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Research paper

Combating P-glycoprotein-mediated multidrug resistance with 10-*O*-phenyl dihydroartemisinin ethers in MCF-7 cells

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ABSTRACT

A series of 10-β-phenyl ethers of dihydroartemisinin (DHA) with piperazine substitutions were synthesized with the goal of overcoming multidrug resistance in cancer therapy. These novel compounds exerted significant antiproliferative activities in breast cancer MCF-7 and MCF-7/Adr cell lines at the submicromolar level and were shown to be approximately 100- to 300-times more potent than the lead compound DHA. Remarkably, the P-gp-overexpressed MCF-7/Adr cell line showed collateral sensitivity towards these derivatives. Furthermore, compounds **3d** and **5c,** with the highest selectivity for MCF-7/Adr towards MCF-7 cells, were free from P-gp efflux in a MDCK-MDR1 assay. Flow cytometry and western blot assays suggested that the antiproliferative effects of **5c** were associated with cell cycle arrest at G1 phase through the downregulation of Cyclin D1 and Cyclin B1.

1. Introduction

The overexpression of P-glycoprotein (P-gp) is one of multiple mechanisms of multidrug resistance (MDR) in cancer, which is a hindrance to successful chemotherapy [1]. Clinical chemotherapeutic agents such as paclitaxel, adriamycin (Adr) [2], and even highly selective kinase inhibitors erlotinib [3] and sorafenib [4] often suffer reduced efficacy as a consequence of the efflux function of P-gp. Despite great efforts to inhibit P-gp directly to overcome P-gp-mediated resistance, none of the current P-gp inhibitors have shown improved clinical efficacy due to inherent side effects [5]. The development of agents to evade the P-gp efflux effect seems to be a practical approach to overcoming P-gp-mediated multidrug resistance in cancer.

Artemisinins (Fig. 1) have been widely used in the treatment of drug resistant malaria. Dihydroartemisinin (DHA), the simplest derivative of artemisinin, retains the endoperoxide bridge as required pharmacophore and displays improved pharmacokinetic profiles. Artemisinins have demonstrated potential anticancer activity, especially in some P-gp-overexpressed MDR cancer cells [6,7]. For instance, the growth inhibitory activities of artesunate (ART) against 55 NCI cell lines were unaffected by the content of P-gp in a microarray analysis, and no cross-resistance occurred with P-gp-overexpressed multidrug resistance in an HL-60 cell line [8]. The preserved anti-cancer potency of ART was also observed in drug resistant neuroblastoma cells [9]. Recently, a series of homodimeric ARTs (e.g.**1**, Fig. 1) displayed great potency in P-gp-overexpressed CEM/ADR5000 leukemia cells [10,11]. These studies informed our hypothesis that P-gp-overexpressed MDR cancer cells might exhibit collateral sensitivity towards DHA derivatives [12].

In a previous study, DHA derivatives with a substituted piperazine moiety exhibited superior antiproliferative activities compared to DHA against mouse lymphoma P388 and adriamycin resistant P388/Adr cells. Particularly, compound **2** (Fig. 2) showed higher efficacy against P388/Adr than the sensitive P388 cells [13]. To improve the antiproliferative effect and sensitivity of DHA against MDR cells, a series of novel 10-phenyl ethers with *N*-aryl piperazines of DHA (**3a**–**3e**, **4a**–**4e** and **5a**–**5e**, Fig. 2) were synthesized based on further modifications of compound **2**. As reported by O'Neill et al. [14], *C*-10-phenyl ethers of DHA contributed

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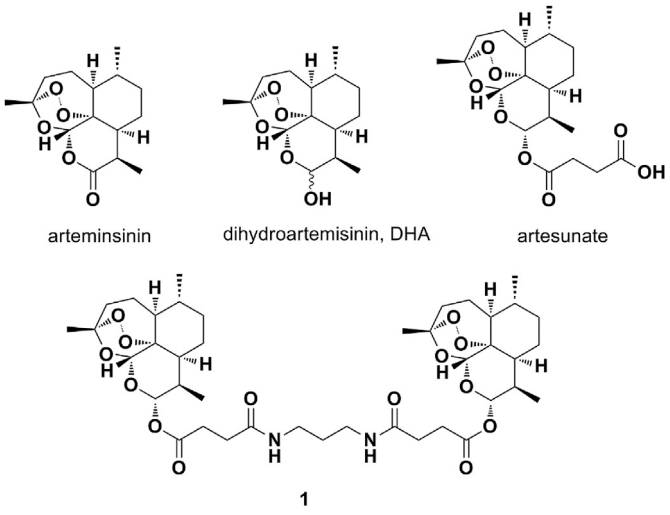


Fig. 1. The chemical structures of reported artemisinin derivatives.

to an improved half-life and metabolic stability and were therefore retained in our design. For systematic SAR studies, the benzoyl linker of **2** was reduced to benzyl or extended to phenylactetyl and α,β-unsaturated cinnamoyl to optimize the linker; additionally, various substituted aryls were introduced into the *N*-4 position of piperazine, as a previous study had showed that a phenyl motif was more preferable than other substitutions [13].

Breast cancer retains the highest morbidity rates of cancer in females, and chemotherapy often fails due to the unavoidable development of drug resistance [15]. Antiproliferative activities of newly synthesized ethers were evaluated against human breast cancer MCF-7 cells and P-gp-overexpressed MCF-7/Adr cells, the latter exhibiting collateral sensitivity to DHA and newly synthesized artemisinin-phenyl ethers. A further mechanism study was also performed and found that the derivatives were unaffected by P-gp efflux; therefore, G1 cell cycle arrest might play a role in their growth inhibition abilities.

2. Results and discussion

2.1. Chemistry

The target artemisinin ethers **3a**–**3e**, **4a**–**4e** and **5a**–**5e** with benzyl, phenylacetyl and cinnamoyl linkers were prepared from DHA and corresponding side chains **6a**–**6e**, **7a**–**7e** and **8a**–**8e**, respectively (Scheme 1) [13]. **6a**–**6e** were prepared through Leuckart-Wallach reductive amination [16], and compounds **7a**–**7e** and **8a**–**8e** were obtained by reacting *p*-hydroxyphenylacetyl and *p*-hydroxycinnamoyl chlorides with *N*-substituted piperazines. The activation of DHA with trifluoroacetic anhydride (TFAA) in the presence of Et_3N at room temperature gave the active ester **9**, and the reaction of **9** with the corresponding side chains **6a**–**6e**, **7a**–**7e**, and **8a**–**8e** in CH_2Cl_2 gave aryl ethers of DHA **3a**–**3e**, **4a**–**4e** and **5a**–**5e** as *C*-10 β-diastereomers.

The chemical structures of the target compounds were confirmed by IR, ^1H NMR, ^{13}C NMR and MS spectroscopy data. The stereochemistry was determined by analyzing the coupling constant values between *H*-9 and *H*-10 (δ= 5.64) in the scaffold of DHA, and all target compounds were β-isomers with similar coupling constant values ($J_{H9\text{-}10}$ = 3.0–3.3 Hz) [17–19]. The stereoselective results were similar to those described in Haynes' study [19] as a kinetic controlled α-ester **9** was exclusively formed and then gave a β-ether through a S_N2 process. The cinnamoyl double bonds of **5a**–**5e** were designed as *trans*-configurations according to the two related H-atoms at about δ = 7.50 and 7.20 (δ = 7.72 and 7.57 for **5e**), displaying a coupling constant value of 15.3 Hz.

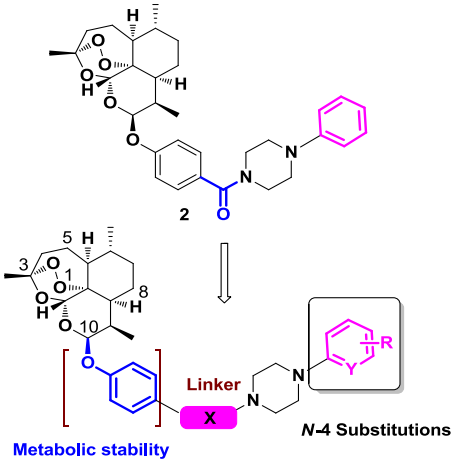
2.2. Pharmacology

2.2.1. Artemisinin-phenyl ethers inhibited cell growth in MCF-7 and MCF-7/Adr cells

The antiproliferative activities of **3a**–**3e**, **4a**–**4e** and **5a**–**5e** were evaluated against the human breast tumor cell line MCF-7 and adriamycin-resistant MCF-7/Adr by the MTT method [20,21]; the results were summarized in Table 1.

