 months.¹ The average occurrence rate of GBM is 3.19/100 000 population, and the median age of diagnosis is 64 years old. The occurrence is higher in men than in women.² Therefore, the discovery of effective therapy for GBM is an area of interest.

Artemisinin (ART) and its derivatives (collectively termed as artemisinins (ARTs)) are sesquiterpene lactones isolated from sweet wormwood (*Artemisia annua*), which are used as a remedy for fevers and chills in Chinese Traditional Medicine.³ The endoperoxide bridge of ARTs is very important for their

activities; they are still the first-line drug for malarial treatment. ARTs have not only antimalarial property but also anticancer effects.^{4,5} It has been confirmed that dihydroartemisinin (DHA), the active metabolite of ARTs, showed cytotoxic activity on ten human glioma cell lines through the induction of autophagy.⁶ DHA also exerted an anti-tumor role through induced ROS-mediated mitochondrion and ER stress apoptotic pathways as well as autophagic cell death in human GBM cells.⁷ Although ART and its derivatives are extremely effective in their antimalarial role, they are less potent as anticancer agents, especially as monotherapy drugs because of their mild activity and short elimination half-life.⁸ Therefore, to improve the clinical outcome of ARTs, future efforts should be focused on the combination of longer-acting drugs for maximum efficacy or the development of potent derivatives.^{9–11}

Naturally occurring isothiocyanates (ITCs), which are generated by the enzymatic hydrolysis of glucosinolates, are abundant in cruciferous vegetables (Fig. 1).^{12–14} It has been proven that some natural and synthetic ITCs have anticancer activities with multiple targets and mechanisms. ITCs can enhance the antitumor efficacy of other drugs, or act as covalent inhibitors.¹⁵ ITCs alone are able to suppress the growth of various cancer cell lines such as non-small-cell lung cancer cells, human malignant astrocytoma cells, and HCT 116 human colon cancer cells.^{16–20} They exert their effects mainly by increasing the production of reactive oxygen species (ROS), induction of apoptosis, induction of cell cycle arrest and so on.^{18–23} However, the exact mechanisms of ITCs against tumors are still not clear. Some research uncovered that aryl

^aSchool of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, China. E-mail: mark07@gzucm.edu.cn

^bSchool of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, China. E-mail: pirb@mail.sysu.edu.cn

^cBiotechnology Research Department, Ministry of Education, Kyaukse, Myanmar

^dGuangdong Provincial Key Laboratory of Brain Function and Disease, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China

^eDepartment of Pharmacy, University of Pisa, Via Bonanno, 6, 56126 Pisa, Italy

^fInterdepartmental Research Center for Biology and Pathology of Aging, University of Pisa, Via Bonanno, 6, 56126 Pisa, Italy

^gDepartment of Pediatrics, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ra08162j

‡ These authors contributed equally to this work.



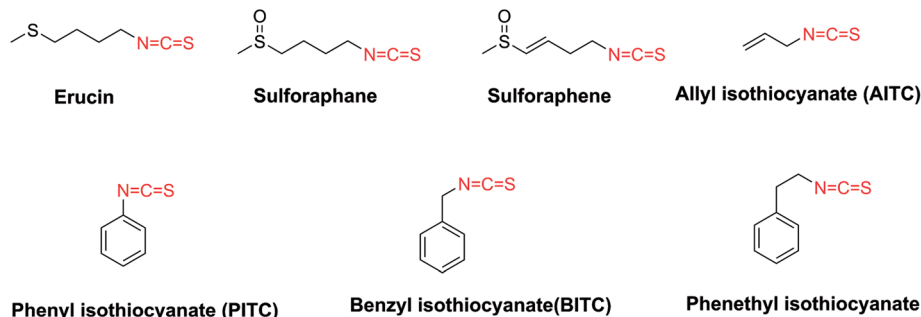


Fig. 1 The representatives of naturally occurring isothiocyanates.

isothiocyanates (e.g., benzyl isothiocyanate (BITC), phenyl isothiocyanate (PITC)) and allyl isothiocyanates (AITC) (Fig. 1) act as H_2S donor agents.^{24,25} H_2S as the third most important gasotransmitter is involved in the development of cancer by shortening the cell cycle and inducing proliferation.^{26,27}

Based on those studies, we hypothesized that artemisinin-based isothiocyanates might be more efficacious compounds towards human GBM cells with multiple mechanisms. In this study, the synthesis, bio-evaluation and mechanism of artemisinin-based isothiocyanates against GBM *in vitro* have been carried out, and we found a novel DHA-ITC derivative **5b** to have potent GBM cell toxicity.

Results and discussion

Chemistry

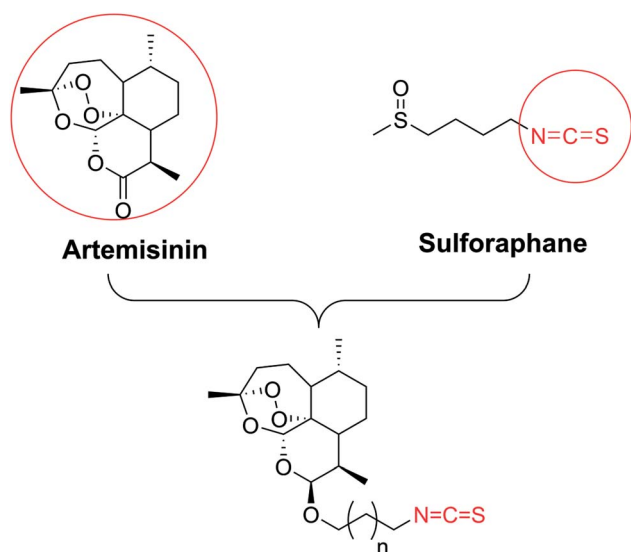
The synthetic strategy employed for the synthesis of the target compounds is depicted in Scheme 1. A series of novel hybrids were afforded by linking DHA at C-10 and an isothiocyanate group. The detailed synthetic route was described in Scheme 2. The DHA derivatives **4a–c** were synthesized according to the literature.^{28,29} The reaction of DHA with the corresponding

bromo-substituted alkanol in CH_2Cl_2 by using boron trifluoride ethyl ether ($\text{BF}_3 \cdot \text{Et}_2\text{O}$) as a catalyst afforded substituted ART enantiomers. **1a–c** were obtained by silica gel column chromatography elution with petroleum ether/ethyl acetate (97 : 3) as the β configuration (indicated the coupling constants between 9-H and 10-H in ^1H NMR, $J_{9,10} = 3\text{--}4$ Hz).³⁰ **1a–c** were then converted to **4a–c** via an azidation and reduction reaction. Finally, the intermediates **4a–c** bearing an amino group reacted with carbon disulfide (CS_2) in dried THF³¹ to afford the target compounds **5a–c**. All the synthesized compounds were purified by silica gel column chromatography.

The structures of compounds **5a–c** were elucidated on the basis of ^1H NMR, ^{13}C NMR and HR-ESI-MS spectra. In a typical example, the HR-ESI-MS of compound **5b** displayed a peak at m/z 384.1802, corresponding to the quasimolecular ions $[\text{M} + \text{H}]^+$, confirming the molecular formula of compound **5b** as $\text{C}_{19}\text{H}_{29}\text{NO}_5\text{S}$. In the ^1H -NMR spectrum of **5b**, the signals of one singlet at δ 1.42 and two doublets at δ 0.89 and 0.94 could be attributed to Me-14, Me-16 and Me-15, respectively. The doublet peak appearing at δ 4.78 (d, $J = 3.2$ Hz) indicated H-10 in the α configuration.³⁰ In the ^{13}C -NMR spectrum, there were 19 peaks appearing in the range from δ 12 to 130 ppm. Among them, a weak peak appearing at δ 129.5 could be ascribed to the carbon of the isothiocyanate ($-\text{N}=\text{C}=\text{S}$). The other NMR characterizations have been assigned through the comparison of relative data in literature.^{32,33}

