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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · NOVEMBER 2011

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2011.11.011 · Source: PubMed

European Journal of Medicinal Chemistry 47 (2012) 424–431

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design, synthesis and biological evaluation of novel glycosylated diphyllin derivatives as topoisomerase II inhibitors

Da-Kuo Shi [a], Wei Zhang [b], Ning Ding [b], Ming Li [a,**], Ying-Xia Li [b,*]

[a] School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China
[b] School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 22 September 2011
Received in revised form
3 November 2011
Accepted 4 November 2011
Available online 13 November 2011

Keywords:
Diphyllin
Topo II inhibitors
Synthesis
Anticancer
Structure–activity relationship

ABSTRACT

Recently, a novel glycosylated diphyllin derivative **11** which exhibiting potent anticancer activity by targeting topoisomerase IIα was reported by our group. In order to provide more molecules for structure-activity relationship (SAR) studies, 12 new glycosylated diphyllin analogs have been designed, synthesized, and evaluated for their biological activities. The SAR analysis revealed that (i) the sugar moiety on the diphyllin is essential for the anticancer activity; (ii) equatorial C4′–OH on the sugar is superior to the axial one, and (iii) a proper cyclic lipophilic group at the C4′ and C6′ of sugar might enhance the anticancer activity.

1. Introduction

Diphyllin (**1**, Fig. 1) is a typical 1-arylnaphthalene lignan isolated from the plant *Justicia procumbens L.* (Acanthaceae), which was found to have a wide spectrum of biological activities, such as cytotoxicity [1,2], antimicrobial [3] and antiviral [4]. Among these, the cytotoxicity is the most attractive, making arylnaphthalene a good lead compound for anticancer drug design and development [5,6]. On the other hand, the role of carbohydrates in biologically active natural products attracts much more attentions than before [7]. With deep insights into the understanding of aglycone and glycoside activities, followed by the interesting stories of morphine-6-glucuronide [8], rebeccamycin analogs with uncommon sugars [9] and subtle sugar modification of digitoxin [10] etc, the emphasis on the key requirement of sugars and in particular the uncommon sugars is emerging as a new driving force in the development of new type distinct from the conventional ones.

In recent years, lots of diphyllin glycoside derivatives have been isolated from the Acanthaceae plants continuously. Sophie Susplugas *et. al.* obtained three kinds of diphyllin glycosides (**2**–**4**, Fig. 1) from *Justicia patentiflor Hemsl* [11], which have attracted us to enjoy these research due to their special structure and biological activities. Such sugar appendages add important features to the shape and the stereoelectronic properties of a molecule and thus wield remarkable influences which range from modulating pharmacology and pharmacokinetic properties to dictating specificity on a tissue, cellular and/or molecular level [12,13]. This concept appeals a surge of interests in the development of novel glycosylated compounds for cancer therapy.

In a previous paper, we reported that **11**(Fig. 1) [14], a novel diphyllin glycoside with acetylated D-quinovose sugar moiety, as a potent topoisomerase IIα (Topo IIα) inhibitior. This peculiar sugar moiety endows **11** an optimal conformation with a high binding affinity for Topo IIα via hydrogen bonding to the entrance of ATPase pocket, thereby helping achieve more potent Topo II inhibition activity compared to the aglycon diphyllin.

With the availability of the synthesized diphyllin in hand [15,16], and in particularly bearing in mind the critical contribution of sugar attachments to the outcome of molecular shape and biological diverse functions, we are encouraged to challenge the glycosylation manipulation to the diphyllin, with the aim to probe insights into the SAR-based nature behind the glycosylated diphyllin analogs via the design, synthesis and biological evaluation.

2. Chemistry

The correlation between the structural feature of sugar moiety and the cytotoxic potency revealed that the glycoside **2** with sugar

* Corresponding author. Tel./fax: +86 21 51980127.
** Corresponding author. Tel.: +86 532 82032150.
E-mail addresses: lmsnouc@ouc.edu.cn (M. Li), liyx417@fudan.edu.cn (Y.-X. Li).

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doi:10.1016/j.ejmech.2011.11.011

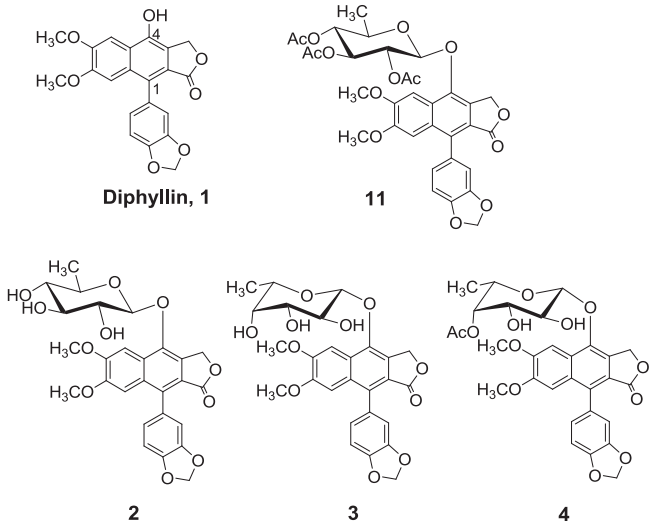


Fig. 1. Structures of diphyllin and its glycosylated analogs.

residue of $^4C_1$ chair conformation was more potent than glycoside **3** and **4** with sugar residue of $^1C_4$ chair conformation, and glycoside **4** with acetylated sugar residue was more potency than **3** [11]. Besides, glycoside **2** showed potent cytotoxicity to drug-resistant cell strain MCF-7R with IC_{50} 40 nM, while glycoside **3** exhibited no activity to this cell strain. Starting from the most potent glycoside **2** and maintaining the 1, 2-*trans* glycosidic bond, first to explore the importance of functional groups of C6 in the sugar, replacing the methyl group (**2**) with CH_2OH (**7**), H (**8**), CH_2OMe (**9**) and CH_2OBn (**13**) groups respectively lead to the first series (Series A) of analogs. In order to determining the effect of the C4–OH orientation of sugar on the bioactivity, the resulting corresponding C4 epimers of Series A lead to the second series (Series B) of analogs. Furthermore, to block simultaneously 4,6-dihydroxy groups of the glucose moiety (**6**), the third series (Series C) of the cyclic acetal derivatives (**15**–**17**) have also been designed and prepared (Fig. 2).

Retrosynthetically, the analogs (**5**–**14**) can be logically disconnected into two fragments—diphyllin and the sugar residues, which are incorporated by β-glycosidic bonds. The 1, 2-*trans*-glycosidic bonds could be formed effectively by glycosylation of diphyllin with peracetylated 1,2-*cis*-glycosyl bromides under phase transfer conditions (TBAB, $CHCl_3$, 0.1 mol L^{-1} NaOH in H_2O, 40 °C) [15]. Subsequently, deacetylation was carried out with K_2CO_3 in methanol to produce target molecule without destroying the lactone residue (Scheme 1 and Table 1). This synthetic method allowed us to obtain analogs **5**–**14** with D-glucosyl (**7**), D-xylosyl(**8**), 6-OMe-D-glucosyl (**9**),6-OBn-D-glucosyl (**13**), D-galactosyl (**5**), L-arabinosyl (**6**), 6-OMe-D-galactosyl (**10**), D-fucosyl (**12**) and 6-OBn-D-galactosyl (**14**) residue respectively.