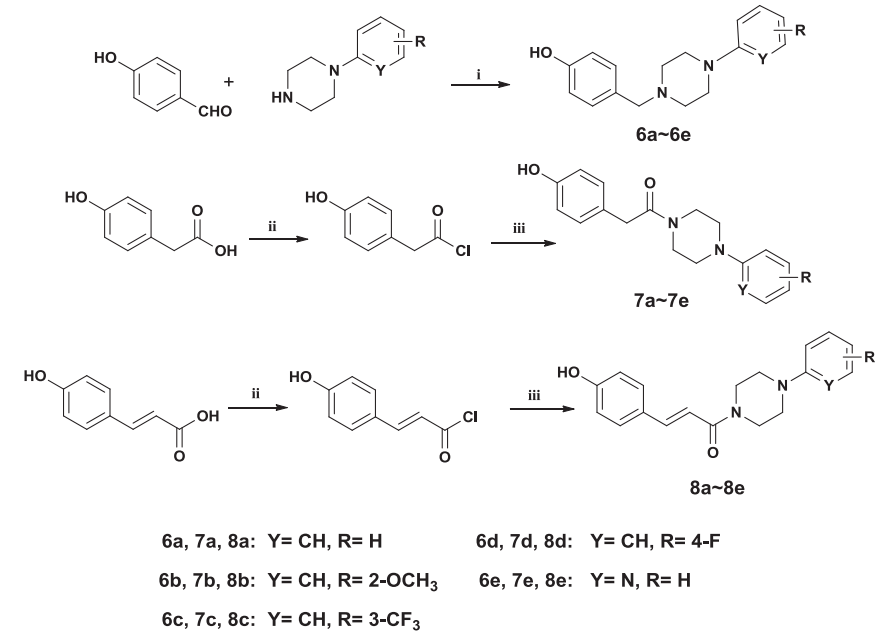
All tested artemisinin ethers showed greater potency against MCF-7 cells than DHA but were less potent than the positive control adriamycin. **2** and **5a** were the most potent artemisinin ethers (GI_{50} = 0.10 μM and 0.11 μM) and displayed 300-fold higher antiproliferative activities than DHA (33.22 μM) against MCF-7 cells. In MCF-7/Adr cells, all artemisinin ethers exhibited significantly improved potency compared to DHA as well as adriamycin. **5c** proved to be the most effective compound against the MCF-7/Adr cell line with a GI_{50} value of 0.021 μM, which was 150- and 25-fold much more potent than DHA (3.32 μM) and adriamycin (0.52 μM), respectively.

Intriguingly, MCF-7/Adr cells showed collateral sensitivity for all novel artemisinin-phenyl ethers, which could be quantitatively

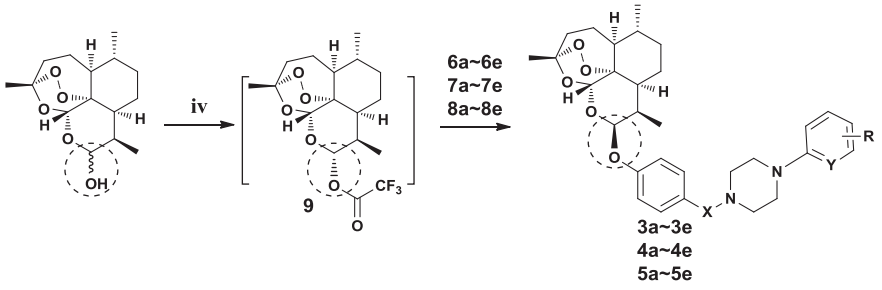


Compd.	X	Y	R
3a		CH	H
3b		CH	2-OCH_3
3c		CH	3-CF_3
3d		CH	4-F
3e		N	H
4a		CH	H
4b		CH	2-OCH_3
4c		CH	3-CF_3
4d		CH	4-F
4e		N	H
5a		CH	H
5b		CH	2-OCH_3
5c		CH	3-CF_3
5d		CH	4-F
5e		N	H

Fig. 2. Design of target compounds.



i: HCOOH, reflux, 2h; **ii**: $SOCl_2$, ET_3N, CH_2Cl_2, reflux, 2h; **iii**: *N*-substituted piperazines, CH_2Cl_2, r.t.



i: HCOOH, reflux, 2h; **ii**: $SOCl_2$, Et_3N, CH_2Cl_2, reflux, 2h; **iii**: *N*-substituted piperazines, CH_2Cl_2, r.t.
iv: TFAA, Et_3N, CH_2Cl_2, r.t.

Scheme 1. The synthetic routes of target compounds.

Table 1
The antiproliferative activities of artemisin ethers in MCF-7 and MCF-7/Adr cells.

Compd.	GI_{50}/μM		SR
	MCF-7	MCF-7/Adr	
2	**0.10 ± 0.12**	0.075 ± 0.017	1.33
3a	0.24 ± 0.19	0.021 ± 0.015	11.43
4a	0.43 ± 0.30	0.090 ± 0.047	4.78
5a	**0.11 ± 0.03**	0.051 ± 0.038	2.16
3b	0.58 ± 0.46	0.15 ± 0.03	3.87
4b	0.47 ± 0.03	0.043 ± 0.020	10.93
5b	0.14 ± 0.04	0.031 ± 0.002	4.52
3c	0.22 ± 0.03	0.033 ± 0.020	6.67
4c	0.26 ± 0.04	0.041 ± 0.006	6.34
5c	0.32 ± 0.03	**0.021 ± 0.007**	**15.24**
3d	0.53 ± 0.10	0.025 ± 0.019	**21.20**
4d	0.29 ± 0.16	0.044 ± 0.028	7.11
5d	0.27 ± 0.06	0.038 ± 0.003	8.57
3e	0.17 ± 0.11	0.055 ± 0.051	3.09
4e	0.96 ± 0.19	0.080 ± 0.048	12.00
5e	0.29 ± 0.07	0.11 ± 0.06	2.64
DHA	33.22 ± 6.33	3.32 ± 0.64	10.00
Adr	0.054 ± 0.020	0.52 ± 0.14	0.10

SR: sensitivity ratio.

evaluated by selectivity ratio value (SR = GI_{50} sensitive/GI_{50} MDR, the ratio of a compound's cytotoxicity over parental cell line) [12]. **3d** had the highest selectivity between MCF-7 (GI_{50} = 0.53 μM) and MCF-7/Adr (GI_{50} = 0.025 μM) with an SR value of 21.20.

A critical SAR analysis illustrated that the introduction of side chains through phenyl ether into DHA was able to improve antiproliferative activity significantly. The alterations of linkers and substituents on the *N*-4-position of piperazine did bring increased selectivities against MCF-7/Adr cells compared with that of compound **2**, while little influence on inhibitory activities was observed. For example, **3d** with a benzyl linker and *N*-4-(*p*-fluorophenyl) piperazine showed the maximum SR value of 21.20. It should be noted that newly synthesized compounds were superior to **2** with a benzoyl linker and *N*-4-phenyl piperazine in selectivity (SR value of 1.33).

2.2.2. ***5c*** *and* ***3d*** *were free from P-gp efflux determined by MDCK-MDR1 and MDCK-WT cell transport assays*

Artemisinin ether derivatives selectively inhibited the proliferation of MCF-7/Adr cells, which are MDR phenotypic cells induced from MCF-7 by adriamycin to overexpress P-gp steadily. To clarify the selectivity and sensitivity for MCF-7/Adr cells, it's necessary to determine whether the novel ethers were transported substrates of P-gp. Madin–Darby canine kidney (MDCK) assays have been widely

utilized in the study of drug transport [22] and for MDCK-MDR1 transfected cells in the study of efflux effects by P-gp [23]. The transcellular permeability of **5c** and **3d** was assessed in MDCK-MDR1 and MDCK-WT cell monolayers, and the experimental results were summarized in Table 2. Apparent permeability coefficients (P_{app}) from A-to-B (apical to basolateral of the cell monolayers, A-B) and B-to-A (basolateral to apical of the cell monolayers, B-A) were obtained by measuring the content of the compounds transported from the donor compartment to the receiver after incubation, with quantification achieved by LC-MS/MS analysis. The P-gp efflux effects were assessed by efflux ratio (the ratio of P_{app} in the direction of B-A over that of A-B). A compound with an efflux ratio value greater than 2.0 was generally considered to be a potential substrate for P-gp [24]. Quinidine (an efflux substrate), Metoprolol (a high permeability control), Atenolol (a low permeability control) and DHA were included as the parallel references in the same experiments.

