European Journal of Medicinal Chemistry 49 (2012) 48–54

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Synthesis and biological evaluation of conjugates of deoxypodophyllotoxin and 5-FU as inducer of caspase-3 and -7

Wen-Ting Huang [a], Jie Liu [a], Jian-Fei Liu [a], Ling Hui [c,*], Yi-Lan Ding [d,**], Shi-Wu Chen [a,b,***]

[a] School of Pharmacy & Key Lab of Preclinical Study for New Drugs of Gansu Province, Lanzhou University, Lanzhou 730000, China
[b] State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, China
[c] Experimental Center of Medicine & Key Laboratory of Stem Cells and Gene Drug of Gansu Province, General Hospital of Lanzhou Military Command, Lanzhou 730050, China
[d] Department of Medicine, Lanzhou Maternal and Child Health Care Hospital, Lanzhou 730000, China

ARTICLE INFO

Article history:
Received 4 November 2011
Received in revised form
30 November 2011
Accepted 3 December 2011
Available online 9 December 2011

Keywords:
Podophyllotoxin
5-FU
Antineoplastic agents
Apoptosis
Caspase

ABSTRACT

In order to generate compounds with superior antitumor activity and reduced toxicity, a series of conjugates of deoxypodophyllotoxin and 5-FU were synthesized by coupling 4′-demethyl-4-dexoypodophyllotoxin with *N*-(5-fluorouracil-N^1-ly acetic)- amino acids (or 5-fluorouracil-N^1-ly acetic acid). The cytotoxic activity of these compounds against four human cancer cell lines (HL-60, A-549, HeLa and SiHa) were evaluated, and results indicated that these compounds were more potent in terms of cytotoxicity than either parent compound DPT or anticancer drug VP-16 and 5-FU. In addition, we found that **14d** induced cell cycle arrest in the G2/M phase accompanied by apoptosis in A-549 cells, and **14d** activated caspase-3 and -7. These results suggested that caspase-mediated pathways are involved in **14d** induced apoptosis.

© 2011 Elsevier Masson SAS. All rights reserved.

1. Introduction

Cancer is a major global health problem, representing the second-leading cause of death worldwide [1]. According to information from the World Health Organization (WHO), it is estimated that there will be 12 million deaths from cancer in 2030. Over the last few years, improvements in treatment and prevention have led to a decrease in cancer deaths, and a number of chemotherapeutic drugs have been developed to treat cancer.

Podophyllotoxin (PPT, **1**), a naturally occurring cyclolignan isolated from *Podophyllum* species, is a well-known cytotoxic derivative that acts as a potent anti-microtubule agent [2]. In spite of potential uses as a medicinal drug, human trials with PPT were discontinued due to its systemic toxicity [3]. Over the last 30 years, extensive structural modifications of podophyllotoxin have led to the development of the clinically valuable anticancer drugs, etoposide (VP-16, **2**), teniposide (VM-26, **3**) and the water-soluble prodrug, etoposide phosphate (**4**). These drugs are presently in clinical use for the treatment of small cell lung cancer, testicular carcinoma, acute leukemia, Kaposi's sarcoma and lymphoma. The cytotoxic mechanism of VP-16 is the inhibition of topoisomerase II (TOP II), unlike the parent compound which inhibits mitosis [4]. VP-16 induces cell death by enhancing the TOP II-mediated DNA cleavage through the stabilization of the transient DNA/TOP II cleavage complex. In such a complex, DNA is cleaved on both strands and covalently linked to the enzyme; the TOP II poison prevents it from dissociating [5]. Recent researches reveal that the antitumor activity of VP-16 is attributed primarily to its inhibition of TOP II α, whereas the carcinogenic effect has been attributed to the β isoform [6]. Besides, after the approval of VP-16, there are several potential drug candidates based on PPT applied in clinical trial, such as, NK-611 (**5**), GL-331 (**6**), tafluposide (F11782, **7**) and F14512 (**8**) (Fig. 1). Among them, F11782 is a dual inhibitor of topoisomerases I and II which impairs the binding of the enzyme to DNA, but does not stabilize the cleavage complex [7,8]. The spermine-conjugated epipodophyllotoxin derivative F14512 is a topoisomerase II poison that exploits the polyamines transport system (PTS) to target preferentially tumor cells [9,10].

Deoxypodophyllotoxin (DPT, **9**), an analog of PPT, possess potential anti-proliferative and antitumor activity in diverse cell types [11–13], as well as anti-inflammatory [14] and anti-viral [15]

* Corresponding author.
** Corresponding author.
*** Corresponding author. School of Pharmacy & Key Lab of Preclinical Study for New Drugs of Gansu Province, Lanzhou University, Lanzhou 730000, China. Tel./fax: +86 931 8915686.
E-mail address: chenshwu@gmail.com (S.-W. Chen).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.12.005

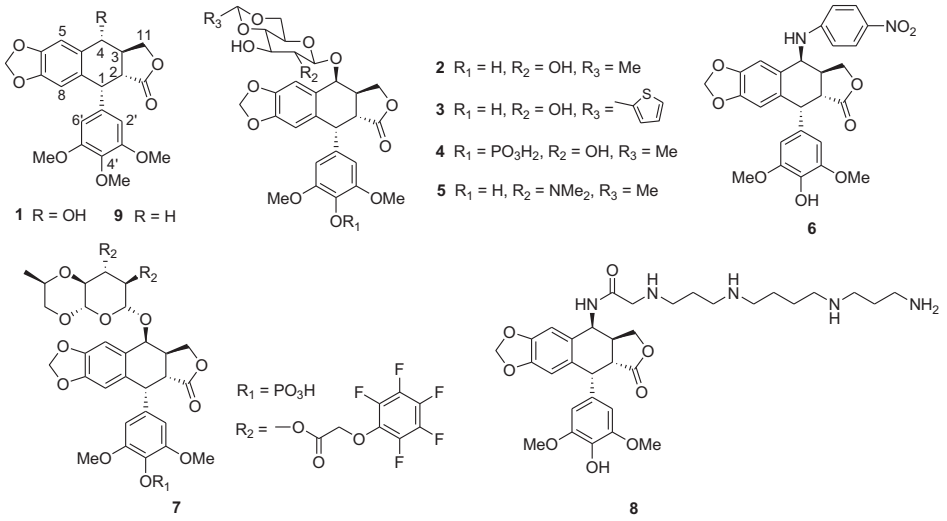


Fig. 1. Structures of podophyllotoxin (**1**), etoposide (**2**), teniposide (**3**), etopophos (**4**), NK-611 (**5**), GL-331 (**6**), tafluposide (**7**), F14512 (**8**) and deoxypodophyllotoxin (**9**).

activity. It has been revealed that DPT inhibits tubulin polymerization and induces G2/M cell cycle arrest followed by apoptosis through multiple cellular processes, involving the activation of ATM, upregulation of p53 and Bax, activation of caspase-3 and -7, and accumulation of PTEN resulting in the inhibition of the Akt pathway [16,17]. Besides, it is found that DPT also inhibits migration and MMP-9 via MAPK pathways in TNF-α-induced HASMC [18].