With glucoside **7** in hand, we further synthesized the cyclic acetals (**15**–**17**), which was smoothly achieved by reaction of β-D-diphyllin glucoside with the corresponding aldehyde or ketone acetal in the presence of catalytic amount of *p*-TsOH·H_2O (Scheme 2).

3. Results and discussion

3.1. In Vitro cytotoxicity

Preliminarily, the cytotoxic activity of all glycosylated diphyllin derivatives were evaluated toward the human colon carcinoma HCT-116, human breast carcinoma MCF-7 and oral epidermoid carcinoma KB cell lines [17,18]. For comparative purposes, compounds **2**, **3**, **4** and Etoposide (**VP-16**) were also included in the assay.

As shown in Table 2, compared with aglycon diphyllin, compounds in Series A and Series C showed more potent cytotoxicities, while compounds in Series B were less potent. Their activity was followed by the order: series C > series A > diphylin > series B. These results indicated the sugar moiety attached on diphyllin is a key element for its cytotoxicity. Series A were much more potent than series B due to the change of 4-equatorial-OH to 4-axial-OH of sugar, implicating that axial-OH at C4 did not favor the activity. Comparing the compounds in series A, it was shown that the C6 functional groups at the sugar strongly affect the bioactivity, the analog **13** with 6-OBn-D-glucosyl moiety showed the most potent cytotoxicity, and in the order **11**(6-Me-D-glucosyl) >**8** (D-xylosyl) >> **7** (D-glucosyl) > **9** (6-OMe-D-glucosyl). More interestingly, the 4, 6-cyclic acetal derivatives of series C (**15**–**17**) showed much more potent cytotoxicity than other analogs by up to 1000-fold,

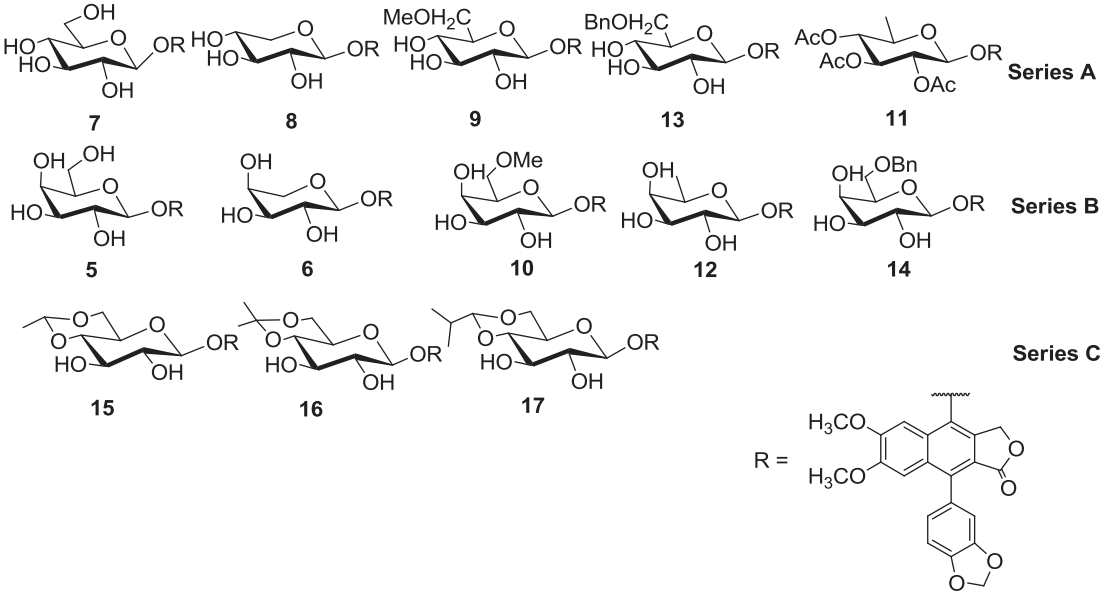
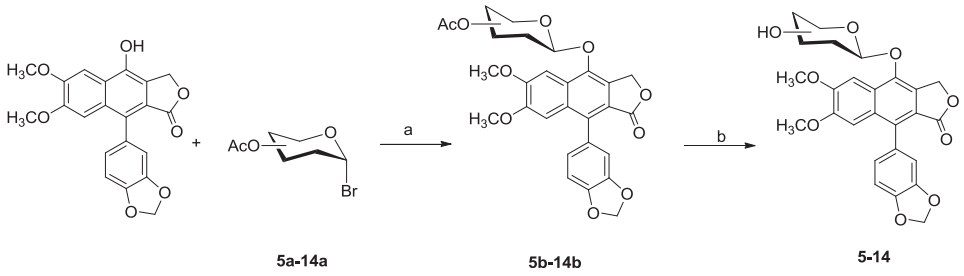


Fig. 2. Structures of designed diphyllin glycosylated derivatives.



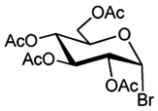
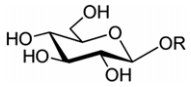
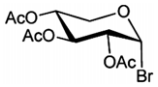
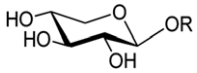
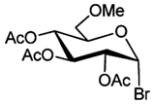
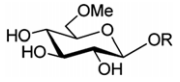
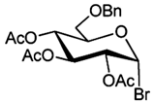
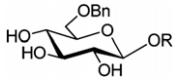
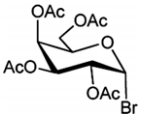
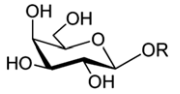
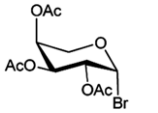
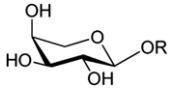
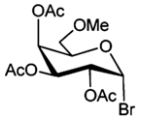
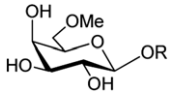
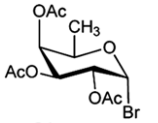
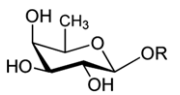
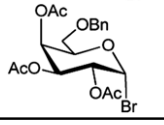
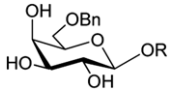
Scheme 1. Reagents and conditions: a) $CHCl_3$, H_2O, TBAB, NaOH, 40 °C; b) K_2CO_3, MeOH.

indicating that a small size lipophilic group masked on the 4–OH and 6–OH favored the bioactivity.

3.2. Multidrug resistance analysis

To further investigate their drug resistance profile, diphyllin, compounds **2**, **8**, **11** and **15** were selected to test against chronic myelogenous leukemia K562 and its adriamycin-selected MDR cell subline K562/A02 as well as oral epidermoid carcinoma KB and its vincristine (VCR)-selected MDR KB/VCR subline [19–21] (Table 3). These compounds retained better inhibition against selected oral epidermoid carcinoma cellular subclones resistant to VCR (KB/VCR) compared to selected leukemia cellular subline resistant to adriamycin (K562/A02). In particular, the KB/VCR cell line exhibited a low level of cross-resistance to **2**, **8** and **11** (RI (resistance index) = 2.9–6.2) except **15** (RI = 25.3), whereas higher cross-resistance to these compounds was observed in the adriamycin resistant subline K562/A02 (RI = 9.5–19.4). These suggest targeting Topo II may contribute to the higher resistant index of diphyllin and its derivates.

Table 1
Results of the glycosylation reaction of donors with diphyllin.

Entry	Donor		Product [a]		Yield (%)[b]
1		**7a**		**7**	71
2		**8a**		**8**	63
3		**9a**		**9**	79
4		**13a**		**13**	67
5		**5a**		**5**	80
6		**6a**		**6**	59
7		**10a**		**10**	58
8		**12a**		**12**	54
9		**14a**		**14**	70

[a] R represents 4-*O*-diphyllin group.
[b] Two steps for glycosylation and deacetylation.