According to the data in Table 2, DHA was highly permeable ($P_{app} > 10.0 \times 10^{-6}$ cm/s) and unaffected by P-gp (Efflux ratio = 0.89). **3d** and **5c** were similar (Efflux ratio < 1.0) in the price of decreased permeabilities. Our results might surmount the previous research by Steglich et al. [25], in which an artemisinin derivative SM616 was found to inhibit P-gp specifically for the reason mentioned above [5]. Our data indicated that DHA, **3d** and **5c** could inhibit cell growth of MCF-7/Adr selectively without the limitation of P-gp, so it was reasonable to conclude that these novel molecules could effectively overcome the P-gp mediated MDR through bypassing the efflux effects of P-gp.

2.2.3. *5c induced G1-phase cell cycle arrest in human MCF-7 and MCF-7/Adr cells*

The growth-inhibitory effects of **5c** towards MCF-7 and MCF-7/Adr cells inspired us to investigate whether **5c** had any effects on cell cycle progression. Treatment of MCF-7 cells with **5c** for 24 h resulted in a higher number of cells in G1 phase at the indicated concentrations [0.25 μM (70.0%), 0.50 μM (73.8%); Fig. 3A], compared with the control (62.5%). A greater accumulation of cells at G1 phase (83.4%) was observed when treated with **5c** for 48 h at 0.50 μM G1-phase arrest also appeared when MCF-7/Adr cells were treated with **5c** (Fig. 3A). Treatment with **5c** at 0.05 μM and 0.25 μM for 24 h led to moderate increases in the number of cells at G1 phase (71.5% and 70.2%, respectively) compared to the untreated cells (54.6%). Treatment with **5c** for a prolonged period (48 h) at 0.25 μM resulted in a smaller increase of cells in G1 phase (62.6%) compared to that of 24 h and untreated cells (70.2%, 56.9%). However, slight apoptosis was observed as sub-G1 phase cells increased from 2.3% to 8.7%, which might explain the decreasing cells in G1 phase during prolonged treatment.

These data demonstrated clearly that **5c** induced a dose-dependent G1 phase arrest in MCF-7 cells, while it caused G1 phase arrest and a slight apoptosis effect in an extended-time manner in MCF-7/Adr cells, which might explain the **5c**-induced growth inhibition in both cell lines.

Cyclins facilitate cell cycle progression and are considered positive regulators, while kinase inhibitor proteins (KIP) are commonly regarded as negative regulators of the cell cycle [26]. To further investigate the potential mechanism for **5c**-induced cell cycle arrest, the effect of **5c** on cell cycle regulatory proteins (Cyclin D1 and B1) in both cell lines was studied. As illustrated in Fig. 3B, treatment with 0.5 μM **5c** decreased the expression of both cyclin D1 and cyclin B1 while increasing the expression of p27 in MCF-7 cells. While in MCF-7/Adr cells, treatment with **5c** at 0.1 μM for 48 h sharply decreased the levels of cyclin D1 and cyclin B1 but not p27. The proteolytic cleavage of Poly (ADP-ribose) polymerase (PARP) [27] was not observed under these conditions, indicating that apoptosis did not dominate in either cell line. These primary experiments demonstrated that **5c**-induced G1 phase arrest was associated with the downregulation of Cyclin D1 and Cyclin B1 in MCF-7 and MCF-7/Adr cells.

Table 2
A–B and B–A P_{app} of DHA, 3d and 5c across MDCK-WT and MDCK-MDR1 monolayers.

Test Articale	Cell Type	Direction	P_{app}(10^{-6} cm/s)		P_{app}(B − A)/ P_{app}(A − B)
				RSD	
3d	MDR1	A–B	<1.34[a]	N/A	N/A
		B–A	<0.85[a]	N/A	
	Wild Type	A–B	<1.33[a]	N/A	N/A
		B–A	<0.83[a]	N/A	
5c	MDR1	A–B	<1.83[a]	N/A	N/A
		B–A	<1.00[a]	N/A	
	Wild Type	A–B	<1.82[a]	N/A	N/A
		B–A	<0.93[a]	N/A	
DHA	MDR1	A–B	27.97	0.06	0.89
		B–A	24.87	0.08	
	Wild Type	A–B	31.37	0.08	0.80
		B–A	25.10	0.01	
Quinidine	MDR1	A–B	4.37	0.05	7.81
		B–A	34.10	0.01	
	Wild Type	A–B	9.98	0.01	2.34
		B–A	23.39	0.03	
Metoprolol	MDR1	A–B	22.19	0.04	1.07
		B–A	23.69	0.00	
	Wild Type	A–B	24.30	0.01	0.76
		B–A	18.54	0.05	
Atenolol	MDR1	A–B	1.25	0.27	0.76
		B–A	0.95	0.14	
	Wild Type	A–B	<0.23[a]	N/A	N/A
		B–A	<0.12[a]	N/A	

N/A: not acquired

[a] P_{app} values were expressed as "<" than the values that were calculated using the minimum concentration of the standards for receiver sides due to the fact that real concentration in the receivers were below quantitation limit.

3. Conclusion

Fifteen novel 10-*O*-dihydroartemisinin derivatives were designed and synthesized, and the structures were confirmed by IR, MS, HR-MS and NMR data. All compounds displayed improved antiproliferative effects in both sensitive MCF-7 and resistant MCF-7/Adr cells compared with DHA. MCF-7/Adr cells revealed collateral sensitivity towards all novel artemisinin-phenyl ethers. **5c**, **3d** and DHA proved to be free from P-gp efflux by MDCK-MDR1 assay. Flow cytometry and western blot assays suggested that the antiproliferative effects of **5c** against both MCF-7 and MCF-7/Adr cells were associated with cell cycle arrest at G1 phase. Our compounds have the potential to combat P-gp-mediated MDR by evading the efflux effect.

4. Experimental section

4.1. Chemistry

Melting points were determined on an X-4 melting point apparatus and uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) and ^{13}C NMR spectra were measured on Bruker ARX-300, AV 400 MHz or ARX-600 spectrometers with tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in δ (ppm). Infrared (IR) spectra were recorded on BRUKER IR-27G or Spectrum100 (PerkinElmer) spectrometers. Mass spectra (MS) were determined on Finnigan MAT/USA spectrometer (LC–MS). High-resolution mass spectra were obtained on BRUKER microOTOF-Q