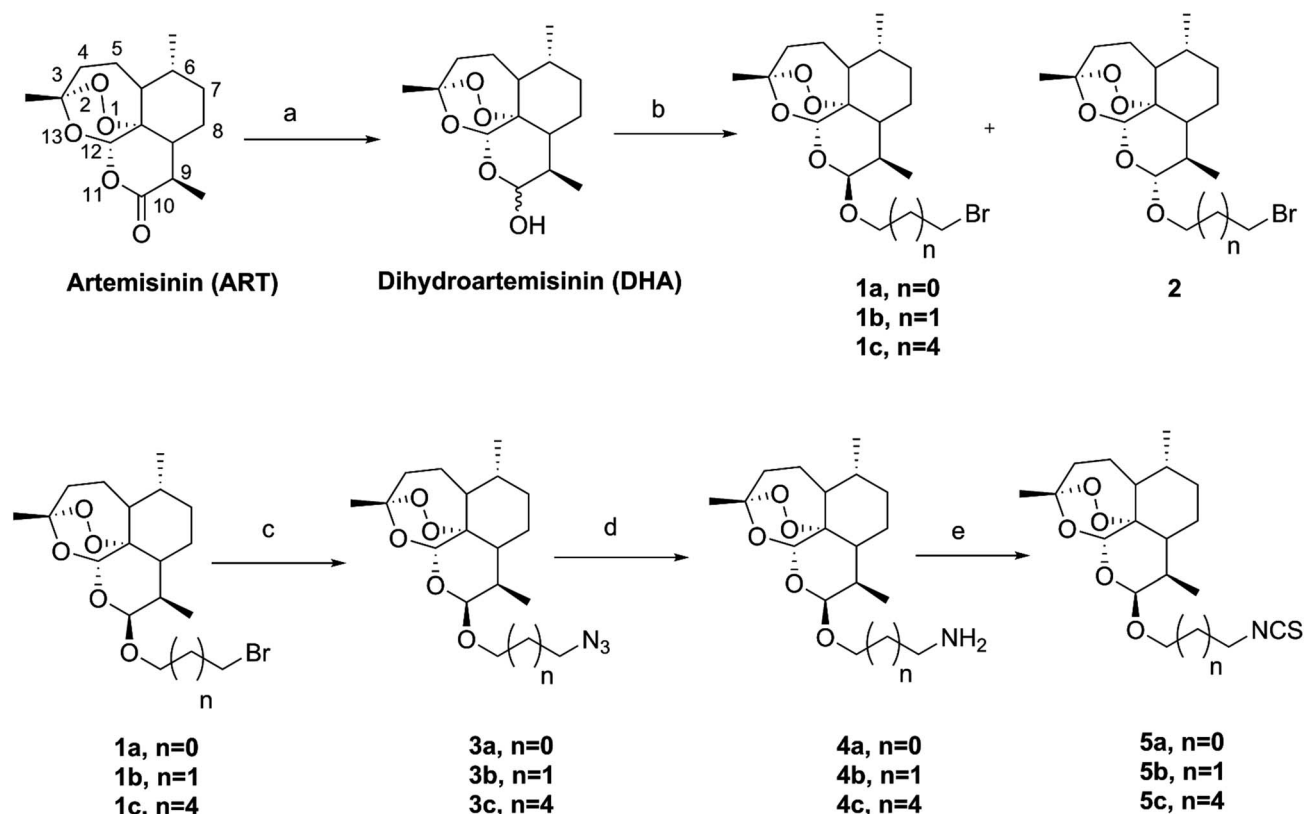
Biological evaluation

Artemisinin-based isothiocyanate derivatives induced cytotoxicity in glioblastoma U87 cells. The cytotoxicity of artemisinin-based isothiocyanate derivatives on human glioblastoma U87 cells was firstly tested by MTT assay. U87 cells were exposed to various concentrations of compounds for 24 h, and all compounds could induce cell death by MTT assay (Table 1). Cells shrunk and fragmented when exposed to compounds under the phase-contrast microscope (Fig. 2). Among all the compounds, **5b** showed the most cytotoxic activity with an IC_{50} value of 7.41 μM and was thus selected for further experiments. As shown in Fig. 3, **5b** reduced the viability of GBM cells in a concentration-dependent manner. To evaluate the effects of **5b** on the proliferative cancer cell line, U87 glioblastoma cells were exposed to various concentrations of **5b** for different durations. **5b** significantly suppressed the survival of U87 cells



Scheme 1 The design strategy.





Scheme 2 The synthetic route of artemisinin-isothiocyanate derivatives **5a–c**. Reagents and conditions: ^aNaBH₄/MeOH, 0 °C, 2 h, 94%; ^bBF₃·Et₂O/CH₂Cl₂, bromo-substituted alkanol; ^cNaN₃/NaI/DMF, 60 °C, 4 h, 85–98%; ^d(1) (Ph)₃P/THF, 60 °C, 2 h; (2) H₂O, 70 °C, 2 h, 65–91%; ^eCS₂/THF/Et₃N, 0 °C–r.t., 30 min, then AcCl, 0 °C–r.t., 30 min, 49–76%.

and the inhibitory efficiency was stronger than that of DHA (see Table 1 and Fig. 3).

Artemisinin-based isothiocyanate derivative 5b inhibits cell migration. Cell migration is a landmark of cancer invasion and metastasis, immune responses, angiogenesis, wound repair, and embryonic morphogenesis.³⁴ Wound-healing assay is

a well-established test of cancer cell migration. To investigate the effects of **5b** on wound-healing phenomena, cells were exposed to 4 μM (around half of the IC₅₀) of **5b** for 0–72 h.³⁵ The migration of U87 cells was reduced significantly by **5b** (Fig. 4).

Artemisinin-based isothiocyanate derivative 5b induces apoptosis in U87 cells. To determine the cell death pathway, the U87 cells were treated with two doses (8 and 16 μM) of **5b**, which were selected to determine the apoptotic effects of **5b** in a concentration-dependent manner. After 16 h of incubation, apoptotic markers of treated cells were analyzed with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI. Annexin V detects phosphatidylserine (PS) on the cell membrane during apoptosis. Propidium iodide (PI) stains the DNA of apoptotic and necrotic cells. As shown in Fig. 5A, **5b** induced a higher proportion of early apoptotic and necrotic cells. The nuclear morphological changes were examined by DAPI staining. Pyknosis and nuclear shrinkage were observed in **5b**-treated cells (Fig. 5B).

To support the fact that apoptosis was involved in the cytotoxic effects of **5b** on GBM cells, we further investigated the caspase family apoptotic proteins and the Bcl2 family apoptotic proteins. As there are three classical apoptotic pathways, we investigated intrinsic and execution pathways. As shown in Fig. 6, the caspase family apoptotic proteins were induced in U87 cells treated with various concentrations of **5b** (4–16 μM) and DHA (120 μM) for 16 h. Caspase 9 and cytochrome *c* were induced in U87 cells treated with **5b**. It was assumed that

Table 1 IC₅₀ values (μM) against human U87 cells

Compounds	<i>n</i>	R	IC ₅₀ ^a
4b	1	–NH ₂	>120
4c	4	–NH ₂	118.26 ± 15.12
5a	0	–N=C=S	27.32 ± 3.65
5b	1	–N=C=S	7.41 ± 1.56
5c	4	–N=C=S	20.29 ± 4.17
Dihydroartemisinin (DHA)			118.95 ± 12.39

^a After 24 h incubation.



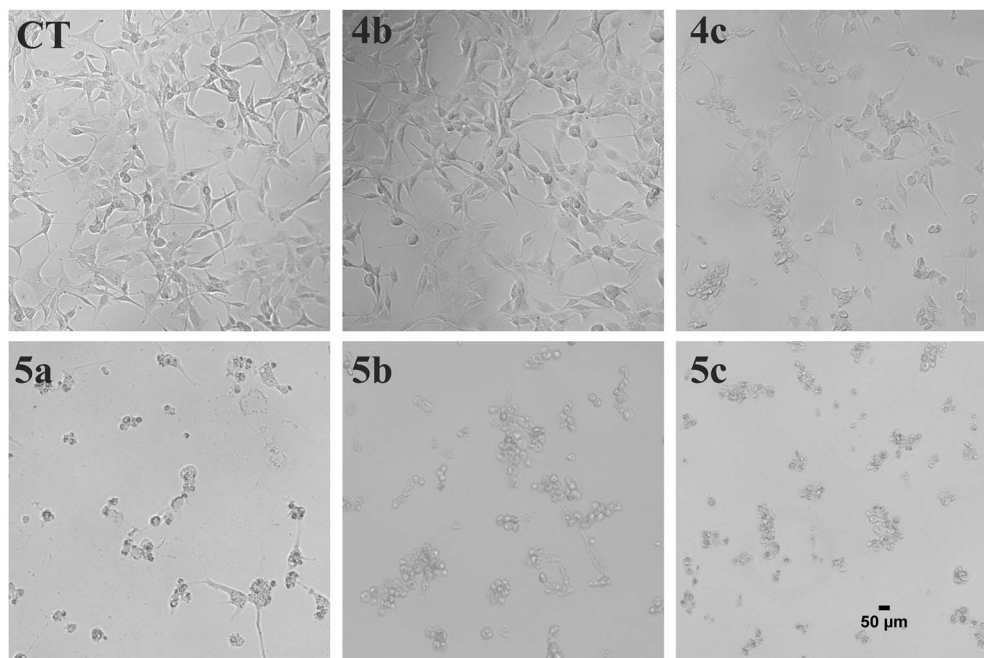


Fig. 2 Morphological examination of U87 cells treated with artemisinin-isothiocyanates (**5a–c**) and the key intermediates (**4b**, **4c**). The cells were treated with 30 μM of the compounds for 24 h and the morphological changes were examined with phase contrast microscope. Scale bar = 50 μm .