In clinical, anticancer drugs are usually used as suitable combinations of different drugs to treat cancer, because only a few tumors are sensitive enough to be cured by single drugs [19]. Many combinations, which were named mutual drug, in clinical use consist of an antimetabolite with one or more other anticancer agents. A mutual drug usually consists of two different synergistic drugs joined together directly or by means of a linker [20]. In order to generate more efficient anticancer drugs based on PPT, many pharmacologist and chemists had synthesized a number of conjugates of PPT with other antitumor agents, such as conjugates of podophyllotoxin-camptothecin [21], taxoid- epipodophyllotoxin [22], polymer-linked podophyllotoxin [23], vinorelbine- podophyllotoxin [24], thiocolchicine-podophyllotoxin [25], the biological evaluation showed that most of these conjugates exhibited superior cytotoxicity *in vitro* than parent compound PPT.

Recently, we have reported some conjugates of podophyllotoxin with antimetabolite 5-FU [26–30] and derivatives of DPT [31,32]. In our continuing efforts to find new compounds with potent activities and low toxicity from natural products, considering amino acids are often used as carrier vehicles for some drugs because of their good water solubility when actively transplanted into mammalian tissue, in this work we described the synthesis of a series of conjugates of DPT and 5-FU joined by suitable amino acid spacers and tested their cytotoxicity against a panel of four human cancer cell lines. Furthermore, compound **14d** was evaluated for its effect on cell cycle progression, apoptosis, and induction to caspase-3 and -7.

2. Results and discussion

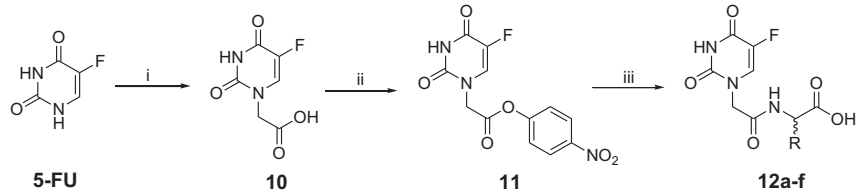
2.1. Chemistry

The synthetic route to the target compounds first involved the generation of 5-Fluorouracil-N^1-ly acetic acid (**10**) and its amino acids derivatives **12a**–**f**, they were synthesized from 5-FU as reference reported (Scheme 1) [30,33]. Briefly, 5-FU was treated initially with 2-chloride acetic acid in the presence of aqueous potassium hydroxide to afford compound **10**. The activated ester **11** was obtained through esterification of **10** with *p*-nitrophenol using *N*,*N*'-dicyclohexylcarbodiimide (DCC) in dry *N*,*N*-dimethylformamide (DMF). The intermediate **11** was further reacted with appropriate amino acids in alkaline DMF-H_2O solvent to yield *N*-(5-fluorouracil-N^1-ly acetyl)-amino acids **12a**–**f**.

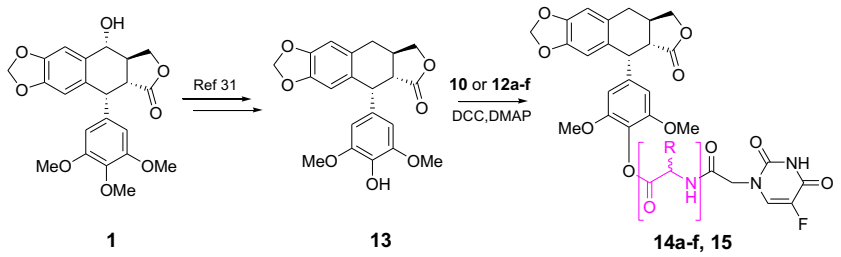
Synthesis of 4-dexoydopodophyllotoxin conjugates was carried out from the key intermediate 4′-demethy-4-deoxypodophyl lotoxin (DDPT, **13**), which was prepared as described in our previously reported procedure [31]. The intermediate DDPT was coupled to various *N*-(5-fluorouracil-N^1-ly acetic)-amino acids (or 5-Fluorouracil-N^1-ly acetic acid) in the presence of DCC and 4-dimethylamino pyridine (DMAP) to provide the corresponding conjugates **14a**–**f** (or **15**) with high or moderate yields as depicted in Scheme 2. All the synthesized compounds were characterized by IR, ^{1}H and ^{13}C NMR spectroscopy, and high-resolution mass spectrometry.

2.2. Biological activities

The biological activities of the DPT conjugates **14a**–**f** and **15** were evaluated by an *in vitro* cytotoxicity test carried out with a panel of four human tumor cell lines (premyelocytic leukemia HL-60, lung carcinoma A-549, cervical carcinoma HeLa, and cervical



Scheme 1. Synthesis of **12a**–**f**. Reagents and conditions: i) 2-chloride acetic acid, 10% KOH, reflux; ii) p-nitro-phenol, DCC/DMF, rt; iii) amino acids, DMF, rt.



Compds	Configuration	R	Compds	Configuration	R
14a	*L*-	Me	**14e**	*L*-	CH_2CH_2SMe
14b	*L*-	$CHMe_2$	**14f**	*D*-	CH_2CH_2SMe
14c	*L*-	CH_2CHMe_2	**15**	--	--
14d	*L*-	$CH_2C_6H_5$			

Scheme 2. Synthesis of **14a**–**f** and **15.**

squamous cell carcinoma SiHa), using VP-16 and 5-FU as reference compounds. The screening procedure was based on the standard MTT or CCK-8 method [34], and the results of these experiments are summarized in Table 1.