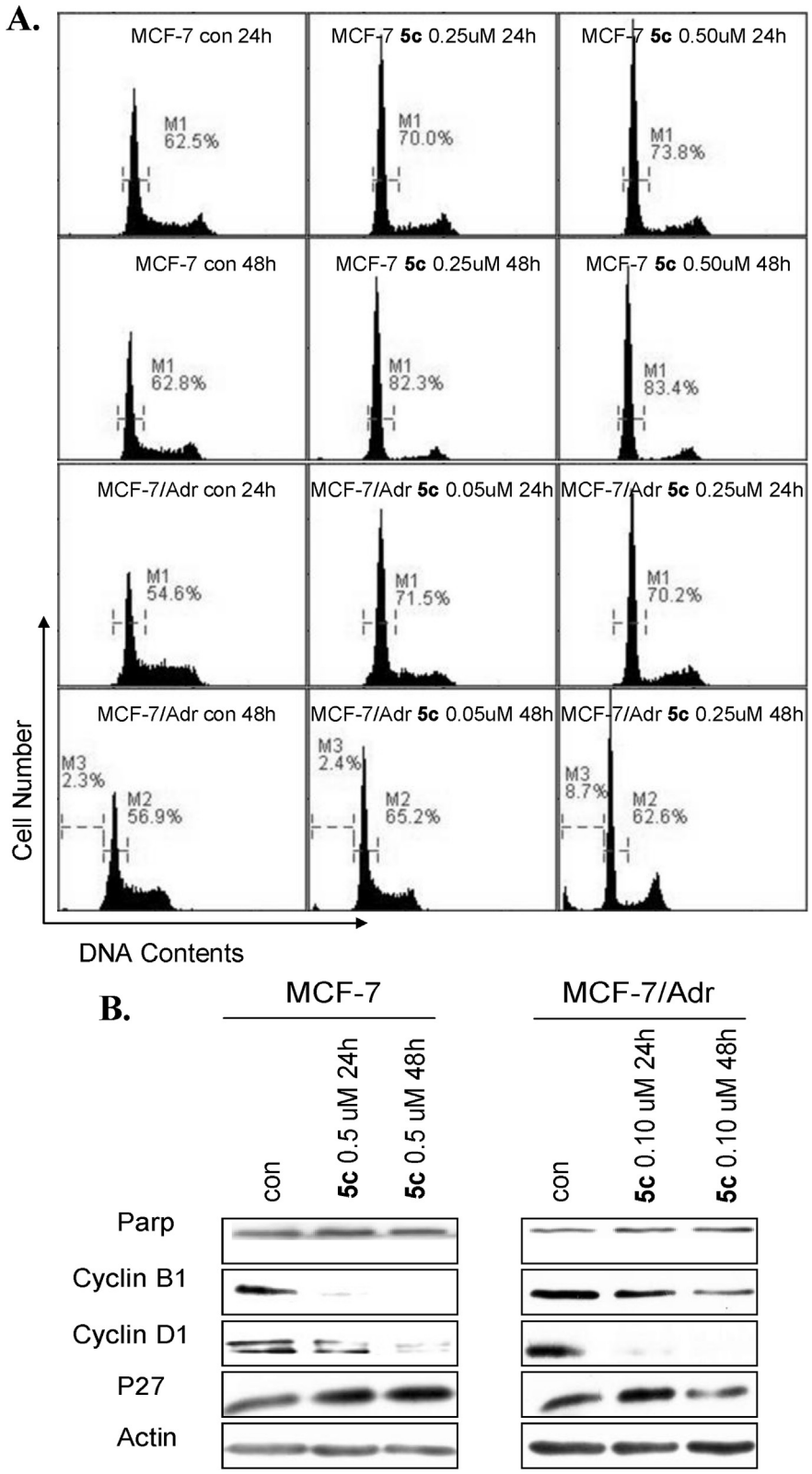


Fig. 3. G1-phase cell cycle arrest in human MCF-7 and MCF-7/Adr induced by **5c**. A: Cell cycle progressions were analyzed by Flow cytometry method. B: Levels of cycle related proteins were tested by Western blot.

in the ESI mode (HR-ESI-MS). DHA was purchased from Jiangsu swellxin bio-pharm. pty. Ltd. China, with purity over 91%. The other reagents were obtained from commercial suppliers and used without purification. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Column chromatography was performed with silica gel 60 (200–300 mesh). All

target compounds are ≥95% purity through HPLC analysis.

4.1.1. General procedure for side chains

4.1.1.1. Compounds **6a**–**6e**

*4.1.1.1.1. 4-((4-phenylpiperazin-1-yl)methyl)phenol (**6a**).* *N*-phenylpiperazine (1.78 g, 11 mmol) and *p*-hydroxybenzaldehyde (1.22 g, 10 mmol) were added into a flask, and heated to 60 °C to generate a eutectic body. Subsequently, formic acid (0.37 mL, 12 mmol) was added drop-wise to the mixture, which was heated and stirred under refluxing for another 2 h. After the solution was cooled to room temperature, about 30 mL ethyl acetate was added, the generated solids were filtrated in vacuum, washed with a small amount of ethyl acetate to obtain brown solid 1.06 g, yield: 39.5%.

*4.1.1.1.2. 4-((4-(2-methoxyphenyl)piperazin-1-yl)methyl)phenol (**6b**).* The product was obtained by the similar procedure of **6a**, from *N*-(2-methoxyphenyl)piperazine and *p*-hydroxybenzaldehyde, as a white powder 2.04 g, yield: 68.3%.

*4.1.1.1.3. 4-((4-(3-trifluoromethylphenyl)piperazin-1-yl)methyl) phenol (**6c**).* The product was obtained by the similar procedure of **6a**, from *N*-(3-trifluoromethylphenyl)piperazine and *p*-hydroxybenzaldehyde, as a white powder 2.40 g, yield: 71.4%.

*4.1.1.1.4. 4-((4-(4-fluorophenyl)piperazin-1-yl)methyl)phenol (**6d**).* The product was obtained by the similar procedure of **6a**, from *N*-(4-fluorophenyl)piperazine and *p*-hydroxybenzaldehyde, as a yellow powder 2.11 g, yield: 73.5%.

*4.1.1.1.5. 4-((4-(pyridin-2-yl)piperazin-1-yl)methyl)phenol (**6e**).* The product was obtained by the similar procedure of **6a**, from *N*-(pyridin-2-yl)piperazine and *p*-hydroxybenzaldehyde, as a white powder 2.21 g, yield: 82.1%.

4.1.1.2. Compounds **7a**–**7e**

*4.1.1.2.1. 2-(4-hydroxyphenyl)-1-(4-phenylpiperazin-1-yl)ethanone (**7a**).* To a solution of *p*-hydroxyphenylacetic acid (1.52 g, 10 mmol) in anhydrous dichloromethane (30 mL), anhydrous Et_3N (1.01 g, 1.39 mL, 10 mmol) was added slowly, the complex was stirred under reflux for 30 min. Then thionyl chloride (0.87 mL, 12 mmol) was added drop-wise to the mixture and stirred for another 2 h. Subsequently, the solution was cooled to room temperature and *N*-phenylpiperazine (1.78 g, 11 mmol) was added and stirred at room temperature for 2 h. The solution was concentrated in vacuum and the residue was purified by silica gel column chromatography eluting with dichloromethane–acetone system, as a light yellow powder 1.06 g, yields: 41.3%.

*4.1.1.2.2. 2-(4-hydroxyphenyl)-1-(4-(2-methoxyphenyl)piperazin-1-yl)ethanone (**7b**).* The product was obtained by the similar procedure of **7a**, from *N*-(2-methoxyphenyl)piperazine and *p*-hydroxyphenylacetic acid, as a yellow powder 0.84 g, yield: 25.7%.

*4.1.1.2.3. 2-(4-hydroxyphenyl)-1-(4-(3-trifluoromethylphenyl) piperazin-1-yl)ethanone (**7c**).* The product was obtained by the similar procedure of **7a**, from *N*-(3-trifluoromethylphenyl)piperazine and *p*-hydroxyphenylacetic acid, as a white powder 1.50 g, yield: 41.2%.

*4.1.1.2.4. 2-(4-hydroxyphenyl)-1-(4-(4-fluorophenyl)piperazin-1-yl)ethanone (**7d**).* The product was obtained by the similar procedure of **7a**, from *N*-(4-fluorophenyl)piperazine and *p*-hydroxyphenylacetic acid. The white solid was precipitated from solvent, 1.78 g. Yield: 56.7%.

*4.1.1.2.5. 2-(4-hydroxyphenyl)-1-(4-(pyridin-2-yl)piperazin-1-yl) ethanone (**7e**).* The product was obtained by the similar procedure of **7a**, from *N*-(pyridin-2-yl)piperazine and *p*-hydroxyphenylacetic acid. The white solid was precipitated from solvent, 2.32 g. Yield: 78.1%.

4.1.1.3. Compounds **8a**–**8e**

*4.1.1.3.1. (E)-3-(4-hydroxyphenyl)-1-(4-phenylpiperazin-1-yl) prop-2-en-1-one (**8a**).* To a solution of *p*-hydroxycinnamic acid (1.64 g, 10 mmol) in anhydrous dichloromethane (30 mL), anhydrous Et_3N (1.01 g, 1.39 mL, 10 mmol) was added slowly, the complex was stirred under reflux for 30 min. Then thionyl chloride (0.87 mL, 12 mmol) was added drop-wise to the mixture and stirred for another 2 h. Subsequently, the solution was cooled to room temperature and concentrated in vacuum. The residue was soluted in 30 mL anhydrous dichloromethane and *N*-phenylpiperazine (1.78 g, 11 mmol) was added and stirred under reflux for 2 h. The solution was cooled to room temperature and a white solid was precipitated from solvent, 0.97 g. Yield: 32.1%.

*4.1.1.3.2. (E)-3-(4-hydroxyphenyl)-1-(4-(2-methoxyphenyl)piperazin-1-yl)prop-2-en-1-one (**8b**).* The product was obtained by the similar procedure of **8a**, from *N*-(2-methoxyphenyl)piperazine and *p*-hydroxycinnamic acid, as a light yellow powder 2.16 g, yield: 63.8%.