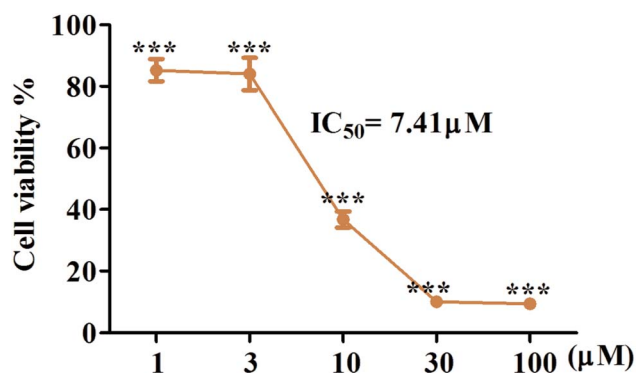


Fig. 3 **5b** possesses the cytotoxic activity on human GBM cell lines, U87. The cells were treated with indicated concentrations of **5b** for 24 h. Cell viability was tested via MTT assay and IC_{50} value was calculated. **5b** reduced the cell viability of U87 cells in a concentration dependent manner. All data were represented as mean $\pm$ SD from three independent experiments; *** $P < 0.001$ versus control.

release of cytochrome *c* activated the caspase-dependent mitochondrial pathway. Cytochrome *c* binds and activates Apaf1 as well as procaspase-9, forming an “apoptosome”. The clustering of procaspase-9 leads to caspase-9 activation.^{36,37} The anti-apoptotic protein Bcl-2 was downregulated and pro-apoptotic protein BAX was upregulated in **5b**-treated cells. The Bcl-2 family of proteins governs mitochondrial membrane permeability *via* the regulation of cytochrome *c* released from the mitochondria.³⁶ Caspase 3 is considered to be the most important of the executioner caspases in apoptotic cell death. Cleaved (active) caspase 3 was increased in **5b**-treated cells (Fig. 6).

Artemisinin-based isothiocyanate derivative 5b induces autophagy in U87 cells. Autophagy assures cellular homeostasis and is gaining increasing importance in cancer treatment, where it impacts carcinogenesis as well as the propagation of the malignant phenotype and the development of resistance.³⁸ To observe whether autophagy was also involved in **5b**-induced cytotoxicity, autophagic markers, such as LC3-II, microtubule-associated proteins 1A/1B light chain 3B, and p62/SQSTM1, were investigated in U87 cells treated with **5b** for 16 h. LC3-II expression increased in the **5b**-treated group compared to the control groups. Moreover, the decrease of p62 was observed in the **5b**-treated group (Fig. 7).

Conclusion

In conclusion, novel ART derivatives containing an ITC group have been synthesized. All the compounds showed more potent activity than that of the parent DHA towards glioblastoma multiforme U87 *in vitro*. The data in the present study demonstrated that **5b** significantly induced cytotoxicity (IC_{50} 7.41 μM) towards human GBM cells through inducing both apoptotic and autophagic cell death and also inhibited the migration of human GBM cells. It was a stronger inducer in GBM cells compared to DHA (about twenty times stronger). Therefore, it suggested that **5b** may be a potential multifunctional small molecule for developing new drugs for the treatment of GBM.

Experimental

Chemistry

Melting points were measured on an XT-4 apparatus (Taikete Corp., Beijing, China) and were uncorrected. ^1H and ^{13}C NMR



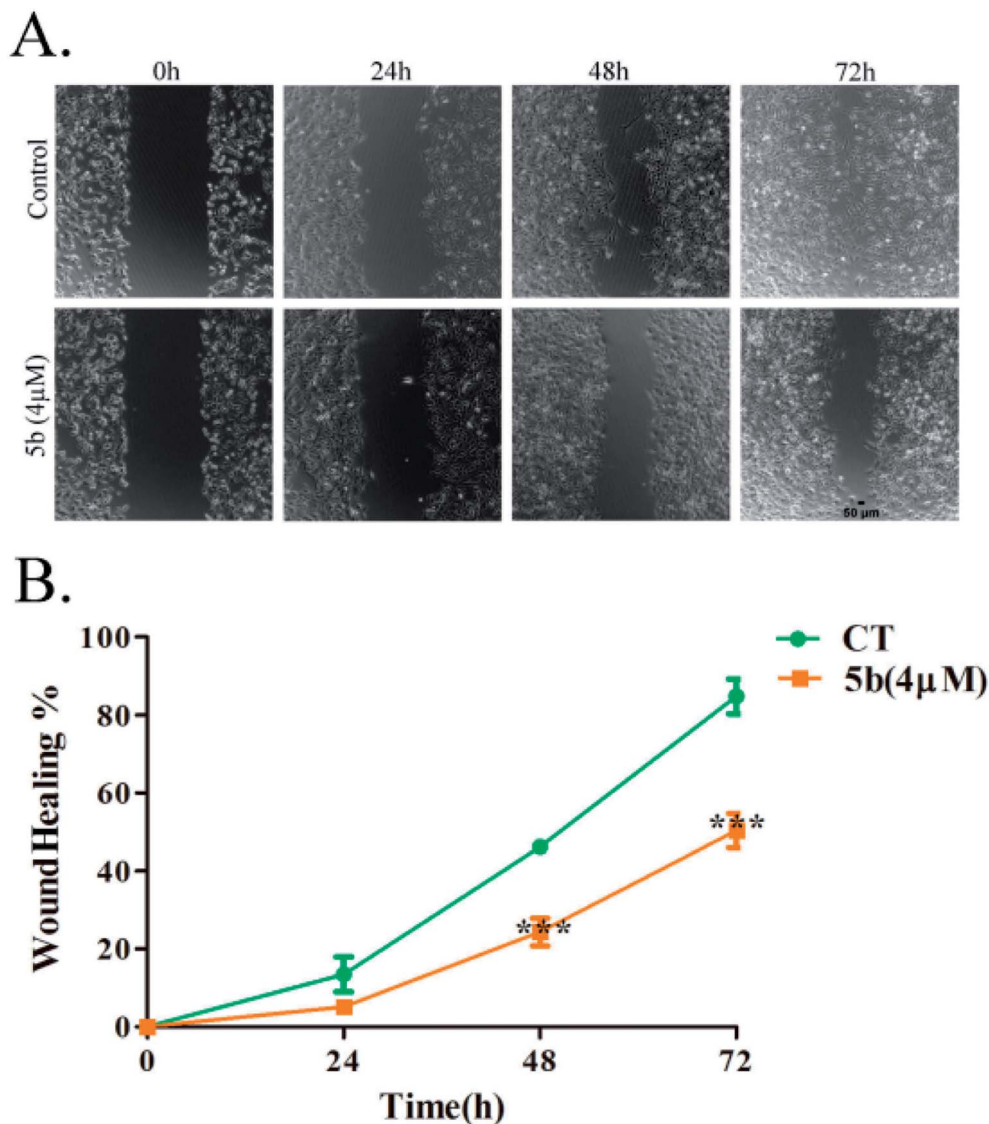


Fig. 4 5b inhibits the migration of human glioblastoma cells, U87. Data were expressed as means $\pm$ SD. *** P < 0.001 versus control. All experiments were performed in triplicates.

spectra were recorded on Bruker 400 MHz, and the data were recorded using CDCl_3 as the solvent. Chemical shifts (δ) are reported in ppm downfield from an internal TMS standard. HR-ESI-MS spectra were determined on an AB Sciex TripleTOF 5600⁺ apparatus. Flash column chromatography on silica gel (200–300 mesh) was used for the routine purification of the reaction products. Analytical TLC was performed on silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany). All solvents and chemical reagents were obtained from commercial sources and used without further purifications.