As illustrated in Table 1, the DPT conjugates, **14a**–**f** and **15**, were generally more potent than 5-FU, DPT, DDPT and VP-16 in their cytotoxicity to these four cell lines. As a group, these compounds were most effective in HL-60 cells, and had lowest potency in HeLa cells, although the order of potency varied in each cell line. The IC_{50} value of compound **14d**, which is the most potent conjugate in this group compounds, is 0.023, 0.56, 0.83 and 0.76 μM for HL-60, A-549, HeLa and SiHa, respectively. In Table 1, we also found that different substituent at the α-carbon of the amino acid in this class of compounds (**14a**–**e**) appeared to have significant effects on their anti-proliferative activity in the *in vitro* assay. Compounds **14c**–**e** exhibited more potent activity against four human cancer cell lines than those of **14a** and **14b**. In addition, the conjugates with L-amino acids incorporated appear to be more potent than those with D-amino acid substituent (**14e** *vs*. **14f**). Notably, conjugates **15** incorporated of DDPT with 5-Fluorouracil- N^1-ly acetic acid also exhibited high cytotoxities against these four tumor cell lines. Results presented herein and elsewhere [35,36] suggested that esters of DDPT will improve the antitumor activity compared with that of the starting compound DDPT. These results also confirmed the assumption that free hydroxyl group at the 4′ position in DDPT was not favorable for antitumor activity [35].

DPT is reported to induce apoptosis and cell cycle arrest in the G2/M phase in HeLa cells [16,17]. To determine whether conjugates of DDPT and 5-FU have similar effects on tumor cells, the effects of **14d** on cell cycle progression were determined by FACS analysis in propidium iodide-stained A-549 cells [37]. As shown in Fig. 2, treatment with 0.5 μM **14d** lead to a time-dependent accumulation of cells in the G2/M phase with a concomitant decrease in the population of G1 phase cells. G2/M phase arrest was initially detectable after 12 h of treatment; 52.6% and 64.8% of the cells were in G2/M phase after **14d** exposure for 12 h (Fig. 2B) and 24 h (Fig. 2C) respectively, compared with 15.6% in untreated cultures (Fig. 2A). These results demonstrate that **14d** interfere with cell proliferation by arresting the cell cycle, and induce G2/M arrest in A549 cells, and that these conjugates of DDPT and 5-FU have similar effects on the cell cycle arrest with that of parent compound DPT [16,17].

As above mentioned, DPT induce apoptosis of tumor cells in a time-dependent manner, however, we did not find obvious apoptosis in FACS analysis of **14d**-treated A-549 cells. To confirm the effect of conjugates of DPT and 5-FU on induction of apoptosis, the morphology of A-549 cells were examined using Hoechst staining. As shown in Fig. 3, negative control (untreated) cells exhibited excellent growth characteristic after 24 h incubation (Fig. 3A). However, A-549 cells treated with 0.5 μM **14d** evoked typical apoptotic features, such as membrane blebbing, cell shrinkage and detachment, and nuclear condensation even fragmentation (Fig. 3B). A similar effect was observed when cells were exposed to 0.5 μM PPT [16].

Caspases are cysteinyl aspartate proteinases that cleave substrate proteins at aspartate residues. Upon receiving an apoptotic signal, the precursor caspases undergo proteolytic processing to generate an active subunit. Among the 11 caspases characterized in humans, caspase-3 and -7 are the main downstream effector caspases that play essential roles in degrading the majority of key cellular components in apoptotic cells [38]. To determine whether caspase is involved in **14d**-induced apoptosis, western blot analysis was performed using antibodies to recognize the cleaved forms of caspase-3 and caspase-7 [39]. We found that treatment of A-549 cells with 0.5 μM **14d** gradually increased the cleavage of both caspase-3 (Fig. 4A) and caspase-7 (Fig. 4B) in a time-dependent manner. These results demonstrate the involvement of caspases-3 or -7 in **14d**-induced apoptosis.

Table 1
Cytotoxicity of compounds **14a**–**d** and **15** with 48 h drug exposure.

Compounds	Cytotoxicity (IC_{50}, μM)[a]			
	HL-60[b]	A−549[c]	HeLa[b]	SiHa[c]
14a	0.19	1.4	0.2	0.6
14b	0.14	0.36	1.23	1.65
14c	0.063	1.07	0.43	0.35
14d	0.023	0.56	0.83	0.76
14e	0.23	0.83	0.78	0.36
14f	0.94	2.6	1.97	1.51
15	0.035	0.66	0.18	0.11
DPT	0.47	1.38	6.01	1.98
DDPT	2.96	1.8	53.3	43
VP-16	2.85	24.9	40.4	2.76
5-Fu	68.3	54.8	82.2	218

[a] Date are the mean of three independent experiment.
[b] CCK-8 method.
[c] MTT method.

3. Conclusions

In summary, the cytoxicity assay for most of these conjugates demonstrated many fold increase of activity in comparison to either parent compound DPT or clinical drug etoposide and 5-FU; Furthermore, the promising conjugate **14d** could induce cell cycle arrest in the G2/M phase and apoptosis by activated caspase-3 and -7 in A-549 cells. Despite these potential therapeutical benefits of combining such agents through suitable amino acid linkers are still unclear, the results presented herein promoted us to believe that the approach should be applicable for other antitumor agents, and it is worthwhile to explore the antitumor potential of these and similar types of compounds. Our current results shed more light on the potential of these conjugated compounds to overcome drug resistance frequently encountered with chemotherapeutic agents.