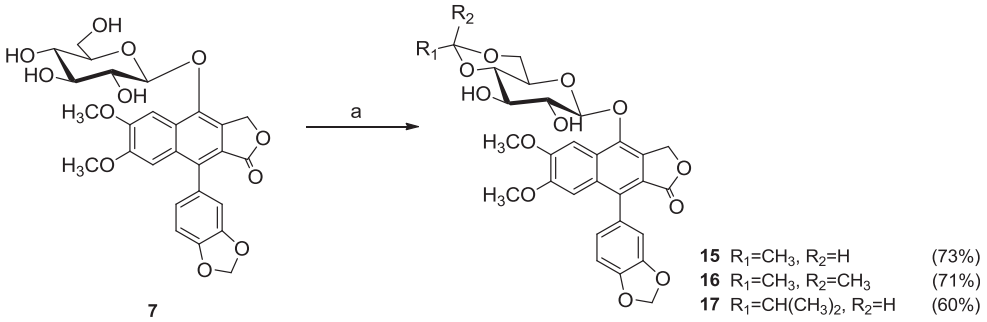
3.3. Drug inhibition of kDNA decatenation induced by topoisomerase II (Topo II)

The relative compound activity in inhibiting Topo II-mediated kDNA decatenation was studied using catenated kinetoplast DNA as a substrate for human Topo II and VP-16 as a Topo II-specific inhibitor [22]. The preliminary experiments indicated that the compounds containing quinovopyranosyl, glucopyranosyl, xylopyranosyl, 6-*O*-methyl-pyranosyl residue (**2**, **4**, **11** and **13**) and acetal glycosides (**15**, **16** and **17**) exhibited potent inhibitory activity (Fig. 3), whereas the remaining compounds did not inhibit Topo II catalytic activity at a concentration of 100 μM. The most potent cytotoxic compounds **13**, **15**, **16**, and **17** inhibited most strongly Topo II catalytic activity. These results demonstrate that there is a good correlation between cytotoxicity in cancer cell cultures and Topo II inhibitory activity.

3.4. Cell cycle arrest and apoptosis

Previous studies have indicated patentiflorin A(**2**) caused G_0/G_1 arrest [11]. On these grounds the action on the cell cycle of these compounds were studied by flow cytometry. To gain further insight into the mode of action of these compounds, we examined the effects of compounds **2**, **8**, **11**, **12** and **15** which were determined cell cycle accumulation at 24 h by propidium iodide staining and flow cytometry quantification in HL-60 cells.

Cell cycle analysis of HL-60 cells showed that cells were arrested at the G0/G1 and G2/M phase following treatment with **2**, **8**, **11** and **15** (Table 4), whereas no cell cycle arrest was observed for **12**. This cell cycle arrest was dose- and time-dependent with a relative potency of the assayed compounds being **15** > **11** > **8** > **12**, which was in agreement with the cytotoxicity results for these compounds. Higher concentrations and longer incubation times are required for the less potent analogs in order to get a similar cell cycle response to that achieved by the most potent compounds. Similar results were obtained with human colon adenocarcinoma HCT-116 cells (data not shown).



Scheme 2. Reagents and conditions: a) $R_1R_2CH(OMe)_2$, *p*-TsOH·H_2O.

3.5. DNA fragmentation during drug-induced apoptosis

To better characterize the biological activity of these compounds, we tested the effects of a selected series of derivatives on programmed cell death (apoptosis), using the human leukemia cell line HL-60 as the experimental system. We examined whether these compounds induced DNA fragmentation, which is considered the end point of the apoptotic pathway [23]. As shown in Fig. 4, all the compounds (10 μM, 36 h) induced DNA fragmentation. Meanwhile **2**, **11** and **12** displayed similar values in the percentage of apoptotic cells, and **15** was the most potent compound to induce apoptosis.

4. Conclusion

In summary, new glycosylated diphyllin derivatives containing different sugars or chemical modified sugars were prepared and evaluated against several human tumor cell lines. All the compounds showed IC_{50} cytotoxicity levels in the μM-nM range. In general, those compounds containing 4′, 6′-cyclic acetal glycosylated derivatives (Series C) were more potent than other series. The results suggest that a moderate size cyclic lipophilic group at the C4′ and C6′ of sugar is very important to the bioactivity. By comparing the three series of compounds, glycosides with equatorial C4′–OH in the sugars are more active than those with axial C4′–OH, indicating the equatorial C4′–OH played a very important role.

Furthermore, some derivatives showed multi-drug resistant (MDR) activity against two MDR cell lines (K562/A02 and KB/VCR), which were much lower than those of ADR, VCR, and VP-16. The inhibitory activity of these compounds toward Topo II investigated by Topo II-induced kDNA decatenation assay matched their cytotoxicity well, suggesting Topo II is one of the main targets of this series of compounds. Using the human leukemia HL-60 cell line, selected compounds were found to induce G0/G1 arrest and DNA fragmentation characteristic of apoptotic cell death. These results suggest that the sugar moiety on the C4 of diphyllin is a key element for its anti-tumor activity.

In addition, all acetals of diphyllin glucoside showed much more potent cytotoxicity and Topo II inhibitory activity compared with the other derivates. Therefore, these derivatives can provide some information about glycoside modification to natural products and should be considered as interesting lead compounds for anticancer compounds research.

Table 2
Inhibitory effect of compounds **5**–**17** response for 50% growth inhibition (IC_{50}, μM) on human tumor cell lines (HCT-116, MCF-7, KB).

Series	Compound	IC_{50}, μM		
		HCT-116	MCF-7	KB
	VP-16	0.91	1.09	0.85
	Diphyllin	3.54	0.54	0.78
	2	0.99	0.44	0.093
	3	>10	1.42	>10
	4	0.27	0.11	0.14
Series A	**7**	9.67	2.44	>10
	8	0.82	1.55	0.41
	9	>10	>10	4.5
	11	0.27	0.38	0.44
	13	0.036	0.028	0.015
Series B	**5**	>10	4.11	4.89
	6	>10	>10	6.88
	10	>10	>10	4.1
	12	6.43	1.81	3.05
Series C	**15**	0.008	0.004	0.003
	16	0.043	0.053	0.010
	17	0.016	0.0002	0.0006

5. Experimental

5.1. Chemistry

Solvents were purified in the usual way. TLC was performed on precoated Merck silica Gel 60 F254 plates. Flash chromatography was performed on silica gel (300–400 mesh Qingdao, China). Optical rotations were determined with a Perkin–Elmer Model 241 MC polarimeter. ^{1}H NMR and ^{13}C NMR spectra were taken on a JEOL JNM-ECP 600 MHz spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts were recorded in δ values. The high resolution mass spectra were obtained on a Q-TOF Global Mass (ESIMS) instrument.

5.1.1. General procedure of the diphyllin glycoside compounds (5–14)

Step A: To the solution of diphyllin (1.0 mmol) and TBAB (1.0 mmol) in $CHCl_3$ (20 mL) was added aqueous 0.1 mol•L^{-1} NaOH (20 mL). After stirring for 10 min at 40 °C, the corresponding substituted sugar bromide (1.5 mmol) was added, and the two-phase reaction mixture was stirred for 6 h at 40 °C. Then $CHCl_3$

Table 3
The effect of **Diphyllin**, **2**, **8**, **11** and **15** to the resistant tumor cell lines.