*4.1.1.3.3. (E)-3-(4-hydroxyphenyl)-1-(4-(3-trifluoromethylphenyl) piperazin-1-yl)prop-2-en-1-one (**8c**).* The product was obtained by the similar procedure of **8a**, from *N*-(3-trifluoromethylphenyl) piperazine and *p*-hydroxycinnamic acid, as a light yellow powder 2.33 g, yield: 61.9%. MS: 377.1 $[M+H]^+$; ^{1}H NMR (DMSO, δ (ppm)): 9.84 (1H, s, Ar–OH),7.57 (2H, d, J = 8.4 Hz, Ar–H), 7.45 (1H, d, J = 15.3 Hz, -CH=CH-), 7.44(1H, s, Ar–H), 7.26 (1H, m, Ar–H), 7.21(1H, s, Ar–H), 7.11 (1H, m, Ar–H),7.09 (1H, d, J = 15.2 Hz, -CH=CH-), 6.79 (2H, d, J = 8.4 Hz, Ar–H) 3.78 (4H, d, -NCH_2-), 3.28 (4H, s, -NCH_2-).

*4.1.1.3.4. (E)-3-(4-hydroxyphenyl)-1-(4-(4-fluorophenyl)piperazin-1-yl)prop-2-en-1-one (**8d**).* The product was obtained by the similar procedure of **8a**, from *N*-(4-fluorophenyl)piperazine and *p*-hydroxycinnamic acid, as a light yellow powder 2.47 g, yield: 75.6%.

*4.1.1.3.5. (E)-3-(4-hydroxyphenyl)-1-(4-(pyridin-2-yl)piperazin-1-yl)prop-2-en-1-one (**8e**).* The product was obtained by the similar procedure of **8a**, from *N*-(pyridin-2-yl)piperazine and *p*-hydroxycinnamic acid, as a light yellow powder 2.21 g, yield: 71.5%.

4.1.2. General procedure for **3a**–**3e**, **4a**–**4e** and **5a**–**5e**

A solution of DHA (0.568 g, 2 mmol) and Et_3N (0.56 g, 4 mmol) in 30 mL anhydrous dichloromethane was stirred under 0 °C for 30 min. Then, trifluoroacetic anhydride (0.57 g, 4 mmol) was added drop-wise, the mixture was allowed to warm to room temperature and stirred overnight, corresponding side chains (**6a**–**6e**, **7a**–**7e** and **8a**–**8e**) (6 mmol) were added to the mixture and reacted for 12 h. Finally, the solution was washed by 5% aqueous sodium hydroxide (30 mL × 3) and saturated brine (30 mL × 3), the organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated in vacuum. The crude product was purified by silica gel column chromatography eluting with petroleum ether–ethyl acetate system.

*4.1.2.1. 10-O-(4-((4-phenylpiperazin-1-yl)methyl)phenyl)-(10S)-dihydroartemisinin (**3a**).* White solid, 0.129 g; yield: 12.1%, mp: 52–53 °C; IR (KBr): 3439, 2921, 2873, 1600, 1507, 1384, 1227, 1035, 959, 876, 837, 758 cm^{-1}; MS: 535.5 $[M+H]^+$; ^{1}H NMR (DMSO, δ (ppm)): 7.27 (2H, d, J = 8.7 Hz, Ar–H), 7.20 (2H, m, Ar–H), 7.04 (2H, d, J = 8.7 Hz, Ar–H), 6.91 (2H, d, J = 8.1 Hz, Ar–H), 6.76 (1H, m, Ar–H), 5.52 (1H, d, J = 3.0 Hz, H-10), 5.44 (1H, s, H-12), 3.47 (2H, s, $ArCH_2$-), 3.12 (4H, s, -NCH_2-), 2.59 (1H, m, H-9), 2.50 (4H, s, -NCH_2-), 2.21 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.40 (2H, m, H-6 and H-8),1.29 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.97 (3H, d, J = 7.2 Hz, H-16), 0.91 (3H, d, J = 6.0 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 156.4, 151.4, 131.8, 130.4, 129.2, 119.1, 117.1, 115.7, 103.9, 99.8, 87.8, 80.8, 61.9, 52.9, 52.4, 48.6, 44.1, 36.9, 36.4, 34.5, 30.8, 26.0, 24.6, 24.4, 20.6, 13.0. HRMS (ESI) m/z calcd for

$C_{32}H_{43}N_2O_5$ $[M+H]^+$ 535.3165, found 535.3166.

*4.1.2.2. 10-O-(4-((4-(2-methoxyphenyl)piperazin-1-yl)methyl) phenyl)-(10S)-dihydroartemisinin (**3b**).* White solid, 0.121 g; yield: 10.7%, mp: 135–137 °C; IR (KBr): 2935, 2816, 1595, 1500, 1373, 1242,1226, 1039, 981, 877, 740 cm^{-1}; MS: 565.1 $[M+H]^+$, 587.2 $[M+Na]^+$, 1165.3 $[2M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 7.26 (2H, d, J = 8.1 Hz, Ar–H), 7.03 (2H, d, J = 8.4 Hz, Ar–H), 6.92 (2H, d, J = 8.1 Hz, Ar–H), 6.87 (2H, d, J = 8.4 Hz, Ar–H), 5.53 (1H, d, J = 3.0 Hz, H-10), 5.43 (1H, s, H-12), 3.76 (3H, s, -OCH_3), 3.48 (2H, s, $ArCH_2$-), 2.95 (4H, s, -NCH_2-), 2.58 (1H, m, H-9), 2.19 (1H, m, H-4), 2.09 (4H, s, -NCH_2-), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.39 (2H, m, H-6 and H-8), 1.29 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.97 (3H, d, J = 7.2 Hz, H-16), 0.91 (3H, d, J = 6.0 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 156.4, 152.4, 141.6, 130.5, 122.7, 121.2, 118.3, 117.1, 115.3, 112.3, 103.9, 99.8, 87.8, 80.8, 61.9, 55.7, 53.1, 52.4, 50.4, 44.1, 36.9, 36.4, 34.5, 30.8, 26.0, 24.6, 24.4, 20.6, 13.0. HRMS (ESI) m/z calcd for $C_{33}H_{45}N_2O_6$ $[M+H]^+$ 565.3272, found 565.3250.

*4.1.2.3. 10-O-(4-((4-(3-trifluoromethylphenyl)piperazin-1-yl)methyl) phenyl)-(10S)- dihydroartemisinin (**3c**).* White solid, 0.116 g; yield: 9.6%, mp: 65–67 °C; IR (KBr): 2950, 1610, 1509, 1450, 1351, 1319, 1228, 1165, 1122, 1035, 979, 957, 877 cm^{-1}; MS: 603.2 $[M+H]^+$, 625.2 $[M+Na]^+$, 641.1 $[M+K]^+$; ^1H NMR (DMSO, δ (ppm)): 7.40 (1H, t, Ar–H), 7.26 (2H, d, J = 8.4 Hz, Ar–H), 7.19 (1H, d, J = 8.4 Hz, Ar–H), 7.13 (1H, s, Ar–H), 7.05 (1H, d, J = 8.4 Hz, Ar–H), 7.03 (2H, d, J = 8.4 Hz, Ar–H), 5.53 (1H, d, J = 3.0 Hz, H-10), 5.43 (1H, s, H-12), 3.47 (2H, s, $ArCH_2$-), 3.21 (4H, s, -NCH_2-), 2.59 (1H, m, H-9), 2.50 (4H, s, -NCH_2-), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.39 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.97 (3H, d, J = 7.2 Hz, H-16), 0.91 (3H, d, J = 6.3 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 156.4, 151.6, 131.8, 130.4, 130.3, 125.7, 123.9, 119.1, 117.1, 114.8, 111.2, 103.9, 99.8, 87.8, 80.8, 61.8, 52.6, 52.4, 48.0, 44.1, 36.9, 36.4, 34.5, 30.8, 26.0, 24.6, 24.4, 20.5, 13.0. HRMS (ESI) m/z calcd for $C_{33}H_{42}F_3N_2O_5$ $[M+H]^+$ 603.3040, found 603.3046.