4. Experimental section

4.1. Chemistry

Melting points were determined in Kofler apparatus and were uncorrected. IR spectra were measured on a Nicolet 5DX-FT-IR spectrometer on neat samples placed between KBr plates. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mecury-400BB spectrometer with TMS as an internal standard, all chemical shift values are reported as δ ppm. Optical rotations were measured on a Perkin Elmer 341 polarimeter in a 1 dm cell at 23 °C. Mass spectra were recorded on a Bruker Daltonics APEX‖49e and VGZAB-HS (70 ev) spectrometer with ESI source as ionization, respectively. All reactions were monitored by thin layer chromatograph (TLC) on silica gel GF_{254} (0.25 mm thick). Column chromatography (CC) was performed on Silica Gel 60 (230–400 mesh, Qingdao Ocean Chemical Ltd., China). Podophyllotoxin was isolated from a Chinesis medicinal herb *Podophyllum emodi* Wall var Chinesis Sprague, other starting materials and regents were purchased commercially and used without further purified, unless otherwise stated.

4.1.1. Procedure of synthesis of ***10***

Into a solution of 5-fluorouracil (6.5 g, 50 mmol) and potassium hydroxide (5.6 g, 100 mmol) in water (40 mL) was added the solution of chloroacetic acid (4.75 g, 50 mmol) in water (20 mL) and stirred for 30 min at room temperature. The pH value of the reaction mixture was adjusted to, and kept at 10 by the drop-by-drop addition of a 10% potassium hydroxide aqueous solution. The mixture was then refluxed for 2 h, cooled, and acidified to pH 2 by the addition of concentrated hydrochloric acid and the crystals of **10** were isolated by suction. Recrystallization was completed by dissolving the product in the saturated aqueous sodium bicarbonate and reprecipitated with concentrated hydrochloric acid to result in white needles.

4.1.2. Procedure of synthesis of ***11***

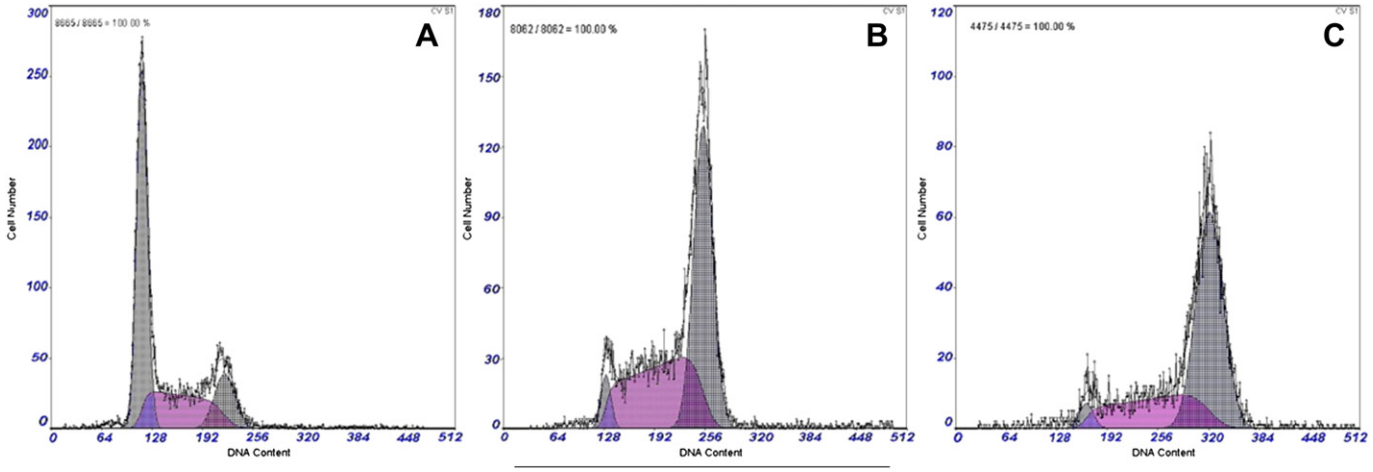
A mixture of 2-N^1-5-FU acetic acid (9.8 g, 0.05 mol), *p*-nitrophenol (13.9 g, 0.1 mol) and DCC (10.3 g, 0.05 mol) was stirred in anhydrous DMF (100 mL) for 4 h at 0 °C, then stirred for another 12 h at room temperature. The reaction mixture was filtered and filtrate was evaporated *in vacuo*, the crude product was further recrystallized in acetonitrile-ethanol to yield compound **11**.

4.1.3. General procedure of synthesis of ***12a–f***

p-Nitrophenyl 2-N^1-5-fluorouracil-ylacetate **11** (1.4 g, 5 mmol) was dissolved in DMF (10 mL), then the solution of aqueous 5% NaOH (5 mL) of appropriate α-amino acid (6 mmol) was added and the mixture stirred for 2 h at 0 °C, then continuously stirred for 10 h at room temperature while the pH value of the reaction mixture was kept at 8–10. The solvent was removed under vacuum, the residue was crystallized from water, and compounds **12a–f** were provided in needle crystal.

4.1.4. General preparation of compounds ***14a–f*** and ***15***

A mixture of **13** (0.5 mmol), **10** (or the appropriate *N*-5-FU-acetic amino acid **12a–f**) (0.5 mmol) and *N,N*-dimethylaminopyridine (DMAP, 20 mg) was stirred in dry dichloromethane (10 mL) for 5 min at room temperature under argon. *N,N′*-dicyclohexyl carbodiimide (DCC, 104 mg) was added and the reaction mixture



Compounds	G1	S	G2/M
Control	54.1%	30.3%	15.6%
12 h	4.84%	42.6%	52.6%
24 h	3.94%	31.3%	64.8%

Fig. 2. Effect of **14d** on cell cycle progression. A) control A-549 cells; B) A-549 cells treated with 0.5 μM **14d** for 12 h; C) A-549 cells treated with 0.5 μM **14d** for 24 h.

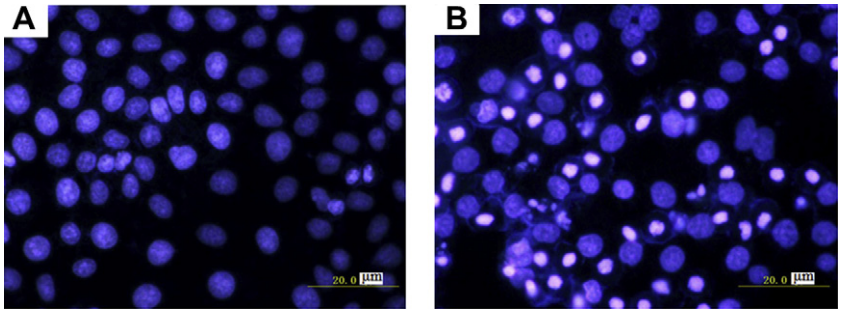


Fig. 3. Hoechst staining in A-549 cell line. A) control A-549 cells; B) A-549 cells treated with 0.5 μM **14d** for 24 h.

was stirred for 3–40 h and monitored by TLC. The reaction mixture was filtered and the filtrate was evaporated. The residue was separated to afford compound **14a**–**f** and **15** by column chromatography on silica gel with dichloromethane-acetone as eluent.