Compd	IC_{50} (μM)					
	KB	KB/VCR	RI	K562	K562/A02	RI
Diphyllin	0.78	3.15	4.0	1.67	23.46	14.1
2	0.093	0.573	6.2	1.43	16.14	11.3
8	0.41	1.19	2.9	4.402	58.82	13.4
11	0.44	1.32	3.0	0.808	7.66	9.5
15	0.003	0.076	25.3	0.044	0.853	19.4
VP-16	0.85	153.26	180.3	5.16	242.8	47.1
ADR	NT	NT	NT	0.43	56.83	132.2
VCR	0.003	1.61	536.7	NT	NT	NT

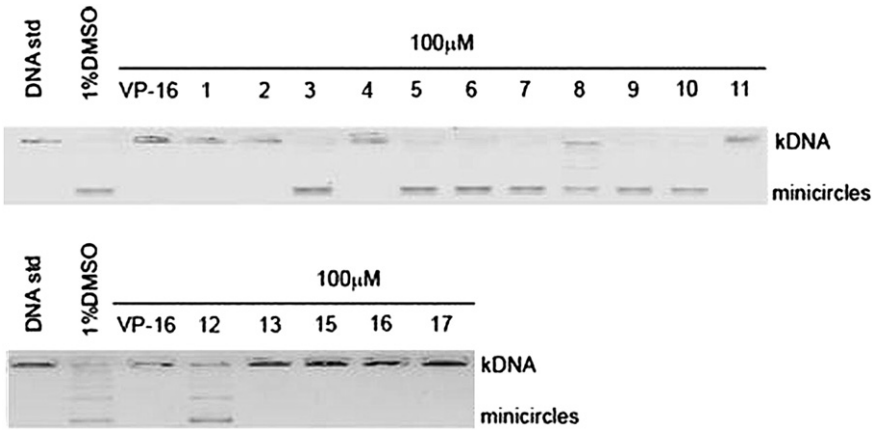


Fig. 3. Drug inhibition of kDNA decatenation induced by topoisomerase II (Topo II).

Table 4
Effect of **2**, **8**, **11**, **12** and **15** on cell cycle in HL-60 cells.

Treatment		% of cells		
Compd	Dose (M)	G0/G1	S	G2/M
Control		39.0	38.4	22.6
2	10^{-6}	45.9	38.0	16.1
	10^{-5}	54.4	22.8	22.8
8	10^{-6}	45.6	35.5	18.9
	10^{-5}	47.0	33.8	19.2
11	10^{-6}	46.4	36.3	17.4
	10^{-5}	55.0	19.3	25.7
12	10^{-6}	38.6	30.0	31.5
	10^{-5}	40.5	34.6	24.9
15	10^{-6}	53.6	23.4	22.9
	10^{-5}	56.2	19.2	24.6

(20 mL) was added and the resulting organic phase was washed with water (2 × 20 mL), dried (Na_2SO_4), and the solvent evaporated under reduced pressure. The residue was purified by flash chromatography (1:1, EtOAc-petroleum ether) to afford a solid.

Step B: The solid above (0.8 mmol) was dissolved in CH_3OH (20 mL), and then K_2CO_3 (138 mg, 1.0 mmol) was added. After stirring for 60 min at room temperature the solution was neutralized with 1 mol•L^{-1} HCl and concentrated under reduced pressure. $CHCl_3$ (20 mL) was added and the resulting organic phase was washed with water (2 × 20 mL) and dried (Na_2SO_4), and the solvent was evaporated under reduced pressure to get a residue, which was purified by flash chromatography (25:1, CH_2Cl_2 :MeOH) to afford a solid.

*5.1.1.1. 4-O-β-D-galactopyranosyldiphyllin (**5**).* White solid, yield 79.9%, $\mathbf{R_f}$ 0.31 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -36.0^\circ$ (*c*1.00, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.20 (d, 1 H, J = 5.0 Hz, ArH), 7.04 (d, 1 H, J = 7.8 Hz, ArH), 6.98 (s, 1 H, ArH), 6.93 (d, 1 H, J = 1.4 Hz, ArH), 6.81–6.78 (dd, 1 H, J = 7.8, 1.4 Hz, ArH), 6.13 (s, 2 H, OCH_2O), 5.80 (d, 1 H, J = 5.0 Hz, OH-2″), 5.70 (d, 1 H, J = 14.6 Hz, H-9a), 5.49 (dd, 1 H, J = 14.6, 3.2 Hz, H-9b), 5.00 (d, 1 H, J = 5.5 Hz, OH-3″), 4.71 (d, 1 H, J = 7.8 Hz, H-1″), 4.63 (d, 1 H, J = 4.6 Hz, OH-4″), 3.95 (s, 3 H, OCH_3), 3.79–3.77 (m, 1 H, H-2″), 3.66 (s, 3 H, OCH_3), 3.66–3.65 (m, 1 H, H-4″), 3.60–3.58 (m, 2 H, H-6″a, 6″b), 3.48–3.44 (m, 1 H, H-5″), 3.43–3.41 (m, 1 H, H-3″); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.1 (C═O), 151.4 (C-5), 149.9 (C-4), 146.9 (C-4′), 146.8 (C-3′), 144.9 (C-7), 134.7 (C-7′), 129.7 (C-1), 129.6 (C-8), 128.2 (C-1′), 126.7 (C-2), 123.5 (C-6′), 118.8 (C-8′), 110.8 (C-2′), 107.9 (C-3), 105.6 (C-1″), 105.3 (C-5′), 101.8 (C-6), 101.0 (OCH_2O), 75.8 (C-5″), 73.0 (C-3″), 70.8 (C-2″), 68.4 (C-4″), 67.3 (C-9), 60.9 (C-6″), 55.7 (OCH_3), 55.1 (OCH_3); HRMS calcd for $C_{27}H_{27}O_{12}$ 543.1503, found 543.1476.

*5.1.1.2. 4-O-α-L-arabinosyldiphyllin (**6**).* White solid, yield 58.8%, $\mathbf{R_f}$ 0.27 ($CHCl_3$:MeOH, 20:1), $[\alpha]_D^{25} = -7.5^\circ$ (*c*1.04, $CHCl_3$); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.17 (s, 1 H, ArH), 7.04 (d, 1 H, J = 8.3 Hz, ArH), 6.98 (s, 1 H, ArH), 6.92 (d, 1 H, J = 1.9 Hz, ArH), 6.80–6.78 (m, 1 H, ArH), 6.13 (s, 2 H, OCH_2O), 5.76 (d, 1 H, J = 5.5 Hz, OH-4″), 5.53 (dd, 1 H, J = 15.1, 2.3 Hz, H-9a), 5.45 (d, 1 H, J = 15.1 Hz, H-9b), 5.04 (d, 1 H, J = 5.5 Hz, OH-3″), 4.71 (d, 1 H, J = 6.8 Hz, H-1″), 4.75 (d, 1 H, J = 3.2 Hz, OH-2″), 3.95 (s, 3 H, OCH_3), 3.85 (dd, 1 H, J = 13.7, 6.4 Hz, H-2″), 3.78 (d, 1 H, J = 11.4 Hz, H-5″a), 3.71 (brs, 1 H, H-4″), 3.67 (s, 3 H, OCH_3), 3.53–3.51 (m, 1 H, H-3″), 3.45 (d, 1 H, J = 11.4 Hz, H-5″b); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.0 (C═O), 151.4 (C-5),