*4.1.2.4. 10-O-(4-((4-(4-fluorophenyl)piperazin-1-yl)methyl)phenyl)-(10S)- dihydroartemisinin (**3d**).* White solid, 0.120 g; yield: 10.9%, mp: 71–73 °C; IR (KBr): 3429, 2972, 1610, 1511, 1453, 1384, 1229, 1094, 1037, 979, 958, 877 cm^{-1}; MS: 553.5 $[M+H]^+$, 575.4 $[M+Na]^+$, 1106.1 $[2M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 7.26 (2H, d, J = 8.7 Hz, Ar–H), 7.04 (2H, d, J = 8.4 Hz, Ar–H), 7.01 (2H, d, J = 8.7 Hz, Ar–H), 6.93 (2H, d, J = 8.4 Hz, Ar–H), 5.53 (1H, d, J = 3.3 Hz, H-10), 5.43 (1H, s, H-12), 3.46 (2H, s, $ArCH_2$-), 3.06 (4H, s, -NCH_2-), 2.60 (1H, m, H-9), 2.50 (4H, s, -NCH_2-), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.39 (2H, m, H-6 and H-8), 1.29 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.97 (3H, d, J = 7.2 Hz, H-16), 0.91 (3H, d, J = 6.3 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 156.7, 156.0, 155.1, 147.9, 130.0, 117.1, 117.0, 116.7, 115.2, 115.1, 103.5, 99.4, 87.4, 80.4, 61.4, 52.4, 52.0, 49.0, 43.7, 36.5, 35.9, 34.1, 30.4, 25.6, 24.1, 24.0, 20.1, 12.6. HRMS (ESI) m/z calcd for $C_{32}H_{42}FN_2O_5$ $[M+H]^+$ 553.3072, found 553.3080.

*4.1.2.5. 10-O-(4-((4-(pyridin-2-yl)piperazin-1-yl)methyl)phenyl)-(10S)- dihydroartemisinin (**3e**).* White solid, 0.095 g; yield: 8.9%, mp: 68–70 °C; IR (KBr): 3437, 2944, 1596, 1509, 1481, 1440, 1384, 1224, 1094, 1034, 978, 941, 877 cm^{-1}; MS: 536.3 $[M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 8.08 (1H, d, J = 1.5 Hz, Ar–H), 7.51 (1H, m, Ar–H), 7.26 (2H, d, J = 8.4 Hz, Ar–H), 7.03 (2H, d, J = 8.4 Hz, Ar–H), 6.78 (1H, d, J = 8.4 Hz, Ar–H), 6.62 (1H, m, Ar–H), 5.53 (1H, d, J = 3.3 Hz, H-10), 5.43 (1H, s, H-12), 3.45 (2H, s, $ArCH_2$-), 3.41 (4H, s, -NCH_2-), 2.60 (1H, m, H-9), 2.43 (4H, m, -NCH_2-), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.39 (2H, m, H-6 and H-8), 1.29 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.97 (3H, d, J = 7.2 Hz, H-16), 0.91 (3H, d, J = 7.5 Hz, H-15). ^{13}C NMR ($CDCl_3$, δ (ppm)): 158.0, 151.7, 139.7, 129.6, 129.4, 123.1, 119.9, 116.3, 115.7, 104.5, 100.8, 88.6, 81.4, 63.2, 53.4, 52.9, 49.3, 44.8, 37.7, 36.7, 35.0, 31.3, 26.4, 25.0, 24.8, 20.7, 13.3. HRMS (ESI) m/z calcd for $C_{31}H_{42}N_3O_5$ $[M+H]^+$ 536.3119, found 536.3115.

*4.1.2.6. 10-O-(4-(2-oxo-2-(4-phenylpiperazin-1-yl)ethyl)phenyl)-(10S)- dihydroartemisinin (**4a**).* White solid, 0.276 g; yield: 24.5%, mp: 122–124 °C; IR (KBr): 3439, 2922, 2872, 1645, 1599, 1509, 1450, 1382, 1342, 1280, 1231, 1095, 1066, 1035, 979, 959, 876 cm^{-1}; MS: 563.4 $[M+H]^+$, 585.4 $[M+Na]^+$; ^1H NMR (DMSO, δ (ppm)): 7.22 (2H, d, J = 8.4 Hz, Ar–H), 7.19 (2H, m, Ar–H), 7.01 (2H, d, J = 8.4 Hz, Ar–H), 6.93 (2H, d, J = 8.1 Hz, Ar–H), 6.80(1H, m, Ar–H), 5.50 (1H, d, J = 3.0 Hz, H-10), 5.42 (1H, s, H-12), 3.70 (2H, s, $ArCH_2$-), 3.62 (4H, m, -NCH_2-), 3.07 (4H, m, -NCH_2-), 2.57 (1H, m, H-9), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.64–1.49 (2H, m, H-5 and H-8a), 1.38 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.96 (3H, d, J = 7.2 Hz, H-16), 0.90 (3H, d, J = 6.0 Hz, H-15); ^{13}C NMR (DMSO, δ (ppm)): 169.6, 156.0, 151.2, 130.4, 129.7, 129.4, 119.8, 117.5, 116.3, 104.0, 100.0, 87.9, 80.9, 52.6, 49.2, 48.7, 44.2, 41.5, 37.0, 36.5, 34.6, 30.9, 26.1, 24.7, 24.5, 20.6, 13.1. HRMS (ESI) m/z calcd for $C_{33}H_{43}N_2O_6$ $[M+H]^+$ 536.3116, found 563.3117.

*4.1.2.7. 10-O-(4-(2-(4-(2-methoxyphenyl)piperazin-1-yl)-2-oxoethyl)phenyl)-(10S)-dihydroartemisinin (**4b**).* White solid, 0.122 g; yield: 10.3%, mp: 99–102 °C; IR (KBr): 2943, 1644, 1509, 1459, 1375, 1241, 1095, 1065, 1035, 979, 959, 877 cm^{-1}; MS: 593.3 $[M+H]^+$, 615.3 $[M+Na]^+$; ^1H NMR (DMSO, δ (ppm)): 7.18 (2H, d, J = 8.7 Hz, Ar–H), 7.01 (2H, d, J = 8.7 Hz, Ar–H), 6.95 (2H, m, Ar–H), 6.86(2H, m, Ar–H), 5.50 (1H, d, J = 3.3 Hz, H-10), 5.43 (1H, s, H-12), 3.78 (3H, s, -OCH_3), 3.69 (2H, s, $ArCH_2$-), 3.61 (4H, m, -NCH_2-), 2.88 (4H, m, -NCH_2-), 2.58 (1H, m, H-9), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.39 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.96 (3H, d, J = 7.2 Hz, H-16), 0.90 (3H, d, J = 6.3 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 169.4, 155.9, 152.4, 141.1, 130.3, 129.7, 123.2, 121.2, 118.6, 117.4, 112.3, 103.9, 99.9, 87.8, 80.8, 55.7, 52.4, 50.9, 50.4, 46.1, 44.1, 41.8, 36.9, 36.4, 34.5, 30.8, 30.0, 26.0, 24.5, 24.4, 20.6, 13.0. HRMS (ESI) m/z calcd for $C_{34}H_{45}N_2O_7$ $[M+H]^+$ 593.3221, found 593.3216.

*4.1.2.8. 10-O-(4-(2-(4-(3-trifluoromethylphenyl)piperazin-1-yl)-2-oxoethyl)phenyl)-(10S)- dihydroartemisinin (**4c**).* White solid, 0.134 g; yield: 10.4%, mp: 96–97 °C; IR (KBr): 2945, 1646, 1610, 1510, 1450, 1348, 1309, 1231, 1121, 1095, 1075, 1035, 979, 958, 877 cm^{-1}; MS: 631.3 $[M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 7.43 (1H, t, Ar–H), 7.20 (4H, m, Ar–H), 7.09 (1H, d, J = 7.6 Hz, Ar–H), 7.01 (2H, d, J = 8.4 Hz, Ar–H), 5.51 (1H, d, J = 3.2 Hz, H-10), 5.42 (1H, s, H-12), 3.71 (2H, s, $ArCH_2$-), 3.63 (4H, m, -NCH_2-), 3.19 (4H, m, -NCH_2-), 2.58 (1H, m, H-9), 2.18 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.39–1.36 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.96 (3H, d, J = 7.2 Hz, H-16), 0.90 (3H, d, J = 6.3 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 169.5, 155.9, 151.3, 130.4, 130.3, 129.6, 125.7, 123.9, 119.4, 117.4, 115.3, 111.6, 103.9, 99.9, 87.7, 80.8, 52.4, 48.3, 47.9, 44.1, 41.2, 36.9, 36.3, 34.5, 30.8, 26.0, 24.5, 24.4, 20.5, 13.0. HRMS (ESI) m/z calcd for $C_{34}H_{41}F_3N_2O_6Na$ $[M+Na]^+$ 653.2809, found 653.2801.

