Bioorganic & Medicinal Chemistry 18 (2010) 6351–6359

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis, biological evaluation, and structure–activity relationship study of novel cytotoxic aza-caffeic acid derivatives

Hongbin Zou [a], Hao Wu [a], Xiangnan Zhang [b], Yu Zhao [a], Joachim Stöckigt [a,c], Yijia Lou [b,*], Yongping Yu [a,*]

[a] Institute of Material Medica, College of Pharmaceutical Sciences, Zhejiang University, 383 Yu Hang Tang Road, Hangzhou 310058, China
[b] Institute of Pharmacology, Toxicology, and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, 383 Yu Hang Tang Road, Hangzhou 310058, China
[c] Lehrstuhl für Pharmazeutische Biologie, Institut für Pharmazie, Johannes-Gutenberg Universität Mainz, Staudinger Weg 5, D-55099 Mainz, Germany

ARTICLE INFO

Article history:
Received 4 May 2010
Revised 7 July 2010
Accepted 8 July 2010
Available online 13 July 2010

Keywords:
Aza-caffeic acid derivatives
Cytotoxicity
Mitochondria-dependent apoptosis
SAR analysis
Linker B

ABSTRACT

Three series of aza-caffeic acid derivatives with different linkers were designed and synthesized. Each of the synthesized derivatives was then used in cytotoxicity screening on either 8 or 12 human cancer cell lines. The structure–activity relationships on three structural regions A, B, and C are analyzed in detail, indicating that a nine bond linker B, containing a piperazine unit, is the most favorable linker leading to the generation of molecules with potent cytotoxicities. Compound (*E*)-1-(4-(3,4-dichlorobenzyl)piperazin-1-yl)-3-(4-(4-ethoxybenzyloxy)-3,5-dimethoxyphenyl)prop-2-en-1-one (**80**) exhibited the most significant and selective cytotoxicity to KB, BEL7404, K562, and Eca109 cell lines, with IC_{50} values of 0.2, 2.0, 1.7, and 1.1 μM, respectively, stronger than that seen for caffeic acid phenethyl ester (CAPE) and cisplatin (CDDP). Flow cytometric and western blot analysis indicate that compound **80** plays a role in mitochondria-dependent apoptosis activity by suppressing K562 cell proliferation in a concentration- and time-dependent manner.

1. Introduction

Caffeic acid phenethyl ester (CAPE, **1**, Fig. 1), which was first isolated from Hungarian propolis,[1] is a well known caffeic acid derivative with strong cytotoxic activity.[2,3] Petasiphenol (**2**, Fig. 1), another caffeic acid derivative isolated from *Petasites japonicum*, also possesses antimutagen activity.[4] Due to their structural simplicity and interesting cytotoxic properties, the synthesis and biological evaluation of CAPE, petasiphenol, and their derivatives have been the subject of studies by several groups.[4–7] In addition to caffeic acid derivatives, sinapyl acid derivatives possessing the same α-, β-unsaturated carbonyl as an important Michael acceptor anticancer pharmacophore have also been previously reported.[8,9] Sinapyl acid **3** (Fig. 1) displays potent cytotoxicity against the KB cell line with an IC_{50} value of 14 μM.[9]

The aza-derivatives of caffeic acid, in particular, the aza-CAPE (*N*-caffeoylphenalkylamide) derivatives have also been found to possess intensive activities.[10–12] However, a cytotoxicity study of the aza-caffeic acid derivatives has rarely been reported.[13] To systematically assess the effect of structural variations on the compound's activity, the structure of CAPE was divided into three regions (A, B, and C, shown in Fig. 1). In order to investigate the structure–cytotoxicity relationship of aza-caffeic acid derivatives, different substituents in region A, C, and variant linker B with a different position and number of nitrogen atoms were synthesized to constitute three series of 32 compounds in total based on the structures of compounds **1**, **2**, and **3** (Fig. 2). In addition, compounds **91** and **88** were synthesized as analogous of series 2 and 3 (Fig. 2). The synthetic procedures are reported herein. The synthesized aza-caffeic acid derivatives were then used to screen 8 (**37**–**51**, **59**–**63**, Table 1) and 12 (**73**–**84**, **88**, **91**, Table 2) cancer cell lines, respectively. The results showed that the compounds have cytotoxic potential. Moreover, the SAR discussion on cytotoxicity effect by structural variations in regions A, B, and C is reported herein.