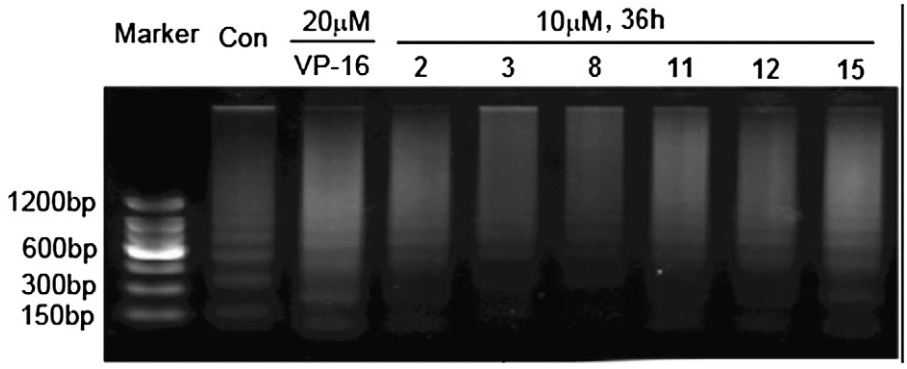


Fig. 4. The diphyllin and its glycoside derivatives caused apoptosis in HL-60 cells by DNA fragmentation electrophoresis.

149.9 (C-4), 146.9 (C-4′), 146.8 (C-3′), 144.5 (C-7), 134.4 (C-7′), 129.6 (C-1), 128.9 (C-8), 128.2 (C-1′), 126.6 (C-2), 123.5 (C-6′), 118.6 (C-8′), 110.7 (C-2′), 107.9 (C-3), 105.4 (C-1″), 104.8 (C-5), 101.8 (C-6), 101.0 (OCH_2O), 72.2 (C-3″), 70.8 (C-2″), 67.2 (C-4″), 67.0 (C-9), 65.7 (C-5″), 55.8 (OCH_3), 55.1 (OCH_3); HRMS calcd for $C_{26}H_{25}O_{11}$ 513.1397, found 513.1409.

*5.1.1.3. 4-O-β-D-glucopyranosyldiphyllin (**7**).* White solid, yield 71.3%, R_f 0.30 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -30.9°$ (*c*1.00, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.18 (d, 1 H, *J* = 5.0 Hz, ArH), 7.04 (d, 1 H, *J* = 7.8 Hz, ArH), 6.98 (s, 1 H, ArH), 6.93 (d, 1 H, *J* = 1.9 Hz, ArH), 6.82–6.78 (dd, 1 H, *J* = 7.8, 1.9 Hz, ArH), 6.13 (s, 2 H, OCH_2O), 5.98 (d, 1 H, *J* = 4.1 Hz, OH-2″), 5.77 (d, 1 H, *J* = 15.1 Hz, H-9a), 5.48 (dd, 1 H, *J* = 15.1, 3.7 Hz, H-9b), 5.25 (d, 1 H, *J* = 4.6 Hz, OH-3″), 5.09 (d, 1 H, *J* = 5.5 Hz, OH-4″), 4.75 (d, 1 H, *J* = 7.8 Hz, H-1″), 4.71–4.68 (m, 1 H, OH-6″), 3.95 (s, 3 H, OCH_3), 3.79–3.77 (m, 1 H, H-6″a), 3.67 (s, 3 H, OCH_3), 3.50–3.44 (m, 2 H, H-6″b, H-2″), 3.31–3.28 (m, 1 H, H-5"), 3.24–3.22 (m, 1 H, H-3″), 3.17–3.16 (m, 1 H, H-4″); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.2 (C═O), 151.4 (C-5), 149.9 (C-4), 146.9 (C-3′), 146.8 (C-4′), 144.8 (C-7), 134.9 (C-7′), 129.9 (C-1) 129.6 (C-8), 128.2 (C-1′), 126.7 (C-2), 123.5 (C-6′), 118.8 (C-8′), 110.9 (C-2′), 107.9 (C-3), 105.4 (C-1″), 105.0 (C-5′), 101.7 (C-6), 101.1 (OCH_2O), 77.2 (C-3″), 76.3 (C-5″), 73.7 (C-2″), 70.0 (C-4″), 67.3 (C-9), 61.2 (C-6″), 55.8 (OCH_3), 55.2 (OCH_3); HRMS calcd for $C_{27}H_{27}O_{12}$ 543.1503, found 543.1481.

*5.1.1.4. 4-O-β-D-xylosyldiphyllin (**8**).* White solid, yield 63.3%, $\mathbf{R_f}$ 0.28 ($CHCl_3$:MeOH, 20:1), $[\alpha]_D^{25} = -24.4°$ (*c*1.00, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.12 (d, 1 H, *J* = 2.3 Hz, ArH), 7.04 (d, 1 H, *J* = 7.8 Hz, ArH), 6.98 (d, 1 H, *J* = 5.5 Hz, ArH), 6.92 (d, 1 H, *J* = 1.4 Hz, ArH), 6.80–6.78 (m, 1 H, ArH), 6.13 (s, 2 H, OCH_2O), 5.76 (dd, 1 H, *J* = 5.5, 4.6 Hz, OH-4″), 5.51 (dd, 1 H, *J* = 15.1, 2.3 Hz, H-9a), 5.45 (d, 1 H, *J* = 15.1 Hz, H-9b), 5.26 (d, 1 H, *J* = 5.0 Hz, OH-3″), 5.10 (d, 1 H, *J* = 5.0 Hz, OH-2″), 4.76 (d, 1 H, *J* = 7.8 Hz, H-1″), 3.94 (s, 3 H, OCH_3), 3.81 (dd, 1 H, *J* = 11.5, 4.6 Hz, H-5″a), 3.67 (s, 3 H, OCH_3), 3.49–3.43 (m, 2 H, H-2″, H-4″), 3.27–3.24 (m, 1 H, H-3″), 3.15–3.11 (m, 1 H, H-5″b); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.0 (C═O), 151.4 (C-5), 149.9 (C-4), 146.9 (C-4′), 146.8 (C-3′), 144.6 (C-7), 134.9 (C-7′), 129.7 (C-1), 129.6(C-8), 128.1 (C-1′), 126.7 (C-2), 123.5 (C-6′) 118.6 (C-8′), 110.8 (C-2′), 107.9 (C-3) 105.7 (C-1″), 105.4 (C-5′), 101.6 (C-6), 101.1 (OCH_2O), 76.3 (C-3″), 73.5 (C-2″), 69.3 (C-4″), 67.0 (C-9), 65.9 (C-5″), 55.8 (OCH_3), 55.2 (OCH_3); HRMS calcd for $C_{26}H_{25}O_{11}$ 513.1397, found 513.1396.