*4.1.4.1. 4′-O-(5-FU- acetic)-L-alanine 4-deoxyl-4′-O-demethylpodophyllic ester (**14a**).* Yield: 70%; m.p.:168–170 °C; $[\alpha]_D^{23}$ -66 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3518, 3316, 3206, 3071, 2920, 2846, 1768, 1696, 1670, 1601, 1505, 1483, 1462, 1422, 1379, 1338, 1227, 1130, 1037, 996; ^1H NMR (400 MHz, $CDCl_3$) δ 9.71 (brs, 1H, NH), 7.36–7.33 (m, 1H, NH), 7.17 (d, J = 7.2 Hz, 1H), 6.66 (s, 1H), 6.49 (s, 1H), 6.37 (s, 2H), 5.93 (d, J = 7.6 Hz, 2H), 4.88–4.83 (m, 1H), 4.60 (d, J = 4.0 Hz, 1H), 4.45 (t, J = 6.4 Hz, 1H), 4.36–4.33 (m, 2H), 3.90 (t, J = 8.8 Hz, 1H), 3.66 (s, 6H, 2 OMe), 3.06 (dd, J = 12.0, 4.0 Hz, 1H), 2.80–2.72 (m, 3H), 1.55 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0 (2C), 170.7, 165.9, 157.4 (d, J = 26 Hz, 1C), 151.1, 151.0, 149.9, 147.0, 146.7, 140.2 (d, J = 236 Hz, 1C), 139.5, 130.0, 129.6 (d, J = 33 Hz, 1C), 128.4, 127.2, 110.3, 108.5, 107.8, 101.2, 72.1, 56.2 (2 OMe), 49.9, 48.4, 47.2, 43.7, 32.9, 32.7, 18.1; HRMS (ESI) 643.2039 for $[M + NH_4]^+$ (calcd 643.2046 for $C_{30}H_{32}FN_4O_{11}$).

*4.1.4.2. 4′-O-(5-FU- acetic)-L-valine 4-deoxyl-4′-O-demethylpodophyllic ester (**14b**).* Yield: 65%; m.p.:183–185 °C;$[\alpha]_D^{23}$ -69 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3316, 3204, 3070, 3002, 2966, 2938, 2844, 1765, 1703, 1601, 1505, 1483, 1465, 1422, 1378, 1338, 1227, 1154, 1131, 1038, 996; ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, J = 5.6 Hz, 1H), 7.05 (br, 1H), 6.67 (s, 1H), 6.50 (s, 1H), 6.37 (s, 2H), 5.94 (d, J = 3.2 Hz, 2H), 4.88–4.84 (m, 1H), 4.61 (d, J = 3.2 Hz, 1H), 4.46 (t, J = 6.8 Hz, 1H), 4.36 (q, J = 3.6 Hz, 2H), 3.90 (t, J = 8.8 Hz, 1H), 3.66 (s, 6H, 2 OMe), 3.07 (dd, J = 13.2, 4.4 Hz, 1H), 2.80–2.67 (m, 3H), 2.46–2.35 (m, 1H), 1.01 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 174.9, 169.4, 166.2, 157.2 (d, J = 27 Hz, 1C), 151.1, 151.0, 149.9, 147.1, 146.7, 140.3 (d, J = 236 Hz, 1C), 139.6, 130.1, 129.5 (d, J = 28 Hz, 1C), 128.4, 126.9, 110.4, 108.5, 107.7, 101.2, 72.1, 57.4, 56.0 (2 OMe), 50.1, 47.3, 43.8, 32.9, 32.7, 31.4, 18.8, 17.1; HRMS (ESI) 671.2348 for $[M + NH_4]^+$ (calcd 671.2359 for $C_{32}H_{36}FN_4O_{11}$).

*4.1.4.3. 4′-O-(5-FU- acetic)-L-leucine 4-deoxyl-4′-O-demethylpodophyllic ester (**14c**).* Yield: 74%; m.p.:173–175 °C;$[\alpha]_D^{23}$-65 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3313, 3207, 3072, 2958, 2926, 2847, 1766, 1699, 1601, 1505, 1482, 1421, 1379, 1337, 1226, 1153, 1130, 1037, 996; ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, J = 5.6 Hz, 1H), 7.05 (br, 1H), 6.66 (s, 1H), 6.49 (s, 1H), 6.36 (s, 2H), 5.93 (d, J = 8.0 Hz, 2H), 4.89–4.86 (m, 1H), 4.59 (d, J = 4.2 Hz, 1H), 4.45 (t, J = 7.2 Hz, 1H), 4.36 (d, J = 3.6 Hz, 2H), 3.90 (t, J = 8.8 Hz, 1H), 3.66 (s, 6H, 2 OMe), 3.06 (dd, J = 16.0, 4.0 Hz, 1H), 2.80–2.72 (m, 3H), 1.90–1.75 (m, 2H), 1.68–1.65 (m, 1H), 0.96 (d, J = 5.2 Hz, 6H, 2 Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 170.3, 165.8, 157.2, 151.1 (2C), 149.7, 147.1, 146.7, 140.4 (d, J = 236 Hz, 1C), 139.5, 130.1, 129.3 (d, J = 33 Hz, 1C), 128.3, 127.1, 110.4, 108.5, 107.8, 101.2, 72.1, 56.2 (2C), 51.2, 50.0, 47.3, 43.8, 41.6, 33.0, 32.7, 24.7, 22.7, 22.0, 21.9; HRMS (ESI) 685.2526 for $[M + NH_4]^+$ (calcd 685.2516 for $C_{33}H_{38}FN_4O_{11}$).