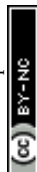
General procedure for the synthesis of compounds 1a–c

The DHA derivatives 1a–c were synthesized *via* the reaction of DHA with the corresponding bromo-substituted alkanol in CH_2Cl_2 using boron trifluoride ethyl ether ($\text{BF}_3 \cdot \text{Et}_2\text{O}$)^{28,29} and isolation in the β configuration by silica gel column

chromatography elution with petroleum ether/ethyl acetate (97 : 3) (indicated are the coupling constants between 9-H and 10-H in ^1H NMR, $J_{9,10} = 3\text{--}4$ Hz).³⁰

The solution of DHA (3.0 mmol) and 3-bromo-1-propanol, or 2-bromo-1-ethanol, or 6-bromo-hexanol (4.6 mmol) in CH_2Cl_2 (20 mL) below 0 °C was added to $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (0.63 mL, 5.0 mmol). The mixture was stirred for about 50 min at the same temperature under nitrogen protection and monitored by TLC. At the end of the reaction, the organic layer was collected and washed with saturated NaHCO_3 solution (1 $\times$ 20 mL), water (1 $\times$ 20 mL) and brine (1 $\times$ 20 mL), dried over anhydrous Na_2SO_4 and then concentrated *in vacuo* to give the crude product, which was purified by silica gel column chromatography (ether/ethyl acetate, 97 : 3, v/v) to afford compounds 1a–c.

1-Bromo-2-(10 β -dihydroartemisinoxy)ethane (1a). Colorless solid; yield: 36%; mp 73–74 °C; ^1H NMR (refer to literature 28); ^{13}C NMR (100 MHz, CDCl_3) δ 104.3(C-3), 102.2(C-10), 88.3(C-12),



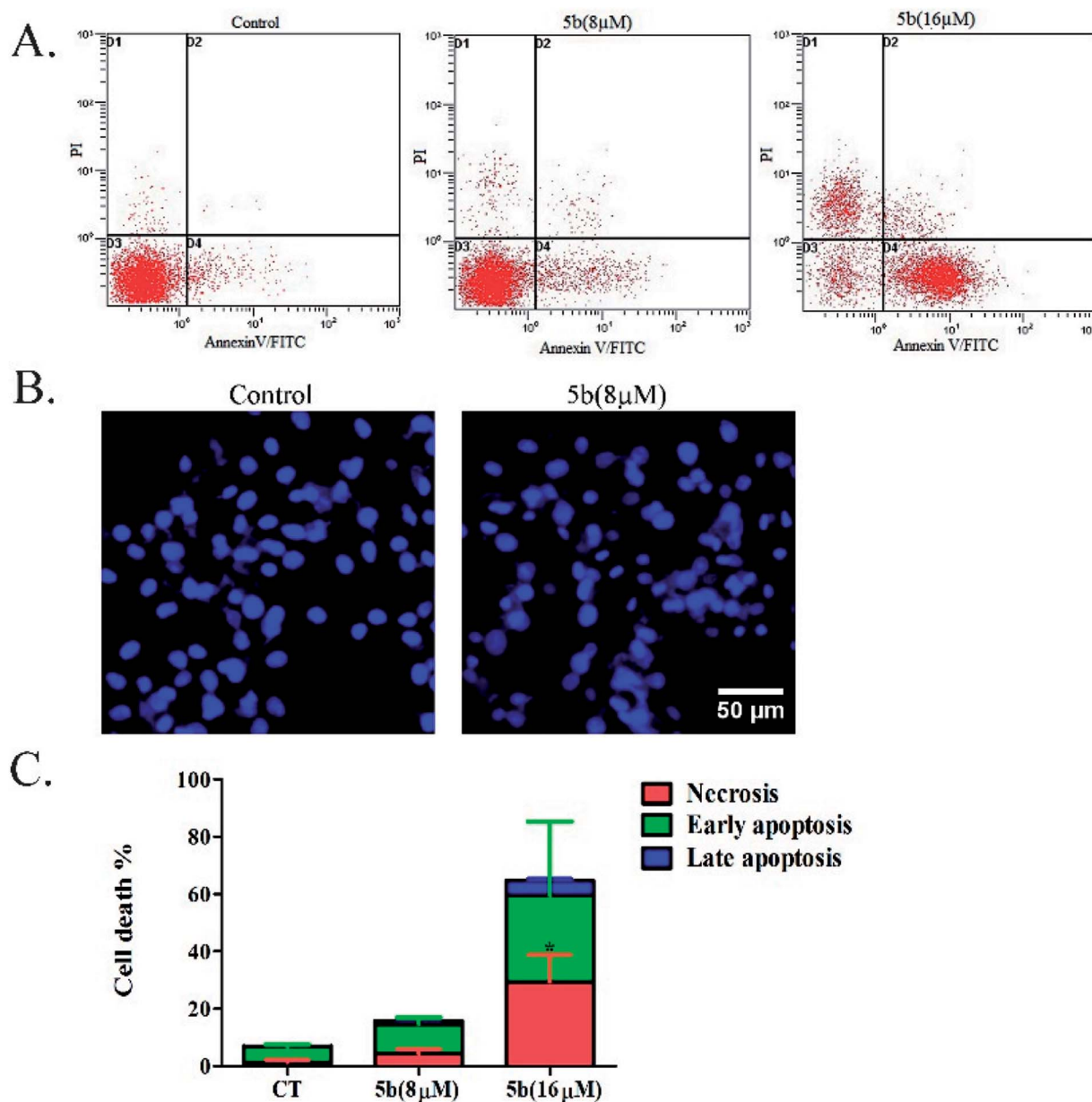


Fig. 5 Apoptotic analysis of **5b** on U87 glioblastoma cells. (A) U87 cells were treated with DMSO or 8 and 16 μM concentrations of **5b** for 16 h, and apoptotic rates were analyzed by flow cytometry after annexin V/PI staining. (B) U87 cells were treated with DMSO or 8 μM of **5b** for 16 h and the nuclear morphological changes were observed using DAPI staining and fluorescence microscope. (C) Data are expressed as mean ± SD of three independent experiments presented in (A). **P* < 0.05 versus control.

81.3(C-12a), 68.4(–OCH₂–), 52.8(C-5a), 44.5(C-8a), 37.6(C-6), 36.6(C-4), 34.9(C-7), 31.6(C-9), 31.1(BrCH₂–), 26.4(C-14), 24.8(C-5), 24.6(C-8), 20.6(C-15), 13.2(C-16).

1-Bromo-3-(10β-dihydroartemisinoxy)propane (1b). Colorless solid; yield: 39%; mp: 69–71 °C; ¹H NMR (refer to literature 29); ¹³C NMR (100 MHz, CDCl₃) δ 104.3(C-3), 102.3(C-10), 88.1(C-12), 81.2(C-12a), 65.8(–OCH₂–), 52.7(C-5a), 44.5(C-8a), 37.6(C-6), 36.6(C-4), 34.8(C-7), 32.7(C-9), 31.1(BrCH₂–), 30.8(BrCH₂CH₂–), 26.4(C-14), 24.8(C-5), 24.7(C-8), 20.5(C-15), 13.2(C-16).

1-Bromo-6-(10β-dihydroartemisinoxy)hexane (1c). Colorless oil; yield: 40%; ¹H NMR (400 MHz, CDCl₃) δ 5.35 (s, 1H, H-12), 4.76 (d, *J* = 3.0 Hz, 1H, H-10), 3.98–3.85 (m, 1H, –OCH₂–), 3.46–3.37 (m, 2H, –CH₂Br), 3.34 (t, *J* = 6.7 Hz, 1H, –OCH₂–), 2.68–2.50 (m, 1H, H-9), 2.34 (td, *J* = 14.1, 3.7 Hz, 1H, H-4), 2.01 (d, *J* = 14.3 Hz, 1H, H-4), 1.66–1.56 (m, 2H), 1.53–1.42 (m, 2H), 1.40 (s, 3H, CH₃-14), 1.36–1.26 (m, 1H), 1.25–1.18 (m, 1H), 0.92 (d, *J* = 6.2 Hz, 3H, CH₃-15), 0.87 (d, *J* = 7.2 Hz, 3H, CH₃-16), 0.87 (m, 1H, H-7); ¹³C NMR (100 MHz, CDCl₃) δ 104.3(C-3), 102.2(C-10), 88.1(C-12), 81.2(C-12a), 68.4(–OCH₂–), 52.8(C-5a), 44.7(C-8a), 37.7(C-6), 36.7(C-4), 34.9(C-7), 34.0, 32.9(C-9), 31.1, 29.7, 28.1, 26.4(C-14), 25.7, 24.9(C-5), 24.7(C-8), 20.6(C-15), 13.1(C-16).