*4.1.2.9. 10-O-(4-(2-(4-(4-fluorophenyl)piperazin-1-yl)-2-oxoethyl) phenyl)-(10S)-dihydroartemisinin (**4d**).* White solid, 0.172 g; yield: 14.8%, mp: 92–94 °C; IR (KBr): 3441, 2922, 2873, 1645, 1510, 1452, 1380, 1231, 1095, 1035, 979, 959, 939, 876, 827 cm^{-1}; MS: 581.8 $[M+H]^+$, 603.8$[M+Na]^+$; ^1H NMR (DMSO, δ (ppm)): 7.17 (2H, d, $J = 8.4$ Hz, Ar–H), 7.00 (4H, m, Ar–H), 6.98 (2H, d, $J = 8.4$ Hz, Ar–H), 5.50 (1H, d, $J = 3.3$ Hz, H-10), 5.42(1H, s, H-12), 3.70 (2H, s, $ArCH_2$-), 3.61 (4H, m, -NCH_2-), 3.00 (4H, m, -NCH_2-), 2.57 (1H, m, H-9), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.38 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.96(3H, d, $J = 7.2$ Hz, H-16), 0.90 (3H, d, $J = 6.3$ Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 169.4, 157.4, 155.9, 148.1, 148.0, 130.3, 129.6, 118.1, 118.0, 117.4, 115.8, 115.6, 103.9, 99.9, 87.8, 80.8, 52.4, 49.9, 49.4, 44.1, 41.4, 36.9, 36.4, 34.5, 30.8, 26.0, 24.5, 24.4, 20.5, 13.0. HRMS (ESI) m/z calcd for $C_{33}H_{42}FN_2O_6$ $[M+H]^+$ 581.3021, found 581.3045.

*4.1.2.10. 10-O-(4-(2-oxo-2-(4-(pyridin-2-yl)piperazin-1-yl)ethyl) phenyl)-(10S)-dihydroartemisinin (**4e**).* White solid, 0.168 g; yield: 14.9%, mp: 116–118 °C; IR (KBr): 2922, 2872, 1646, 1593, 1510, 1480, 1435, 1376, 1230, 1095, 1035, 979, 959, 877 cm^{-1}; MS: 564.8 $[M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 8.11 (1H, m, Ar–H), 7.56 (1H, m, Ar–H), 7.19 (2H, d, $J = 8.4$ Hz, Ar–H), 6.99 (2H, d, $J = 8.4$ Hz, Ar–H), 6.80 (1H, m, Ar–H), 6.65 (1H, m, Ar–H), 5.50 (1H, d, $J = 3.0$ Hz, H-10), 5.42 (1H, s, H-12), 3.71 (2H, s, $ArCH_2$-), 3.58 (4H, m, -NCH_2-), 3.45 (4H, m, -NCH_2-), 2.57 (1H, m, H-9), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.66–1.50 (2H, m, H-5 and H-8a), 1.38 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.96 (3H, d, $J = 7.2$ Hz, H-16), 0.90 (3H, d, $J = 6.0$ Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 169.6, 159.1, 155.9, 147.9, 138.0, 130.3, 129.6, 117.4, 113.6, 107.6, 103.9, 99.9, 87.8, 80.8, 52.4, 45.4, 44.8, 44.1, 41.2, 36.9, 36.4, 34.5, 30.8, 26.0, 24.5, 24.4, 20.6, 13.0. HRMS (ESI) m/z calcd for $C_{32}H_{42}N_3O_6$ $[M+H]^+$ 564.3068, found 564.3066.

*4.1.2.11. 10-O-((E)-4-(3-oxo-3-(4-phenylpiperazin-1-yl)prop-1-enyl) phenyl)-(10S)-dihydro artemisinin (**5a**).* White solid, 0.238 g; yield: 20.7%, mp: 88–89 °C; IR (KBr): 3444, 2922, 2872, 1731, 1647, 1602, 1508, 1384, 1210, 1035, 957, 876, 830, 742, 700 cm^{-1}; MS: 575.4 $[M+H]^+$, 597.3 $[M+Na]^+$; ^1H NMR (DMSO, δ (ppm)): 7.71 (2H, d, $J = 8.4$ Hz, Ar–H), 7.50 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.24 (2H, m, Ar–H), 7.20 (1H, d, $J = 15.3$ Hz, -CH=CH-),7.11 (2H, d, $J = 8.4$ Hz, Ar–H), 6.97 (2H, d, $J = 8.1$ Hz, Ar–H), 6.81 (1H, t, Ar–H), 5.64 (1H, d, $J = 3.3$ Hz, H-10), 5.41 (1H, s, H-12), 3.84–3.73 (4H, m, -NCH_2-), 3.17 (4H, s, -NCH_2-), 2.62 (1H, m, H-9), 2.20 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.67–1.50 (2H, m, H-5 and H-8a), 1.39–1.35 (2H, m, H-6 and H-8), 1.29 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.97 (3H, d, $J = 6.9$ Hz, H-16), 0.91 (3H, d, J = 5.7 Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 165.0, 158.4, 151.2, 141.8, 130.0, 129.4, 119.7, 117.4, 116.4, 116.2, 104.0, 99.5, 87.8, 80.7, 52.4, 49.4, 48.8, 44.0, 36.9, 36.3, 34.5, 30.7, 26.0, 24.5, 24.3, 20.5, 12.9. HRMS (ESI) m/z calcd for $C_{34}H_{43}N_2O_6$ $[M+H]^+$ 575.3116, found 575.3133.

*4.1.2.12. 10-O-((E)-4-(3-oxo-3-(4-(2-methoxyphenyl)piperazin-1-yl) prop-1-enyl)phenyl)-(10S)-dihydro- artemisinin (**5b**).* White solid, 0.115 g; yield: 9.5%, mp: 144–146 °C; IR (KBr): 2941, 2845, 1646, 1599, 1508, 1435, 1374, 1227, 1097, 1036, 979, 955, 876 cm^{-1}; MS: 605.3 $[M+H]^+$, 627.3 $[M+Na]^+$, 1029.7 $[2M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 7.69 (2H, d, $J = 8.4$ Hz, Ar–H), 7.47 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.18 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.10 (2H, d, $J = 8.4$ Hz, Ar–H), 6.96 (2H, m, Ar–H), 6.90 (2H, m, Ar–H), 5.64 (1H, d, $J = 2.4$ Hz, H-10), 5.41 (1H, s, H-12), 3.80 (3H, s, OCH_3), 3.71 (4H, m, -NCH_2-), 2.97 (4H, s, -NCH_2-), 2.61 (1H, m, H-9), 2.19 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.67–1.50 (2H, m, H-5 and H-8a), 1.39–1.35 (2H, m, H-6 and H-8), 1.29 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.96 (3H, d, $J = 7.2$ Hz, H-16), 0.90 (3H, d, $J = 6.0$ Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 165.0, 158.4, 152.4, 141.7, 141.2, 129.9, 129.4, 123.2, 121.2, 118.7, 117.4, 116.4, 112.3, 104.0, 99.5, 87.8, 80.7, 55.7, 52.4, 51.2, 50.6, 44.0, 36.9, 36.3, 34.5, 30.7, 26.0, 24.5, 24.3, 20.5, 12.9. HRMS (ESI) m/z calcd for $C_{35}H_{45}N_2O_7$ $[M+H]^+$ 605.3221, found 605.3218.

*4.1.2.13. 10-O-((E)-4-(3-oxo-3-(4-(3-trifluoromethylphenyl)piperazin-1-yl)prop-1-enyl)phenyl)-(10S)-dihydro- artemisinin (**5c**).* White solid, 0.114 g; yield: 8.9%, mp: 100–102 °C; IR (KBr): 2941, 2872, 1649, 1602, 1509, 1448, 1349, 1231, 1122, 1097, 1035, 978, 956, 876 cm^{-1}; MS: 643.4 $[M+H]^+$, 665.4 $[M+Na]^+$, 681.4 $[M+K]^+$; ^1H NMR (DMSO, δ (ppm)): 7.70 (2H, d, $J = 8.7$ Hz, Ar–H), 7.49 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.20 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.08 (4H, m, Ar–H), 7.00 (2H, m, Ar–H), 5.64 (1H, d, $J = 3.0$ Hz, H-10), 5.41 (1H, s, H-12), 3.78 (4H, d, -NCH_2-), 3.10 (4H, s, -NCH_2-), 2.60 (1H, m, H-9), 2.20 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.67–1.50 (2H, m, H-5 and H-8a), 1.39–1.35 (2H, m, H-6 and H-8), 1.29 (3H, s, H-14), 1.25–1.10 (2H, m H-5a and H-7), 0.98 (3H, d, $J = 7.5$ Hz, H-16), 0.92 (3H, d, $J = 6.3$ Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 165.2, 158.5, 151.5, 141.9, 130.5, 130.0, 129.4, 126.2, 123.5, 119.5, 117.5, 116.4, 115.4, 111.7, 104.0, 99.6, 87.9, 80.8, 52.5, 48.7, 48.1, 44.1, 37.0, 36.4, 34.6, 30.8, 26.0, 24.6, 24.4, 20.6, 13.0. HRMS (ESI) m/z calcd for $C_{35}H_{42}F_3N_2O_6$ $[M+H]^+$ 643.2989, found 643.3001.