*5.1.1.5. 6′-O-Methyl-4-O-β-D-glucopyranosyldiphyllin (**9**).* White solid, yield 78.6%, $\mathbf{R_f}$ 0.26 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -75.5°$ (*c*1.00, CH_2Cl_2); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.16 (d, 1 H, *J* = 3.7 Hz, ArH), 7.04 (d, 1 H, *J* = 7.8 Hz, ArH), 6.98 (s, 1 H, ArH), 6.92 (d, 1 H, *J* = 1.8 Hz, ArH), 6.82–6.78 (m, 1 H, ArH), 6.13 (s, 2 H, OCH_2O), 6.01 (d, 1 H, *J* = 5.5 Hz, OH-2″), 5.67 (dd, 1 H, *J* = 15.1, 2.8 Hz, H-9a), 5.46 (dd, 1 H, *J* = 15.1, 2.3 Hz, H-9b), 5.30 (d, 1 H, *J* = 5.0 Hz, OH-4″), 5.22 (d, 1 H, *J* = 5.5 Hz, OH-3″), 4.76 (d, 1 H, *J* = 7.8 Hz H-1″), 3.95 (s, 3 H, OCH_3), 3.67 (s, 3 H, OCH_3), 3.64–3.62 (m, 1 H, H-6″a), 3.49–3.43 (m, 2 H, H-2″, H-6″b), 3.41–3.39 (m, 1 H, H-5″), 3.31–3.28 (m, 1 H, H-3″), 3.26 (s, 3 H, OCH_3), 3.18–3.14 (m, 1 H, H-4″); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.1 (C═O), 151.4 (C-5), 149.9 (C-4), 146.8 (C-4′), 146.7 (C-3′), 144.8 (C-7), 134.9 (C-7′), 129.8 (C-1), 129.6 (C-8), 128.2 (C-1′), 126.8 (C-2), 123.5 (C-6′), 118.7 (C-8′), 110.8 (C-2′), 107.9 (C-3), 105.3 (C-1″), 105.0 (C-5), 101.7 (C-6), 101.1 (OCH_2O), 76.2 (C-3″), 74.9 (C-5″), 73.6 (C-2″), 72.2 (C-6″), 69.9 (C-4″), 67.2 (C-9), 58.3 (OCH_3), 55.7 (OCH_3), 55.2 (OCH_3); HRMS calcd for $C_{28}H_{29}O_{12}$ 557.1659, found 557.1651.

*5.1.1.6. 6′-O-Methyl-4-O-β-D-galactopyranosyldiphyllin (**10**).* White solid, yield 58.2%, $\mathbf{R_f}$ 0.29 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -65.7°$ (*c*1.00, CH_2Cl_2); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.19 (d, 1 H, *J* = 3.2 Hz, ArH), 7.04 (dd, 1 H, *J* = 7.7, 1.9 Hz, ArH), 6.98 (d, 1 H, *J* = 2.6 Hz, ArH), 6.92 (d, 1 H, *J* = 1.9 Hz, ArH), 6.81–6.78 (m, 1 H, ArH), 6.12 (s, 2 H, OCH_2O), 5.65 (dd, 1 H, *J* = 14.8, 2.6 Hz, H-9a), 5.46 (dd, 1 H, *J* = 14.8, 1.9 Hz, H-9b), 4.71 (d, 1 H, *J* = 8.3 Hz, H-1″), 3.95 (s, 3 H, OCH_3), 3.78 (dd, 1 H, *J* = 9.3, 8.3 Hz, H-2″), 3.66 (s, 3 H, OCH_3), 3.66–3.65 (m, 1 H, H-5″), 3.63 (d, 1 H, *J* = 2.6 Hz, H-4″), 3.56–3.49 (m, 2 H, H-6a, 6b), 3.43 (dd, 1 H, *J* = 9.3, 3.2 Hz, H-3″), 3.23 (s, 3 H, OCH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.1 (C═O), 151.4 (C-5), 149.9 (C-4), 146.8 (C-4′), 146.7 (C-3′), 144.9 (C-7), 134.8 (C-7′), 129.8 (C-1), 129.6 (C-8), 128.2 (C-1′), 126.8 (C-2), 123.5 (C-6′), 118.7 (C-8′), 110.8 (C-2′), 107.9 (C-3), 105.6 (C-1″), 105.3 (C-5), 101.8 (C-6), 101.1 (OCH_2O), 73.3 (C-5″), 72.9 (C-3″), 72.0 (C-6″), 70.6 (C-2″), 68.6 (C-4″), 67.2 (C-9), 58.2 (OCH_3), 55.7 (OCH_3), 55.1 (OCH_3); HRMS calcd for $C_{28}H_{29}O_{12}$ 557.1659, found 557.1663.

*5.1.1.7. 4-O-β-D-fucopyranosyldiphyllin (**12**).* White solid, yield 53.8%, $\mathbf{R_f}$ 0.40 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -57.3°$ (*c*1.00, CH_2Cl_2); ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.18 (d, 1 H, *J* = 2.8 Hz, ArH), 7.03 (dd, 1 H, *J* = 7.8, 1.9 Hz, ArH), 6.98 (d, 1 H, *J* = 3.2 Hz, ArH), 6.93 (d, 1 H, *J* = 1.9 Hz, ArH), 6.81–6.78 (m, 1 H, ArH), 6.12 (s, 2 H, OCH_2O), 5.77 (brs, 1 H, OH-2″), 5.52 (dd, 1 H, *J* = 14.8, 3.7 Hz, H-9a), 5.47 (d, 1 H, *J* = 14.8 Hz, H-9b), 4.96 (brs, 1 H, OH-3″), 4.71 (d, 1 H, *J* = 7.7 Hz, H-1″), 4.68 (brs, 1 H, OH-4″), 3.94 (s, 3 H, OCH_3), 3.76–3.72 (m, 1 H, H-2″), 3.66 (s, 3 H, OCH_3), 3.63–3.60 (m, 1 H, H-5″), 3.47–3.46 (m, 1 H, H-4″), 3.43–3.42 (m, 1 H, H-3″), 1.18 (dd, 3 H, *J* = 6.4, 1.2 Hz, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.1 (C═O), 151.3 (C-5), 149.9 (C-4), 146.9 (C-4′), 146.8 (C-3′), 145.0 (C-7), 134.8 (C-7′), 129.9 (C-1), 129.7 (C-8), 128.2 (C-1′), 126.9 (C-2), 123.5 (C-6′), 118.6 (C-8′), 110.8 (C-2′), 107.9 (C-3), 105.7 (C-1″), 105.3 (C-5), 101.8 (C-6), 101.1 (OCH_2O), 73.2 (C-3″), 70.8 (C-4″), 70.5 (C-2″), 70.4 (C-5″), 67.1 (C-9), 55.7 (OCH_3), 55.1 (OCH_3), 16.5 (CH_3); HRMS calcd for $C_{27}H_{27}O_{11}$ 527.1553, found 527.1576.

*5.1.1.8. 6′-O-Benzyl-4-O-β-D-glucopyranosyldiphyllin (**13**).* White solid, yield 66.6%, $\mathbf{R_f}$ 0.25 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -36.5°$ (*c*1.00, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) : δ 7.91 (s, 1 H, ArH), 7.34–7.32 (m, 2 H, ArH), 7.29–7.27 (m, 2 H, ArH), 7.26 (s, 1 H, ArH), 7.05 (d, 1 H, *J* = 2.2 Hz, ArH), 6.93 (dd, 1 H, *J* = 7.7, 5.0 Hz, ArH), 6.81–6.74 (m, 2 H, ArH), 6.07 (s, 1 H, OCH_2O), 6.04 (d, 1 H, *J* = 3.8 Hz, OCH_2O), 5.54 (d, 1 H, *J* = 14.8 Hz, H-9a), 5.40 (d, 1 H, *J* = 14.8, 2.2 Hz, H-9b), 4.81 (d, 1 H, *J* = 7.7 Hz, H-1″), 4.54 (dd, 2 H, *J* = 20.3, 12.1 Hz, $PhCH_2$), 4.00 (s, 3 H, OCH_3), 3.86 (t, 1 H, *J* = 8.8 Hz, H-5″), 3.78 (s, 3 H, OCH_3), 3.71 (d, 2 H, *J* = 3.3 Hz, H-2″, H-4″), 3.67–3.66 (m, 1 H, H-6″a), 3.60 (t, 1 H, *J* = 8.8 Hz, H-6″b); ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.1 (C═O), 152.0 (C-5), 150.1 (C-4), 147.5 (C-3′), 144.2 (C-4′), 137.0 (C-7), 136.7 (C-7′), 130.7 (C-1), 130.4 (C-8), 128.6 (C-1′), 128.5 (C-Bn), 128.4 (C-Bn), 128.2 (C-Bn), 128.0 (C-Bn), 127.9 (C-Bn), 127.9 (C-2), 127.1 (C-Bn), 123.5 (C-6′), 119.0 (C-8′), 110.6 (C-2′), 108.1 (C-5′), 106.1 (C-3), 104.5 (C-1″), 101.2 (C-6), 100.8 (OCH_2O), 77.2 (C-$PhCH_2$), 73.8 (C-3″), 73.6 (C-5″), 71.7 (C-2″), 70.1 (C-4″), 67.5 (C-9), 56.2 (OCH_3-5), 55.8 (OCH_3-4); HRMS (ESI) calcd for $C_{34}H_{33}O_{12}$ $(M + H)^+$ *m*/*z* 633.1972 found 633.1968.