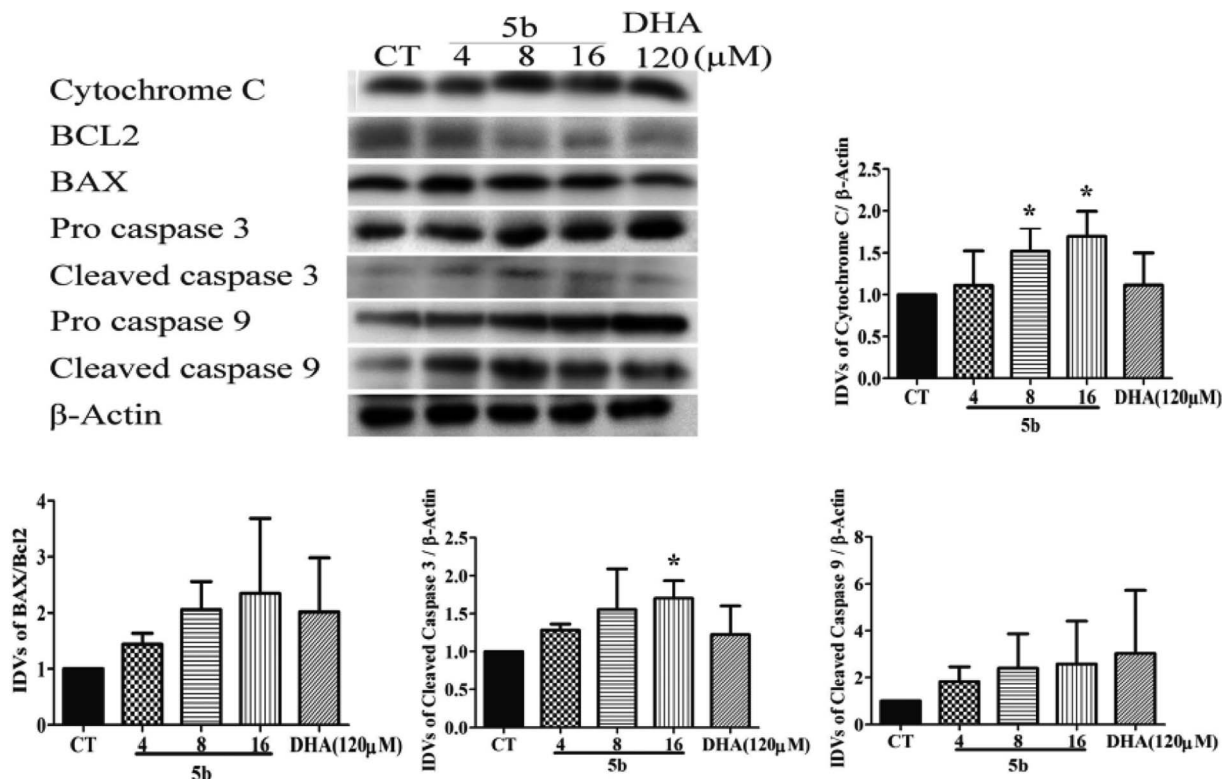


Fig. 6 5b induces apoptosis by regulating caspase family proteins and Bcl2 family proteins in human GBM cells. Cells were treated with the indicated concentrations of 5b and DHA for 16 h. The proteins were collected for western blot assay and β-actin was used as protein loading control. The result was obtained by three different independent experiments.

General procedure for the synthesis of compounds 3a–c

The intermediate 3a–c was synthesized from 1a–c according to the procedure previously reported.^{28,29} To the solution of 1a–c (0.89 mmol) and NaI (10.0 mg) in DMF (5.0 mL), NaN₃ (2.7 mmol) was added. The reaction mixture was heated to 60 °C for 4 h. After the disappearance of the starting material, the solution was poured into ice water (20 mL), stirred for 1 h, and then extracted with CH₂Cl₂ (3 × 20 mL), dried over anhydrous Na₂SO₄ and concentrated to give a light yellow oil. The crude product was further purified by silica gel column chromatography (petroleum ether/ethyl acetate, 9 : 1, v/v) to provide 3a–c.

Table 2 Information of antibodies for western blot

Antibody name	Manufacturer	Catalog	Dilutions
Primary antibody			
Bax	ABclonal	A2211	1 : 1000
BCL2	ABclonal	A0208	1 : 1000
Caspase 3	Cell signaling technology	9665	1 : 1000
Caspase 9	Cell signaling technology	9508	1 : 1000
Cytochrome C	Cell signaling technology	11 940	1 : 1000
LC3B	Cell signaling technology	3868	1 : 1000
P62	MBL	PM045	1 : 1000
β-Actin	Thermo Fisher	MA5-11869	1 : 2500
Secondary antibody			
Anti-rabbit	Beyotime	A0208	1 : 1000
Anti-mouse	Beyotime	A0216	1 : 1000

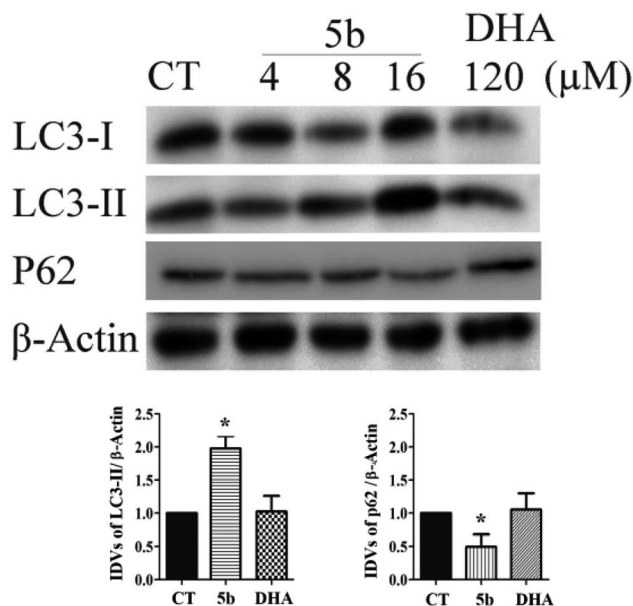


Fig. 7 5b induces autophagy in human GBM cells. Cells were treated with the indicated concentrations of 5b and DHA for 16 h. The proteins were collected for western blot assay with LC3B, p62 and β-actin antibodies. The result was obtained by three different independent experiments.



2-(10 β -Dihydroartemisininoxy)ethyl azide (3a). Colorless solid; yield: 85%; mp: 86–87 °C; ^1H NMR (refer to literature 28); ^{13}C NMR (100 MHz, CDCl_3) δ 104.34(C-3), 102.59(C-10), 88.14(C-12), 81.22(C-12a), 67.47(–OCH₂–), 52.70(C-5a), 51.33(–CH₂N₃), 44.54(C-8a), 37.57(C-6), 36.56(C-4), 34.75(C-7), 30.93(C-9), 26.32(C-14), 24.84(C-5), 24.56(C-8), 20.53(C-15), 13.10(C-16).

3-(10 β -Dihydroartemisininoxy)propyl azide (3b). Colorless solid; yield: 94%; mp: 80–82 °C; ^1H NMR (400 MHz, CDCl_3) δ 5.35 (s, 1H, H-12), 4.76 (d, J = 3.1 Hz, 1H, H-10), 3.97–3.84 (m, 1H, –OCH₂–), 3.49–3.38 (m, 1H, –OCH₂–), 3.34 (t, J = 6.7 Hz, 2H, –CH₂N₃), 2.68–2.53 (m, 1H, H-9), 2.34 (td, J = 14.0, 3.8 Hz, 1H, H-4), 2.00 (m, 1H, H-4), 1.83 (m, 3H), 1.77–1.68 (m, 2H), 1.66–1.56 (m, 2H), 1.54–1.42 (m, 2H), 1.40 (s, 3H, CH₃-14), 1.35–1.24 (m, 1H), 1.24–1.12 (m, 1H), 0.92 (d, J = 6.2 Hz, 3H, CH₃-15), 0.87 (d, J = 7.3 Hz, 3H, CH₃-16), 0.87 (m, 1H, H-7); ^{13}C NMR (100 MHz, CDCl_3) δ 104.3(C-3), 102.3(C-10), 88.1(C-12), 81.2(C-12a), 65.2(–OCH₂–), 52.7(C-5a), 48.8(–CH₂N₃), 44.5(C-8a), 37.6(C-6), 36.6(C-4), 34.8(C-7), 31.1(C-9), 29.3(N₃CH₂CH₂–), 26.8(C-14), 24.8(C-5), 24.7(C-8), 20.5(C-15), 13.2(C-16).