*4.1.4.4. 4′-O-(5-FU-acetic)-L-phenylalanine 4-deoxyl-4′-O-demethyl podophyllic ester (**14d**).* Yield: 48%; m.p.:186–187 °C; $[\alpha]_D^{23}$ -71 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3304, 3201, 3067, 3032, 3006, 2934, 2846, 1768, 1702, 1668, 1602, 1539, 1505, 1483, 1462, 1421, 1378, 1338, 1227, 1155, 1131, 1094, 1037, 996; ^1H NMR (400 MHz, $CDCl_3$) δ 9.52 (brd, 1H, NH), 7.24–7.13 (m, 6H), 7.07 (t, J = 8.8 Hz, 1H), 6.66 (s, 1H), 6.48 (s, 1H), 6.38 (s, 2H), 5.93 (d, J = 7.6 Hz, 2H), 5.17 (q, J = 6.0 Hz, 1H), 4.60 (d, J = 3.2 Hz, 1H), 4.45 (t, J = 8.0 Hz, 1H), 4.36 (ddq, J = 16, 3.6 Hz, 2H), 3.89 (t, J = 8.8 Hz, 1H), 3.67 (s, 6H, 2 OMe), 3.39–3.32 (m, 1H), 3.21–3.15 (m, 1H), 3.08 (dd, J = 12.8, 4.4 Hz, 1H), 2.79–2.69 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.9, 174.8, 169.2, 165.7, 157.0 (d, J = 26 Hz, 1C), 151.1, 149.6, 147.1, 146.7, 140.2 (d, J = 234 Hz, 1C), 139.7, 135.6, 130.1, 129.7, 129.0 (d, J = 37 Hz, 1C), 128.4 (2C), 127.0, 110.4, 108.5, 107.7, 101.2, 72.1, 69.5, 56.0 (2C, 2OMe), 53.8, 53.1, 49.9, 47.3, 43.8, 37.5, 33.0, 32.7, 31.6, 29.6, 29.2; HRMS (ESI) 719.2345 for $[M + NH_4]^+$ (calcd 719.2359 for $C_{36}H_{36}FN_4O_{11}$).

*4.1.4.5. 4′-O-(5-FU- acetic)-L-methine 4-deoxyl-4′-O-demethylpodophyllic ester (**14e**).* Yield: 56%; m.p.:168–169 °C;$[\alpha]_D^{23}$ -83 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3304, 3201, 3068, 3003, 2918, 2844, 1767, 1701, 1601, 1505, 1483, 1463, 1379, 1338, 1227, 1154, 1130, 1037, 996; ^1H NMR (400 MHz, $CDCl_3$) δ 7.37–7.31 (m, 2H), 6.67 (s, 1H), 6.49 (s, 1H), 6.37 (s, 2H), 5.94 (d, J = 4.6 Hz, 2H), 5.01 (q, J = 6.8 Hz, 1H), 4.59 (d, J = 3.2 Hz, 1H), 4.45 (t, J = 6.8 Hz, 1H), 4.38–4.31 (m, 2H), 3.90 (t, J = 8.8 Hz, 1H), 3.67 (s, 6H, 2 OMe), 3.07 (dd, J = 15.6, 4.0 Hz, 1H), 2.80–2.72 (m, 3H), 2.64 (t, J = 7.6 Hz, 2H), 2.35–2.27 (m, 1H), 2.21–2.12 (m, 1H), 2.09 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 169.5, 166.0, 157.3 (d, J = 26 Hz, 1C), 151.0 (2C), 149.9, 147.1, 146.7, 140.3 (d, J = 236 Hz, 1C), 139.7, 130.0, 129.5 (d, J = 32 Hz, 1C), 128.3, 126.9, 110.3, 108.5, 107.1, 101.2, 72.1, 56.1 (2C), 51.9, 50.1, 47.3, 43.8, 32.9, 32.7, 31.7, 29.5, 15.2; HRMS (ESI) 703.2072 for $[M + NH_4]^+$ (calcd 703.2080 for $C_{32}H_{36}FN_4O_{11}S$).

*4.1.4.6. 4′-O-(5-FU- acetic)-D-methine 4-deoxyl-4′-O-demethylpodophyllic ester (**14f**).* Yield: 48%; m.p.:169–171 °C;$[\alpha]_D^{23}$ -77 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3303, 3225, 3069, 3303, 2918, 2844, 1766, 1698, 1601, 1505, 1483, 1379, 1338, 1227, 1154, 1130, 1037, 996; ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, J = 5.6 Hz, 1H), 7.22 (d, J = 8.0 Hz, 1H), 6.67 (s, 1H), 6.50 (s, 1H), 6.37 (s, 2H), 5.94 (d, J = 8.8 Hz, 2H), 5.02 (q,

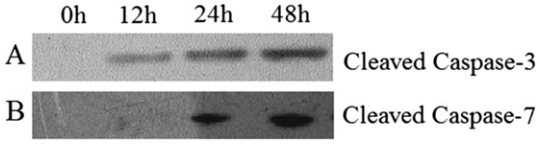


Fig. 4. Effects of **14d** on the activation of caspase-3 and caspase-7.

J = 6.8 Hz, 1H), 4.61 (d, J = 2.4 Hz, 1H), 4.46 (t, J = 6.4 Hz, 1H), 4.37 (d, J = 4.4 Hz, 2H), 3.92 (t, J = 8.8 Hz, 1H), 3.67 (s, 6H, 2 OMe), 3.08 (dd, J = 16.0, 4.4 Hz, 1H), 2.80–2.72 (m, 3H), 1.90–1.75 (m, 2H), 0.96 (d, J = Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 169.4, 166.0, 157.3 (d, J = 26 Hz, 1C), 151.0 (2C), 149.8, 147.1, 146.7, 140.3 (d, J = 236 Hz, 1C), 139.7, 130.0, 129.5 (d, J = 32 Hz, 1C), 128.4, 126.8, 110.3, 108.5, 107.1 (2C), 101.2, 72.1, 56.1 (2C, 2OMe), 51.9, 50.1, 47.3, 43.8, 32.9, 32.7, 31.7, 29.5, 15.2; HRMS (ESI) 703.2070 for $[M + NH_4]^+$ (calcd 703.2080 for $C_{32}H_{36}FN_4O_{11}S$).