*5.1.1.9. 6′-O-Benzyl-4-O-β-D-galactopyranosyldiphyllin (**14**).* White solid, yield 70.2%, $\mathbf{R_f}$ 0.60 ($CHCl_3$:MeOH, 10:1), $[\alpha]_D^{25} = -25.4°$ (*c*1.00, $CHCl_3$), ^{1}H NMR (600 MHz, $CDCl_3$) : δ 7.94 (s, 1 H, ArH), 7.33–7.31 (m, 2 H, ArH), 7.26 (s, 2 H, ArH), 7.02 (d, 1 H, *J* = 6.4 Hz, ArH), 6.93 (t, 1 H, *J* = 6.9 Hz, ArH), 6.80–6.72 (m, 3 H, ArH), 6.07 (s, 1 H, OCH_2O), 6.04 (d, 1 H, *J* = 4.1 Hz, OCH_2O), 5.58 (d, 1 H, *J* = 15.1 Hz, H-9a), 5.45 (dd, 1 H, *J* = 15.1, 2.3 Hz, H-9b), 4.77 (d, 1 H, *J* = 7.8 Hz, H-1″), 4.52 (dd, 2 H, *J* = 11.5, 1.4 Hz, OCH_2), 4.16–4.13 (m, 1 H, H-2″), 4.05 (brs, 1 H, H-4″), 3.99 (s, 3 H, OCH_3), 3.78 (s, 3 H, OCH_3), 3.75 (m, 1 H, H-3″), 3.63–3.62 (m, 2 H, H-6a, H-6b), 3.58 (t, 1 H, *J* = 5.0 Hz, H-5″); HRMS (ESI) calcd for $C_{34}H_{33}O_{12}$ $(M + H)^+$ *m*/*z* 633.1972 found 633.1975.

*5.1.2. General procedure of the diphyllin glycoside compounds (**15**–**17**)*

To the solution of **7** (108 mg, 0.2 mmol) in dried CH_3CN (10 mL) was added TsOH (2 mg, 0.01 mmol) and corresponding acetals (0.3 mmol). The mixture stirred at room temperature for 5 h, quenched by Et_3N (2 drops), The solvent was removed under vacuum, and the resdue was chromatographed on silica gel (EtOAc : petroleum ether, 2:1) to get the corresponding derivatives.

*5.1.2.1. 4′, 6′-ethydenide-4-O-β-D-glucopyranosyldiphyllin (**15**).* White solid, yield 73.2%, $\mathbf{R_f}$ 0.26 (EtOAc:petroleum ether, 2:1), $[\alpha]_D^{25} = -67.7^\circ$ (*c*1.00, CH_2Cl_2), ^{1}H NMR (600 MHz, $CDCl_3$) : δ 7.92 (s, 1 H, ArH), 7.02 (d, 1 H, $J = 2.8$ Hz, ArH), 6.91 (d, 1 H, $J = 7.8$ Hz, ArH), 6.79–6.75 (m, 1 H, ArH), 6.67–6.66 (m, 1 H, ArH), 6.08 (d, 1 H, $J = 1.4$ Hz, OCH_2O), 6.03 (d, 1 H, $J = 1.4$ Hz, OCH_2O), 5.36 (s, 2 H, H-9a, 9b), 4.88 (d, 1 H, $J = 7.3$ Hz, H-1″), 4.76–4.74 (m, 1 H, CH), 4.11–4.08 (m, 1 H, H-6″a), 4.01 (s, 3 H, OCH_3), 3.91–3.89 (m, 1 H, H-2″), 3.81–3.80 (m, 1 H, H-3″), 3.78 (s, 3 H, OCH_3), 3.61–3.57 (m, 1 H, H-6″b), 3.46–3.43 (m, 1 H, H-4″), 3.24–3.20 (m, 1 H, H-5″), 1.36 (d, 3 H, $J = 5.0$ Hz, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ 169.9 (C=O), 151.9 (C-5), 150.1 (C-4), 147.4 (C-3′), 147.3 (C-4′), 144.8 (C-7), 136.7 (C-7′), 130.9 (C-1) 130.6 (C-8), 128.0 (C-1′), 127.0 (C-2), 123.5 (C-6′), 118.8 (C-8′), 110.6 (C-2′), 108.1 (C-5′), 106.0 (C-3), 104.9 (C-1″), 101.2 (C-6), 100.8 (OCH_2O), 99.7 (C–CH), 79.4 (C-4″), 74.7 (C-3″), 73.7 (C-2″), 67.8 (C-9), 67.2 (C-5″), 66.4 (C-6″), 56.2 (OCH_3), 55.7 (OCH_3), 20.1 (C–CH_3); HRMS calcd for $C_{29}H_{29}O_{12}$ 569.1654, found 569.1661.

*5.1.2.2. 4′, 6′-Acetonide-4-O-β-D-glucopyranosyldiphyllin (**16**).* White solid, yield 71.4%, $\mathbf{R_f}$ 0.21 (EtOAc:petroleum ether, 2:1), $[\alpha]_D^{25} = 23.1^\circ$ (*c*1.00, CH_2Cl_2), ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.14 (d, 1 H, $J = 4.1$ Hz, ArH), 7.04 (dd, 1 H, $J = 7.8$, 2.8 Hz, ArH), 6.98 (d, 1 H, $J = 3.2$ Hz, ArH), 6.93 (d, 1 H, $J = 1.4$ Hz, ArH), 6.81–6.78 (m, 1 H, ArH), 6.21 (d, 1 H, $J = 5.2$ Hz, OH-2″), 6.13 (s, 2 H, OCH_2O), 5.48–5.42 (m, 3 H, H-9a, H-9b, OH-3″), 4.91 (d, 1 H, $J = 7.8$ Hz, H-1″), 3.94 (s, 3 H, OCH_3), 3.86–3.83 (m, 1 H, H-6″a), 3.78–3.74 (m, 1 H, H-5″), 3.66 (s, 3 H, OCH_3), 3.57–3.52 (m, 2 H, H-6″b, H-2″), 3.45–3.41 (m, 1 H, H-4″), 3.32–3.27 (m, 1 H, H-3″), 1.48 (s, 3 H, CH_3), 1.31 (s, 3 H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.9 (C=O), 151.9 (C-5), 150.1 (C-4), 147.4 (C-3′), 147.3 (C-4′), 144.1 (C-7), 136.7 (C-7′), 131.0 (C-1) 130.7 (C-8), 128.0 (C-1′), 127.0 (C-2), 123.5 (C-6′), 118.9 (C-8′), 110.6 (C-2′), 108.1 (C-5′), 106.1 (C-3), 104.8 (C-1″), 101.2 (C-6), 100.8 (OCH_2O), 100.1 (C–CH), 79.5 (C-4″), 74.6 (C-3″), 73.9 (C-2″), 67.8 (C-9), 67.2 (C-5″), 66.7 (C-6″), 56.2 (OCH_3), 55.8 (OCH_3), 32.1 (C–CH_3), 30.6 (C–CH_3), 24.9 (C–CH_3); HRMS calcd for $C_{30}H_{31}O_{12}$ 583.1816, found 583.1810.