6-(10 β -Dihydroartemisininoxy)hexyl azide (3c). Colorless oil; yield: 98%; ^1H NMR (400 MHz, CDCl_3) δ 5.36 (s, 1H, H-12), 4.75 (d, J = 3.0 Hz, 1H, H-10), 3.89–3.74 (m, 1H, –OCH₂–), 3.40–3.30 (m, 1H, –OCH₂–), 3.23 (t, J = 6.5 Hz, 2H, –CH₂N₃), 2.59 (m, 1H, H-9), 2.34 (t, J = 13.9 Hz, 1H, H-4), 2.00 (m, 1H, H-4), 1.80 (m, 3H), 1.51 (m, 5H), 1.41 (s, 3H, CH₃-14), 1.38 (m, 7H), 1.26–1.12 (m, 3H), 0.93 (d, J = 6.0 Hz, 3H, CH₃-15), 0.87 (d, J = 6.6 Hz, 3H, CH₃-16), 0.85–0.78 (m, 1H, H-7); ^{13}C NMR (100 MHz, CDCl_3) δ 104.3(C-3), 102.2(C-10), 88.1(C-12), 81.3(C-12a), 68.4(–OCH₂–), 52.8(C-5a), 51.6(–CH₂N₃), 44.7(C-8a), 37.7(C-6), 36.7(C-4), 34.9(C-7), 31.1(C-9), 29.7, 29.0, 26.7, 26.4(C-14), 26.1, 24.9(C-5), 24.7(C-8), 20.6(C-15), 13.2(C-16).

General procedure for the synthesis of compounds 4a–c

The key intermediates **4a–c** were prepared by the reduction of **3a–c** according to literature.^{28,29} Triphenylphosphine (0.85 mmol) was added to the solution of **3a–c** (0.71 mmol) in THF (5.0 mL). The solution was stirred at 55 °C for 2 h. Then, 1.0 mL of water was added, and stirred at room temperature for about 5 h. After removal of the solvent, 20 mL of water was added and then extracted with dichloromethane (3 $\times$ 20 mL). The organic layers were combined, dried over anhydrous Na₂SO₄, and concentrated to afford the crude product, which was further purified by silica gel column chromatography (methanol/dichloromethane, 5 : 95, v/v) to provide a colorless oil.

2-(10 β -Dihydroartemisininoxy)ethylamine (4a). Colorless oil; yield: 65%; HR-ESI-MS (m/z) [$\text{M} + \text{H}$]⁺ calcd for: 328.2124, found: 328.2094; ^1H NMR (refer to literature 28); ^{13}C NMR (100 MHz, CDCl_3) δ 104.34(C-3), 102.39(C-10), 88.11(C-12), 81.27(C-12a), 71.08(–OCH₂–), 52.76(C-5a), 44.60(C-8a), 42.26(–CH₂NH₂), 37.69(C-6), 36.62(C-4), 34.81(C-7), 31.15(C-9), 26.40(C-14), 24.88(C-5), 24.82(C-8), 20.55(C-15), 13.27(C-16).

3-(10 β -Dihydroartemisininoxy)propylamine (4b). Colorless oil; yield: 91%; HR-ESI-MS (m/z) [$\text{M} + \text{H}$]⁺ calcd for: 342.2280, found: 342.2245; ^1H NMR (400 MHz, CDCl_3) δ 5.36 (s, 1H, H-12), 4.76 (d, J = 3.1 Hz, 1H, H-10), 3.96–3.84 (dt, J = 10.9, 6.0 Hz, 1H, –OCH₂–), 3.42 (dt, J = 10.9, 6.0 Hz, 1H, –OCH₂–), 2.77 (t, J =

6.9 Hz, 2H, –CH₂NH₂), 2.66–2.52 (m, 1H, H-9), 2.34 (td, J = 14.0, 3.7 Hz, 1H, H-4), 2.01 (m, 1H, H-4), 1.86 (m, 1H), 1.79–1.66 (m, 4H), 1.66–1.60 (m, 1H), 1.53–1.42 (m, 2H), 1.41 (s, 3H, CH₃-14), 1.30 (m, 1H), 1.26–1.16 (m, 2H), 0.92 (d, J = 6.1 Hz, 3H, CH₃-15), 0.86 (d, J = 7.4 Hz, 3H, CH₃-16), 0.86 (m, 1H, H-7); ^{13}C NMR (100 MHz, CDCl_3) δ 104.3(C-3), 102.3(C-10), 88.1(C-12), 81.3(C-12a), 66.6(–OCH₂–), 52.8(C-5a), 44.6(C-8a), 39.9(–CH₂NH₂), 37.7(C-6), 36.6(C-4), 34.8(C-7), 33.8(–CH₂CH₂NH₂), 31.1(C-9), 26.4(C-14), 24.9(C-5), 24.7(C-8), 20.6(C-15), 13.2(C-16).

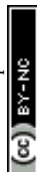
6-(10 β -Dihydroartemisininoxy)hexylamine (4c). Colorless oil; yield: 86%; HR-ESI-MS (m/z) [$\text{M} + \text{H}$]⁺ calcd for: 384.2750, found: 384.2711; ^1H NMR (400 MHz, CDCl_3) δ 5.34 (s, 1H, H-12), 4.73 (d, J = 3.0 Hz, 1H, H-10), 3.78 (dt, J = 15.9, 6.6 Hz, 1H, –OCH₂–), 3.41–3.24 (dt, J = 15.9, 6.6 Hz, 1H, –OCH₂–), 2.64 (t, J = 7.0 Hz, 2H, –CH₂NH₂), 2.59–2.51 (m, 1H, H-9), 2.32 (td, J = 14.0, 3.7 Hz, 1H, H-4), 1.99 (m, 1H, H-4), 1.91–1.64 (m, 7H), 1.64–1.41 (m, 7H), 1.40 (s, 3H, CH₃-14), 1.38 (m, 1H), 1.29 (m, 6H), 1.24–1.11 (m, 2H), 0.91 (d, J = 6.2 Hz, 3H, CH₃-15), 0.88 (m, 1H, H-7), 0.85 (d, J = 7.2 Hz, 3H, CH₃-16); ^{13}C NMR (100 MHz, CDCl_3) δ 104.2(C-3), 102.1(C-10), 88.1(C-12), 81.3(C-12a), 68.5(–OCH₂–), 52.7(C-5a), 44.7(C-8a), 41.8(–CH₂NH₂), 37.7(C-6), 36.6(C-4), 34.8(C-7), 32.9(–CH₂CH₂NH₂), 31.1(C-9), 29.8, 26.8, 26.4(C-14), 26.3, 24.9(C-5), 24.6(C-8), 20.6(C-15), 13.2(C-16).

General procedure for the synthesis of compounds 5a–c

The desired compounds **5a–c** were prepared according to the literature. CS₂ (47 μL , 0.77 mmol) was added dropwise to a solution of compounds **4a–c** (0.64 mmol) and Et₃N (0.24 mL, 1.9 mmol) in anhydrous THF (5.0 mL) in an ice bath. Then, the solution was stirred for 30 min at r.t. and AcCl (54 μL , 0.77 mmol) was added into the solution at 0 °C. After 5 min at the same temperature, it was warmed to r.t. and stirred for 15–30 min. Then, the reaction was quenched with 2.0 mL of 5% HCl (aq.) and extracted with ethyl acetate three times (3 $\times$ 10 mL). The organic layers were combined, dried over anhydrous Na₂SO₄, and concentrated to afford the crude product, which was purified by silica gel column chromatography (petroleum ether/ethyl acetate 97 : 3, v/v) to provide **5a–c** as a colorless oil.