*4.1.4.7. 4′-O-5-FU- acetic acid 4-deoxyl-4′-O-demethylpodophyllic ester (**15**).* Yield: 62%; m.p.:180–182 °C;$[\alpha]_D^{23}$ -50 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3194, 3072, 3004, 2943, 2915, 2844, 1773, 1706, 1670, 1601, 1505, 1483, 1463, 1420, 1378, 1337, 1227, 1159, 1130, 1093, 1037, 996; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.40 (br, 1H, NH), 7.31 (d, J = 5.6 Hz, 1H), 6.67 (s, 1H), 6.51 (s, 1H), 6.39 (s, 2H), 5.95 (d, J = 7.6 Hz, 2H), 4.77 (s, 2H), 4.63 (d, J = 4.4 Hz, 1H), 4.46 (t, J = 8.8 Hz, 1H), 3.92 (t, J = 8.8 Hz, 1H), 3.70 (s, 6H, 2 OMe), 3.07 (dd, J = 15.6, 4.8 Hz, 1H), 2.80–2.67 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.8, 165.2, 157.1 (d, J = 26 Hz, 1C), 150.9 (2C), 149.4, 147.1, 146.7, 140.4 (d, J = 237 Hz, 1C), 139.8, 130.1, 129.5 (d, J = 32 Hz, 1C), 128.3, 126.7, 110.4, 108.5, 107.6, 101.2, 72.0, 56.1 (2 OMe), 48.0, 47.3, 43.7, 33.0, 32.7, 30.7; HRMS (ESI) 572.1668 for $[M + H]^+$ (calcd 572.1675 for $C_{27}H_{277}FN_3O_{11}$).

4.2. Biological evaluation

4.2.1. Cytotoxicity assays

Cells were incubated at 37 °C in a 5% CO_2 atmosphere. The MTT and Cell Counting Kit-8 (CCK-8) colorimetric assay were used to determined growth inhibition [34]. The synthetic conjugates and reference compounds were dissolved in saline for five concentrations (0.005–50 μM). For the HL-60 and HeLa, cells were plated in 96-well plates and exposed in quadplex well for 48 h. Then the CCK-8 was added to each well. After 4 h of incubation, the absorbance at λ_{450} was determined with a plate reader. For the A-549 and SiHa, cells were plated in 96-well plates and allowed to attach for 24 h, then exposed in quadplex well for 48 h. The media was aspirated, and 10 μL of 5 mg/mL MTT solution (dilute in sterile PBS) diluted in serum-free media was added to each well. After 4 h of incubation, the solution was centrifuged for 10 min under 2000 rpm. The supernatant was mixed with 150 μL DMSO, then was shaken on an oscillator. The absorbance at λ_{570} was determined on a plate reader. IC_{50} values were determined from a log plot of percent of control versus concentration.

4.2.2. Analysis of cell cycle by flow cytometry

For cell cycle analysis, we used the human lung carcinoma A-549 cell line grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 24 μg/mL gentamicin and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Untreated and drug-treated cells ($(3–5) \times 10^5$) were harvested and fixed overnight in 70% ethanol at 4 °C. Cells were then washed three times with PBS, incubated for 1 h with 1 mg/mL RNase A and 20 μg/mL propidium iodide at room temperature, and analyzed with a flow cytometer (COULTER EPICS XL, USA) as described previously [37].

4.2.3. Detection of apoptosis by fluorescence microscopy

The Hoechst apoptosis detection kit (Beyotime, Jiangsu, China) was used for the detection of apoptotic cells. Briefly, A549 cells were grown to about 70–80% confluence on slides and then treated with **14d** (0.5 μM) or vehicle for 24 h. Subsequently, cells were fixed, washed twice with PBS and stained with Hoechst 33258 staining solution according to the manufacturer's instructions. Chromosomal condensation and morphological changes were observed under a fluorescence microscope image system (Olympus, Tokyo Japan).

4.2.4. Western blot analysis

A-549 Cells (1×10^6 cells) exposed to compound **14d** were collected into tubes and then washed with PBS. Cell pellets were lysesed with lyses buffer (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.2M NaCl, 1.5 mM PMSF and 1% SDS). Cell lysates were boiled for 10 min, centrifuged and stored at −20 °C. Cell lysates containing 10–20 μg protein were separated and transferred to nitrocellulose filters. The blots were incubated with the corresponding antibodies and developed [39].

Acknowledgments

This work was financially supported by the Fundamental Research Funds for the Central Universities, Lanzhou University (lzujbky-2010-136), and the Open Funds of Key Lab of Preclinical Study for New Drugs of Gansu Province (GSKFKT-00801).