*4.1.2.14. 10-O-((E)-4-(3-oxo-3-(4-fluorophenyl)piperazin-1-yl)prop-1-enyl)phenyl)-(10S)-dihydroartemisinin (**5d**).* Yellow solid, 0.245 g; yield: 20.7%, mp: 88–89 °C; IR (KBr): 2926, 1647, 1602, 1509, 1436, 1375, 1229, 1095, 1035, 978, 957, 877, 829 cm^{-1}; MS: 593.3 $[M+H]^+$; ^1H NMR (DMSO, δ (ppm)): 7.70 (2H, d, $J = 8.7$ Hz, Ar–H), 7.49 (1H, d, $J = 15.2$ Hz, -CH=CH-), 7.20 (1H, d, $J = 15.2$ Hz, -CH=CH-), 7.08 (4H, m, Ar–H), 7.01 (2H, m, Ar–H), 5.64 (1H, d, $J = 3.0$ Hz, H-10), 5.41 (1H, s, H-12), 3.78 (4H, d, -NCH_2-), 3.10 (4H, s, -NCH_2-), 2.60 (1H, m, H-9), 2.20 (1H, m, H-4), 2.03–1.90 (2H, m, H-4 and H-5), 1.85–1.81 (2H, m, H-7 and H-8), 1.67–1.50 (2H, m, H-5 and H-8a), 1.39–1.35 (2H, m, H-6 and H-8), 1.28 (3H, s, H-14), 1.25–1.07 (2H, m H-5a and H-7), 0.98 (3H, d, $J = 7.5$ Hz, H-16), 0.92 (3H, d, $J = 6.3$ Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 165.0, 158.4, 155.9, 148.1, 141.8, 130.0, 129.4, 118.2, 117.4, 116.4, 115.6, 104.0, 99.5, 87.8, 80.7, 52.4, 50.2, 49.6, 44.0, 36.9, 36.3, 34.5, 30.7, 26.0, 24.5, 24.3, 20.5, 12.9. HRMS (ESI) m/z calcd for $C_{34}H_{42}FN_2O_6$ $[M+H]^+$ 593.3021, found 593.3019.

*4.1.2.15. 10-O-((E)-4-(3-oxo-3-(4-(pyridin-2-yl)piperazin-1-yl)prop-1-enyl)phenyl)-(10S)-dihydroartemisinin (**5e**).* White solid, 0.094 g; yield: 8.2%, mp: 67–69 °C; IR (KBr): 2943, 2859, 1726, 1647, 1598, 1509, 1481, 1435, 1375, 1229, 1207, 1097, 1037, 979, 957, 876 cm^{-1}; MS: 576.3 $[M+H]^+$, 1152.5 $[2M+H]^+$; ^1H NMR ($CDCl_3$, δ (ppm)): 8.23 (1H, d, $J = 3.3$ Hz, Ar–H), 7.72 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.57 (1H, d, $J = 15.3$ Hz, -CH=CH-), 7.54 (3H, m, Ar–H), 7.14 (2H, d, $J = 8.4$ Hz, Ar–H), 6.69 (2H, d, $J = 8.4$ Hz, Ar–H), 5.56 (1H, d, $J = 3.0$ Hz, H-10), 5.48 (1H, s, H-12), 3.85 (4H, m, -NCH_2-), 3.65 (4H, m, -NCH_2-), 2.84 (1H, m, H-9), 2.40 (1H, m, H-4), 2.24–2.18 (1H, m, H-4), 2.07–1.80 (3H, m, H-5, H-7 and H-8), 1.76–1.70 (2H, m, H-5 and H-8a), 1.62 (2H, m, H-6 and H-8), 1.46 (3H, s, H-14), 1.34–1.27 (2H, m H-5a and H-7), 0.96 (3H, d, $J = 7.2$ Hz, H-16), 0.91 (3H, d, $J = 6.0$ Hz, H-15). ^{13}C NMR (DMSO, δ (ppm)): 167.3, 159.1, 158.4, 148.0, 141.8, 138.0, 130.0, 122.6, 117.4, 116.4, 113.6, 107.6, 104.0, 87.8, 80.7, 52.4, 45.4, 44.9, 44.0, 36.9, 36.3, 34.5, 30.7, 26.0, 24.5, 24.3, 20.5, 12.9. HRMS (ESI) m/z calcd for $C_{33}H_{42}N_3O_6$ $[M+H]^+$ 576.3068, found 576.3061.

4.2. Biological assays

Reagents and chemicals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Quinidine, Metoprolol and Atenolol were obtained from Sigma and penicillin/streptomycin was purchased from Invitrogen. Fetal bovine serum (FBS), phosphate buffered saline (PBS), DMEM and trypsin were purchased from Biosera. Adriamycin and dimethyl sulfoxide were obtained from EBEWE Pharma and Merck, respectively. Propidium iodide (PI) was purchased from BD Biosciences (San Diego, CA). Antibodies to Parp, p27, cyclin D1, cyclin B1 and β-actin were obtained from Santa Cruz Biotechology, Inc. (Santa Cruz, CA).

4.2.1. The antiproliferative activity assay

Cell cultures. MCF-7 and MCF-7/Adr cell lines were maintained at 37 °C in humidified air containing 5% CO_2. Both cell lines were maintained in DMEM supplemented with 10% FBS, and 100 units/ml penicillin-G and 100 μg/ml streptomycin and 0.37% $NaHCO_3$.

Method. MCF-7 and MCF-7/Adr cells were planted into 96-well micro-plates at a density of 1.5–4.0 × 10^4 cells/mL (100 μL per well). After overnight incubation at 37 °C, 5–9 different concentrations of test compounds were added to the wells. Compounds were dissolved in DMSO before being diluted in the growth medium. The concentration of DMSO in the wells did not exceed 0.5%. cells were further incubated for 96 h, at the end of the incubation time, fresh medium containing 0.5 mg/mL of MTT were added. Plates were incubated for another 4 h at 37 °C, the media was removed and formazan crystals formed in the cells were dissolved in 200 μL of DMSO. Optical density was measured at 570 nm with DMSO as blank using a Bio-Rad microplate reader (Model 680). The inhibition ratio of viability for each concentration of the compounds was calculated with respect to the control and GI_{50} values were estimated with the software CurveExpert version 1.34 for Windows. Each experiment was repeated 3–5 times and the results were summarized in Table 1 as mean ± SD (in μM).

4.2.2. MDCK-MDR1 cells and MDCK-WT cells transport [28]

MDCK-MDR1 cells were seeded onto polycarbonate filter Transwells, and confluent MDCK-MDR1 monolayers expressing P-gp were obtained 5 days postseeding. The trans epithellal electrical resistance (TEER) of MDCK-MDR1 monolayers from randomly selected wells was higher than 1000 Ω cm^2 before use in transport experiments. MDCK-WT cells were seeded at a density of 5.0 × 10^4 cells/well, and matured exhibiting a TEER higher than 200 Ω cm^2 before use in transport experiments after 6 days. Cell monolayer integrity was evaluated by measurement of Lucifer yellow permeability by monitoring the change in TEER over the course of the experiment. Transport experiments of the test compounds were performed at 37 °C Cin HBSS at pH 7.4. Separate the apical plate from the basolateral plate after 90 min incubation. A 100 μL sample was taken from the donor compartment to determine the concentration of the compound remaining in the donor chamber at the end of the experiment.

4.2.3. Flow cytometry and western blot analysis

Flow cytometry. After treatment with **5c** for 24 h or 48 h, cells were trypsinized, fixed in ice-cold 70% ethanol, and stained with 50 μg/mL propidium iodide (PI) in the presence of RNase at 37 °C for 30 min. DNA content was measured using a FACS Calibur flow cytometer (BD Accuri C6), and the percentage of cells in each phase of the cell cycle was analyzed by the Accuri C6 software.

Western blot analysis. Protein extracts (50 μg) prepared with RIPA lysis buffer [50 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 μmol/L leupeptin, and 2 μg/mL aprotinin (pH, 8.0)] were separated on 10% or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes loading protein were incubated with specific antibodies overnight at 4 °C. Immunocomplexes were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences Inc., Piscataway, NJ) [29].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.10.040.

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