2-(10 β -Dihydroartemisininoxy)ethyl isothiocyanate (5a). Colorless oil; yield: 49%; HR-ESI-MS (m/z) [$\text{M} + \text{H}$]⁺ calcd for: 370.1688, found: 370.1643; ^1H NMR (400 MHz, CDCl_3) δ 5.43 (s, 1H, H-12), 4.81 (d, J = 2.9 Hz, 1H, H-10), 4.00 (dt, J = 10.3, 5.1 Hz, 1H, –OCH₂–), 3.65 (t, J = 5.1 Hz, 2H, –CH₂NCS), 3.56 (dt, J = 9.7, 4.7 Hz, 1H, –OCH₂–), 2.69–2.57 (m, 1H, H-9), 2.33 (td, J = 14.1, 3.7 Hz, 1H, H-4), 2.00 (d, J = 14.5 Hz, 1H, H-4), 1.90–1.79 (m, 1H), 1.75 (m, 2H), 1.64 (m, 1H), 1.45 (m, 3H), 1.40 (s, 3H, CH₃-14), 1.28–1.14 (m, 2H), 0.91 (d, J = 6.4 Hz, 3H, CH₃-15), 0.91 (d, J = 6.4 Hz, 3H, CH₃-16), 0.88–0.79 (m, 1H, H-7); ^{13}C NMR (100 MHz, CDCl_3) δ 128.99(–N=C=S), 104.35(C-3), 102.57(C-10), 88.24(C-12), 81.21(C-12a), 66.65(–OCH₂–), 52.68(C-5a), 45.65(–CH₂NCS), 44.45(C-8a), 37.42(C-6), 36.55(C-4), 34.74(C-7), 30.88(C-9), 26.30(C-14), 24.82(C-5), 24.64(C-8), 20.50(C-15), 13.16(C-16).

3-(10 β -Dihydroartemisininoxy)propyl isothiocyanate (5b). Colorless oil; yield: 53%; HR-ESI-MS (m/z) [$\text{M} + \text{H}$]⁺ calcd for:



384.1845, found: 384.1802; ^1H NMR (400 MHz, CDCl_3) δ 5.36 (s, 1H, H-12), 4.78 (d, $J = 3.2$ Hz, 1H, H-10), 4.02–3.88 (dt, $J = 9.5$, 6.4 Hz, 1H, $-\text{OCH}_2-$), 3.60 (t, $J = 6.6$ Hz, 2H, $-\text{CH}_2\text{NCS}$), 3.53–3.41 (dt, $J = 9.5$, 6.4 Hz, 1H, $-\text{OCH}_2-$), 2.70–2.57 (m, 1H, H-9), 2.35 (td, $J = 14.1$, 3.8 Hz, 1H, H-4), 2.01 (m, 1H, H-4), 1.97–1.91 (m, 2H), 1.87 (m, 1H), 1.81–1.67 (m, 2H), 1.63 (m, 2H), 1.49 (m, 2H), 1.42 (s, 3H, CH_3 -14), 0.94 (d, $J = 6.3$ Hz, 3H, CH_3 -15), 0.89 (d, $J = 7.3$ Hz, 3H, CH_3 -16), 0.87–0.80 (m, 1H, H-7); ^{13}C NMR (100 MHz, CDCl_3) δ 129.5 ($-\text{N}=\text{C}=\text{S}$), 104.4 (C-3), 102.4 (C-10), 88.1 (C-12), 81.2 (C-12a), 64.8 ($-\text{OCH}_2-$), 52.8 (C-5a), 44.5 (C-8a), 42.5 ($-\text{CH}_2\text{NCS}$), 37.7 (C-6), 36.6 (C-4), 34.8 (C-7), 31.0 (C-9), 30.3 ($-\text{CH}_2\text{CH}_2\text{NCS}$), 26.4 (C-14), 24.9 (C-5), 24.8 (C-8), 20.5 (C-15), 13.2 (C-16).

6-(10 β -Dihydroartemisininoxy)hexyl isothiocyanate (5c). Colorless oil; yield: 76%; HR-ESI-MS (m/z) [$\text{M} + \text{H}$] $^+$ calcd for: 426.2314, found: 426.2302; ^1H NMR (400 MHz, CDCl_3) δ 5.35 (s, 1H, H-12), 4.74 (d, $J = 3.2$ Hz, 1H, H-10), 3.80 (dt, $J = 9.5$, 6.4 Hz, 1H, $-\text{OCH}_2-$), 3.48 (t, $J = 6.6$ Hz, 2H, $-\text{CH}_2\text{NCS}$), 3.33 (dt, $J = 9.5$, 6.4 Hz, 1H, $-\text{OCH}_2-$), 2.65–2.47 (m, 1H, H-9), 2.33 (td, $J = 14.0$, 3.8 Hz, 1H, H-4), 2.06–1.94 (m, 1H, H-4), 1.92–1.80 (m, 1H), 1.80–1.70 (m, 2H), 1.70–1.61 (m, 3H), 1.53 (m, 4H), 1.41 (s, 3H, CH_3 -14), 1.35 (m, 3H), 1.20 (m, 2H), 0.92 (d, $J = 6.2$ Hz, 3H, CH_3 -15), 0.89 (m, 1H, H-7), 0.86 (d, $J = 7.2$ Hz, 3H, CH_3 -16); ^{13}C NMR (100 MHz, CDCl_3) δ 129.8 ($-\text{N}=\text{C}=\text{S}$), 104.2 (C-3), 102.2 (C-10), 88.1 (C-12), 81.3 (C-12a), 68.4 ($-\text{OCH}_2-$), 52.7 (C-5a), 45.2 ($-\text{CH}_2\text{NCS}$), 44.6 (C-8a), 37.7 (C-6), 36.6 (C-4), 34.8 (C-7), 31.1 (C-9), 30.1, 29.6, 26.5, 26.4 (C-14), 25.7, 24.9 (C-5), 24.7 (C-8), 20.6 (C-15), 13.2 (C-16).

Cell lines and reagents

In the present study, a U87 cell line (from Prof. Wenbo Zhu, Zhongshan Medical School, Sun Yat-sen University) was used. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Scientific, China) supplemented with 10% fetal bovine serum (PAN BIOTECH) in a humidified incubator at 37 °C with 5% CO_2 . Artemisinin-based isothiocyanate derivatives were dissolved in dimethyl sulfoxide and stored at -20 °C in the dark. Annexin V-FITC apoptosis detection kits were from Best Bio(China). The antibodies used are listed in Table 2.

Cytotoxic assay

The cells were seeded in 96-well plates at a density of 1×10^4 cells/100 μL and treated with different concentrations of the compounds ranging from 1 to 250 μM . After 24 hours of incubation, MTT (Sigma-Aldrich) was added to cells following the treatment and incubated for 4 h at 37 °C. DMSO was subsequently added, and the absorbance was measured with a microplate reader after shaking for ~ 30 s. The half maximal inhibitory concentration (IC_{50}) was calculated. The cytotoxicity of **5b** and **DHA** were investigated for the 24 and 72 h incubations.

Wound healing assay

The wound healing assay was performed in 6-well tissue culture plates. After the cells were allowed to attach and reach 80%

confluence, a scratch in the cell monolayer was made using a sterilized pipette tip (10 μL). Thereafter, all wound-healing processes were performed in serum-free conditions. After washing with 0.01 M phosphate-buffered saline (PBS, pH 7.4), the plates were incubated with **5b** or with DMSO, and images were captured at 0, 24, 48 and 72 h using an EVOS® Digital microscope. Cell death would affect the wound healing results. Thus, to avoid too much cell death, half of the IC_{50} (4 μM) of **5b** was used. The distance of wound healing was calculated with the MRI wound healing tool of the Image J software (National Institutes of Health, MD, USA).

DAPI staining

Morphological changes of the nuclear chromatin of apoptotic cells were identified by staining with the DNA binding dye 4',6-diamidino-2-phenylindole (DAPI, cat. no. D8417, Sigma Aldrich). Cells were grown in 12-well plates at a density of 1×10^5 cells per well followed by the desired treatment. After 16 h of incubation, the cells were washed with cold PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, rewashed and then stained with DAPI solution (1 : 1000 in PBS) at 37 °C for 5 min. After removing the staining solution, apoptotic cells were visualized using a fluorescence microscope.

Apoptotic analysis by flow cytometry

For this assay, U87 cancer cells were seeded at a density of 2×10^6 cells per mL and then incubated with 0, 8 and 16 μM of **5b** for 16 h. The cells were harvested using trypsinization and then washed with ice-cold PBS and resuspended in binding buffer. After adding Annexin V-FITC and PI, the cells were incubated at 4 °C in the dark for 15 minutes according to the manufacturer instructions. The samples were analyzed by flow cytometer (Beckman Coulter, Epics XL) for the percentage of apoptotic and necrotic cells.