*5.1.2.3. 4′, 6′-Isobutydenide-4-O-β-D-glucopyranosyldiphyllin (**17**).* White solid, yield 60.3%, $\mathbf{R_f}$ 0.30 (EtOAc: petroleum ether, 2:1), $[\alpha]_D^{25} = -20.2^\circ$ (*c*1.00, CH_2Cl_2), ^{1}H NMR (600 MHz, $CDCl_3$) : δ 7.95 (s, 1 H, ArH), 7.08 (brs, 1 H, ArH), 6.96–6.93 (m, 1 H, ArH), 6.84–6.82 (m, 1 H, ArH), 6.81–6.77 (m, 1 H, ArH), 6.09 (s, 1 H, OCH_2O), 6.05 (d, 1 H, $J = 3.7$ Hz, OCH_2O), 5.40 (s, 2 H, H-9a, 9b), 4.93 (d, 1 H, $J = 7.4$ Hz, H-1″), 4.32 (d, 1 H, $J = 4.6$ Hz, CH), 4.13–4.11 (m, 1 H, H-6″a), 4.04 (s, 3 H, OCH_3), 3.95–3.92 (m, 1 H, H-2″), 3.82–3.81 (m, 1 H, H-3″), 3.80 (s, 3 H, OCH_3), 3.59–3.56 (t, 1 H, $J = 10.1$ Hz, H-6″b), 3.23–3.21 (m, 2 H, H-4″, H-5″), 1.88–1.82 (m, 1 H, CH), 0.94 (t, 6 H, $J = 7.3$ Hz, 2 × CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ 169.9 (C=O), 151.9 (C-5), 150.1 (C-4), 147.4 (C-3′), 147.3 (C-4′), 144.1 (C-7), 136.7 (C-7′), 131.0 (C-1) 130.7 (C-8), 128.0 (C-1′), 127.0 (C-2), 123.5 (C-6′), 118.9 (C-8′), 110.6 (C-2′), 108.1 (C-5′), 106.1 (C-3), 106.0 (C–CH), 104.8 (C-1″), 101.2 (C-6), 100.8 (OCH_2O), 79.5 (C-4″), 74.6 (C-3″), 73.9 (C-2″), 67.8 (C-9), 67.2 (C-5″), 66.7 (C-6″), 56.2 (OCH_3), 55.8 (OCH_3), 32.1 (C–CH_3), 17.2 (C–CH_3), 16.8 (C–CH_3); HRMS calcd for $C_{31}H_{33}O_{12}$ 597.1972, found 597.1964.

5.2. Biology evaluation

5.2.1. Cell growth inhibition assays and multidrug resistance analysis

Sulforhodamine B (SRB) assay [18]: cells in the logarithmic phase were seeded to 6 well plate and cultured over night, followed treating with the corresponding drug for 72 h (Setting 5 above concentration gradient and 3 double-pored holes for each concentration). Culture solution was dumped after the drug treatment, and the cells were then treated with 10% precooled TCA in each well. After that, cells were fixed for 1 h at 4 °C, then washed 5 times with distilled water and dried in the air. 100 μL of 4 mg/ml SRB (Sigma) in 1% acetic acid was added to each well for staining over 15 min. Then plate was washed 5 times with 1% acetic acid and dried. SRB in the cells was dissolved in 150 μL of 10 mM Tris–HCl and was measured at 520 nm using a multiwell spectrophotometer. The inhibition rate on cell proliferation was calculated as (OD_{520} control - OD_{520} treated)/OD_{520} control × 100%. IC_{50} value was obtained by Logit method and was determined at least 3 independent tests. The resistance factor (RF) to each drug was calculated as the ratio of the IC_{50} value of resistant cells to that of parental cells.

Methyl Thiazolyl Tetrazolium (MTT) Assay [17]: cells in the logarithmic phase were transferred into 96 well plates in 100 μL of medium and incubated for 24 h. Followed by treating the corresponding drug similar as above. After 72 h of drug exposure, 20 μL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. 100 μL of Tris buffer (10% SDS-5% isobutanol-0.01 mol/L HCl) was added to each microtiter well and incubated for 12–20 h at the same temperature, then measured at 570 nm using a multiwell spectrophotometer. The IC_{50} value and resistance factor (RF) calculation was similar as the above.

5.2.2. Drug inhibition of kDNA decatenation induced by topoisomerase II

Topo II activity was measured by the ATP-dependent decatenation of kDNA [22]. The standard reaction mixture was a total volume of 15 μL which containing with 50 mM Tris–HCl (pH 7.7), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 50 mg/mL of BSA, 20 μg/mL of kDNA, 1 unit of Topo II and the corresponding compound. After incubation at 37 °C for 15 min, the reaction was terminated by addition of 1 μL of 10% SDS. Electrophoresis was carried out in a 1% agarose gel in 1 × TAE (40 mM Tris base, 40 mM acetate acid and 1 mM EDTA) at 4 V/cm for 1 h. DNA bands were stained with 0.5 mg/mL of ethidium bromide (E.B.) solution and photographed through a Gel Document System.

5.2.3. Cell cycle analysis of HL-60 cells [24]

HL-60 cells in the logarithmic phase were seeded to 6 well plate and treated with the corresponding drug for 24 h. Trypsin was digested and collected into 1.5 mL centrifuge tubes, Cells were harvested, followed by washing once with cold phosphate buffer saline (PBS). Adding 300 μL PBS and the cells were resuspended, and fixed overnight in 70% ethanol at 4 °C. The mixture was spun for 5 min at 300 g, resuspended with 500 μL PBS containing 10 μg/mL RNase for 15 min at 37 °C, and then added 2 μg/mL PI. Staining at room temperature in dark for 30 min. Filtered and then analyzed using an FACS-Calibur cytometer. At least 10,000 events were counted for each sample.

5.2.4. DNA fragmentation electrophoresis

The HL-60 cells were seeded into a six well plate. The corresponding drugs was added at the 10 μM concentrations for 36 h. DNA fragmentation was extracted using the method as described [23,24]. Concisely, harvested cells were lysed by the addition of an equal volume of 1.2% SDS. By adding 350 μL precipitation solution

(3 M CsCl, 1 M potassium acetate, 0.67 M acetic acid) and the mixture was spun for 15 min at 14,000 g at room temperature. The clear supernatant was then absorbed by a miniprep spin column. Finally, DNA was eluted with 50 μL Tris–EDTA buffer. This production was run into the gel at 30 V for about 10 min and then after completely covering the gel with TAE buffer separated for 50 min at 80 V.

Acknowledgments

Authors are really grateful to Prof. Geng, Mei-Yu and Dr. Gui, Min, Division of Antitumor Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, for providing the biological activities assays.

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