References

[1] I. Caleta, M. Kralj, M. Marjanovic, B. Bertosa, S. Tomic, G. Pavilovic, K. Pavelic, G. Karminski-Zamola, J. Med. Chem. 52 (2009) 1744–1756.
[2] M. Gordaliza, P.A. García, J.M.M. del Corral, M.A. Castro, M.A. Gómez-Zurita, Toxicon 44 (2004) 441–459.
[3] T.F. Imbert, Biochimie 80 (1998) 207–222.
[4] E.L. Baldwin, N. Osheroff, Curr. Med. Chem. Anti-Cancer Agents 5 (2005) 363–372.
[5] J.L. Nitiss, Nat. Rev. Cancer 9 (2009) 338–350.
[6] J. Yang, A. Bogni, E.G. Schuetz, M. Ratain, M.E. Dolan, H. McLeod, L. Gong, C. Thorn, M.V. Relling, T.E. Klein, R.B. Altman, Pharmacogenet. Genom. 19 (2009) 552–553.
[7] D. Perrin, B. van Hille, J.-M. Barret, A. Kruczynski, C. Etiévant, T. Imbert, B.T. Hill, Biochem. Pharmacol. 59 (2000) 807–819.
[8] J.M. Barret, C. Etievant, C. Baudouin, K. Skov, M. Charveron, B.T. Hill, Anticancer Res. 22 (2002) 187–192.
[9] J.M. Barret, A. Kruczynski, S. Vispé, J. Annereau, V. Brel, Y. Guminski, J.-G. Delcros, A. Lansiaux, N. Guilbaud, T. Imbert, C. Bailly, Cancer Res. 68 (2008) 9845–9853.
[10] A. Kruczynski, I. Vandenberghe, A. Pillon, S. Pesnel, L. Goetsch, J.M. Barret, Y. Guminski, A. Le Pape, T. Imbert, C. Bailly, N. Guilbaud, Invest. New Drugs 29 (2011) 9–21.
[11] Y. Kim, S.B. Kim, Y.J. You, B.Z. Ahn, Planta Med. 68 (2002) 271–274.
[12] T. Masuda, Y. Oyama, S. Yonemori, Y. Takeda, Y. Yamazaki, S. Mizuguchi, M. Nakata, T. Tanaka, L. Chikahisa, Y. Inabak, Y. Okada, Phytother. Res. 16 (2002) 353–358.
[13] N. Muto, T. Tomokuni, M. Haramoto, H. Tatemoto, T. Nakanishi, Y. Inatomi, H. Murata, A. Inada, Biosci. Biotechnol. Biochem. 72 (2008) 477–484.
[14] S.H. Lee, M.J. Son, H.K. Ju, C.X. Lin, T.C. Moon, H.G. Choi, J.K. Son, H.W. Chang, Biol. Pharm. Bull. 27 (2004) 786–788.
[15] K. Sudo, K. Konno, S. Shigeta, T. Yokota, Antivir. Chem. Chemother. 9 (1998) 263–267.
[16] Y.J. Yong, S.Y. Shin, Y.H. Lee, Y.H. Lim, Bioorg. Med. Chem. Lett. 19 (2009) 4367–4371.
[17] S.Y. Shin, Y. Yong, C.G. Kim, Y.H. Lee, Y. Lim, Cancer Lett. 287 (2010) 231–239.
[18] S.J. Suh, J.R. Kim, U.H. Jin, H.S. Choi, Y.C. Chang, Y.C. Lee, S.H. Kim, I.S. Lee, T.S. Moon, H.W. Chang, C.H. Kim, Vasc. Pharmcol. 51 (2009) 13–20.
[19] E. Frei III, A. Elias, C. Wheeler, P. Richardson, W. Hryniuk, Clin. Cancer Res. 4 (1998) 2027–2037.
[20] G.J. Peters, C.L. Van der Wilt, C.J.A. Van Moorsel, J.R. Kroep, A.M. Bergman, S.P. Ackland, Pharmacol. Ther. 87 (2000) 227–253.
[21] J.-Y. Chang, X. Guo, H.-X. Chen, Z. Jiang, Q. Fu, H.-K. Wang, K.h F. Bastow, X.-K. Zhu, J. Guan, K.-H. Lee, Y.-C. Cheng, Biochem. Pharmacol. 59 (2000) 497–508.
[22] Q. Shi, H.-K. Wang, K.F. Bastow, Y. Tachibana, K. Chen, F.-Y. Lee, K.-H. Lee, Bioorg. Med. Chem. 9 (2001) 2999–3004.
[23] N.-J. Lee, I.-C. Jeong, M.-Y. Cho, C.-W. Jeon, B.-C. Yun, Y.-O. Kim, S.-H. Kim, I. Chung, Eur. Polym. J. 42 (2006) 3352–3359.
[24] D. Passarella, A. Giardini, B. Peretto, G. Fontana, A. Sacchetti, A. Silvani, C. Ronchi, G. Cappelletti, D. Cartelli, J. Borlak, B. Danieli, Bioorg. Med. Chem. 16 (2008) 6269–6285.
[25] D. Passarella, B. Peretto, R.B. Yepes, G. Cappelletti, D. Cartelli, C. Ronchi, J. Snaith, G. Fontana, B. Danieli, J. Borlak, Eur. J. Med. Chem. 45 (2010) 219–226.
[26] S.-W. Chen, X. Tian, Y.Q. Tu, Bioorg. Med. Chem. Lett. 14 (2004) 5063–5066.
[27] S.-W. Chen, R. Xiang, J. Liu, X. Tian, Bioorg. Med. Chem. 17 (2009) 3111–3115.

[28] S.-W. Chen, R. Xiang, X. Tian, Helv. Chim. Acta 92 (2009) 1568–1574.
[29] F.M. Zhang, X.J. Yao, X. Tian, Y.Q. Tu, Molecular 11 (2006) 849–857.
[30] Y.Q. Liu, H. Yang, X. Tian, Chin. J. Chem. 24 (2006) 785–790.
[31] Y. Jin, J. Liu, W.T. Huang, S.-W. Chen, L. Hui, Eur. J. Med. Chem. 46 (2011) 1673–1677.
[32] S.-W. Chen, Y.Y. Gao, N.N. Gao, J. Liu, W.T. Huang, L. Hui, Y. Jin, Y.X. Jin, Bioorg. Med. Chem. Lett. 21 (2011) 7355–7358.
[33] R.X. Zhuo, C.L. Fan, R.L. Zhao, Chem. J. Chin. Univ. (in Chinese) 10 (1986) 508–511.
[34] Z.W. Zhang, J.Q. Zhang, L. Hui, S.-W. Chen, X. Tian, Eur. J. Med. Chem. 45 (2010) 1673–1677.
[35] Y.J. You, Y. Kim, N.H. Nam, S.C. Bang, B.Z. Ahn, Eur. J. Med. Chem. 39 (2004) 189–193.
[36] Y. Kim, Y.J. You, N.H. Nam, B.Z. Ahn, Bioorg. Med. Chem. Lett. 12 (2002) 3435–3438.
[37] C. Gajate, I. Barasoain, J.M. Andreu, F. Mollinedo, Cancer Res. 60 (2000) 2651–2659.
[38] S.A. Lakhani, A. Masud, K. Kuida, G.A. . Porter Jr., C.J. Booth, W.Z. Mehal, I. Inayat, R.A. Flavell, Science 311 (2006) 847–851.
[39] S.Y. Shin, Y.Y. Bahk, J. Ko, I.Y. Chung, Y.S. Lee, J. Downward, H. Eibel, P.M. Sharma, J.M. Olefsky, Y.H. Kim, B. Lee, Y.H. Lee, EMBO J. 25 (2006) 1093–1103.