Western blot assay

Treated cells were washed with cold PBS and lysed in a radio-immunoprecipitation assay (RIPA) buffer supplemented with a proteinase inhibitor for extracting total proteins. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay. After denaturation, the proteins were separated in SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Nonspecific binding was blocked with 5% milk in TBST buffer for 2 h, followed by incubation with primary antibodies at 4 °C overnight and secondary antibodies at room temperature for 2 h. Blots were visualized using ECL detection reagents.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was supported by the Guangdong Provincial International Cooperation Project of Science & Technology (No.



2013B051000038), the National Natural Science Foundation of China (No. 31371070, No. 81671264), Grant (No. 201704020222, No. 201807010094) from Guangzhou Science, Technology and Innovation Commission and the Fundamental Research Funds for the Central Universities (No. 15ykjc08b) to R. Pi., the Science and Technology Project of Guangzhou City (201607010293) to X. He, (2014Y2-00500) to M. Li. The authors also thank the Talented Young Scientist Program supported by the China Science and Technology Exchange Center for the fellowship.

Notes and references

- R. Stupp, W. P. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. J. Taphoorn, K. Belanger, A. A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J. G. Cairncross, E. Eisenhauer and R. O. Mirimanoff, *N. Engl. J. Med.*, 2005, **352**, 987–996.
- J. P. Thakkar, T. A. Dolecek, C. Horbinski, Q. T. Ostrom, D. D. Lightner, J. S. Barnholtz-Sloan and J. L. Villano, *Cancer Epidemiol., Biomarkers Prev.*, 2014, **23**, 1985–1996.
- M. A. van Agtmael, T. A. Eggelte and C. J. van Bostel, *Trends Pharmacol. Sci.*, 1999, **20**, 199–205.
- L. Gang, S. Song, X. Liu, A. Zhang, Z. Miao and C. Ding, *RSC Adv.*, 2016, **6**, 98975–98984.
- N. H. Zuma, F. J. Smit, C. de Kock, J. Combrinck, P. J. Smith and D. D. N'Da, *Eur. J. Med. Chem.*, 2016, **122**, 635–646.
- Z. S. Zhang, J. Wang, Y. B. Shen, C. C. Guo, K. E. Sai, F. R. Chen, X. Mei, F. U. Han and Z. P. Chen, *Oncol. Lett.*, 2015, **10**, 379–383.
- C. Qu, J. Ma, X. Liu, Y. Xue, J. Zheng, L. Liu, J. Liu, Z. Li, L. Zhang and Y. Liu, *Front. Cell. Neurosci.*, 2017, **11**, 310.
- Y. K. Wong, C. Xu, K. A. Kalesh, Y. He, Q. Lin, W. S. F. Wong, H. M. Shen and J. Wang, *Med. Res. Rev.*, 2017, **37**, 1492–1517.
- X. Wu, Q. H. Zhou and K. Xu, *Acta Pharmacol. Sin.*, 2009, **30**, 501–512.
- M. L. Abba, N. Patil, J. H. Leupold, M. E. M. Saeed, T. Efferth and H. Allgayer, *Cancer Lett.*, 2018, **429**, 11–18.
- Y. Zhang, G. Xu, S. Zhang, D. Wang, P. Saravana Prabha and Z. Zuo, *Nat. Prod. Bioprospect.*, 2018, **8**, 303–319.
- J. W. Fahey, A. T. Zalcemann and P. Talalay, *Phytochemistry*, 2001, **56**, 5–51.
- N. K. Amaglo, R. N. Bennett, R. B. L. Curto, E. A. Rosa, V. L. Turco, A. Giuffrida, A. L. Curto, F. Crea and G. M. Timpo, *Food Chem.*, 2010, **122**, 1047–1054.
- C. Müller, J. Van Loon, S. Ruschioni, G. R. De Nicola, C. E. Olsen, R. Iori and N. Agerbirk, *Phytochemistry*, 2015, **118**, 139–148.
- S. M. Lewis, Y. Li, M. J. Catalano, A. R. Laciak, H. Singh, D. R. Seiner, T. J. Reilly, J. J. Tanner and K. S. Gates, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 4549–4552.
- Y. J. Jeong, H. J. Cho, F. L. Chung, X. Wang, H. S. Hoe, K. K. Park, C. H. Kim, H. W. Chang, S. R. Lee and Y. C. Chang, *Oncotarget*, 2017, **8**, 63949–63962.
- Y. T. Yang, Y. Shi, M. Jay and A. J. Di Pasqua, *Chem. Res. Toxicol.*, 2014, **27**, 946–948.
- T. S. Rajan, G. R. De Nicola, R. Iori, P. Rollin, P. Bramanti and E. Mazzon, *Fitoterapia*, 2016, **110**, 1–7.
- K. C. Liu, T. Y. Shih, C. L. Kuo, Y. S. Ma, J. L. Yang, P. P. Wu, Y. P. Huang, K. C. Lai and J. G. Chung, *Am. J. Chin. Med.*, 2016, **44**, 1289–1310.
- R. K. Singh, T. S. Lange, K. Kim, Y. Zou, C. Lieb, G. L. Sholler and L. Brard, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 5846–5852.
- P. Sestili and C. Fimognari, *BioMed Res. Int.*, 2015, **2015**, 402386.
- R. Grzywa, Ł. Winiarski, M. Psurski, A. Rudnicka, J. Wietrzyk, T. Gajda and J. Oleksyszyn, *Bioorg. Med. Chem. Lett.*, 2016, **26**, 667–671.
- A. Minarini, A. Milelli, C. Fimognari, E. Simoni, E. Turrini and V. Tumiatti, *Expert Opin. Drug Metab. Toxicol.*, 2014, **10**, 25–38.
- V. Citi, A. Martelli, L. Testai, A. Marino, M. C. Breschi and V. Calderone, *Planta Med.*, 2014, **80**, 610–613.
- L. D. C. Mannelli, E. Lucarini, L. Micheli, I. Mosca, P. Ambrosino, M. V. Soldovieri, A. Martelli, L. Testai, M. Taglialatela, V. Calderone and C. Ghelardini, *Neuropharmacology*, 2017, **121**, 49–59.
- S. Huang, J. H. Chua, W. S. Yew, J. Sivaraman, P. K. Moore, C. H. Tan and L. W. Deng, *J. Mol. Biol.*, 2010, **396**, 708–718.
- Z. Ma, Q. Bi and Y. Wang, *Oral Dis.*, 2015, **21**, 156–162.
- Y. Liu, Z. Liu, J. Shi, H. Chen, B. Mi, P. Li and P. Gong, *Molecules*, 2013, **18**, 2864–2877.
- Y. Tian, Z. Liang, H. Xu, Y. Mou and C. Guo, *Molecules*, 2016, **21**, 758.
- J. M. Wu, F. Shan, G. S. Wu, Y. Li, J. Ding, D. Xiao, J. X. Han, G. Atassi, S. Leonce, D. H. Caignard and P. Renard, *Eur. J. Med. Chem.*, 2001, **36**, 469–479.
- B. Luo, J. Wang, X. Li, W. Lu, J. Yang, Y. Hu, P. Huang and S. Wen, *Molecules*, 2017, **22**, 773.
- A. K. Pathak, D. U. Jain and R. F. Sharma, *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.*, 1995, **34B**, 992–993.
- A. R. Butler, L. Conforti, P. Hulme, L. M. Renton and T. J. Rutherford, *J. Chem. Soc., Perkin Trans. 1*, 1999, **2**, 2089–2092.
- A. Grada, M. Otero-Vinas, F. Prieto-Castrillo, Z. Obagi and V. Falanga, *J. Invest. Dermatol.*, 2017, **137**, e11–e16.
- L. Jia, Q. Song, C. Zhou, X. Li, L. Pi, X. Ma, H. Li, X. Lu and Y. Shen, *PLoS One*, 2016, **11**, e0147157.
- S. Elmore, *Toxicol. Pathol.*, 2007, **35**, 495–516.
- N. Morishima, K. Nakanishi, H. Takenouchi, T. Shibata and Y. Yasuhiko, *J. Biol. Chem.*, 2002, **277**, 34287–34294.
- A. M. Schläfli, S. Berezowska, O. Adams, R. Langer and M. P. Tschan, *Eur. J. Histochem.*, 2015, **59**, 2481.