2. Results and discussion

2.1. Chemistry

The synthesis of target compounds was accomplished according to the reaction sequence reported in Schemes 1–3. The aza-caffeic acid derivatives (**31**–**44**, **59**–**63**) with an eight bond linker (B) were coupled by potassium salts of substituted cinnamic acids (**12**–**14**, **56**–**58**) and N-substituted 2-chloro acetamides (**23**–**30**) under the activation of potassium iodine with yields of 56–87% (Scheme 1).[14] Compounds **9**–**11**, which were further hydrolyzed by potassium

* Corresponding authors. Tel./fax: +86 571 88208452 (Y.Y.); tel./fax: +86 571 88208403 (Y.L.).
E-mail addresses: yijialou@zju.edu.cn (Y. Lou), yyu@zju.edu.cn, yyongping@tpims.org (Y. Yu).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.07.016

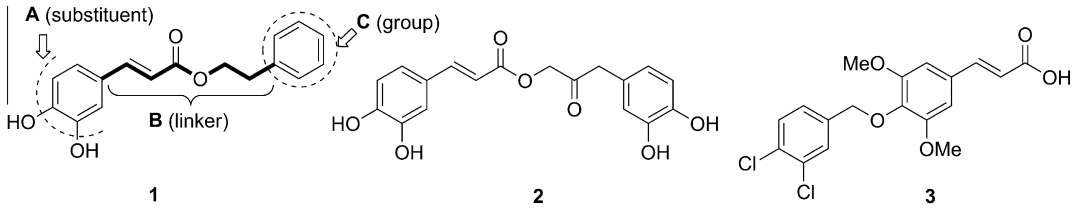


Figure 1. Caffeic acid derivatives and regions A, B, and C for structure modulation.

hydroxide to afford the intermediate potassium salts (**12**–**14**, **56**–**58**), were prepared by a Wittig reaction from **5**, **6**, and **8** as previously described.[9] The aldehydes **5**–**7** were synthesized by alkylation of the hydroxy group of protocatechualdehyde (**4**) with methyl, methoxymethyl, and benzyl substituents, respectively, in the presence of potassium carbonate in which **7** was further alkylated by methoxymethyl to afford **8**.[9] N-substituted 2-chloro acetamides (**23**–**30**) were synthesized by coupling the corresponding amines (**15**–**22**) to chloroacetyl chloride.[15] The preparation of **53**–**55** was achieved as previously described by the appropriate substitution of benzylbromides and syringaldehyde (**52**).[9] Hydrolyzation of compounds **31**–**36** and **44** were conducted in the presence of hydrochloric acid (1 M) to afford compounds **46**–**51** with two free hydroxy groups and **45** with one free hydroxy.[16]

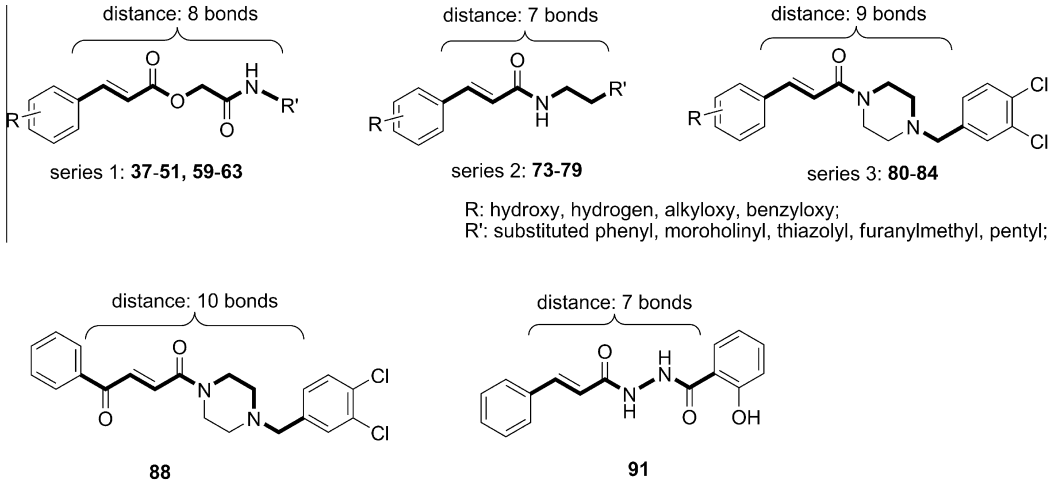


Figure 2. Designed and synthesized aza-caffeic acid derivatives.

Table 1
Inhibitory results of **37**–**51** and **59**–**63** to 8 human cancer cell lines[a]

Compound	IC_{50} (μM)							
	BEL7404	HL-60	HeLa	CNE	PC-3	K562	Eca109	LS174T
37	—[b]	46.4 ± 4.1	193 ± 14.3	—	—	—	—	—
38	120 ± 9.3	31.9 ± 2.7	45.4 ± 3.7	—	—	60.2 ± 4.8	81.9 ± 6.3	—
39	—	92.5 ± 7.3	—	—	—	0.8 ± 0.04	—	—
40	—	—	—	—	—	—	—	—
41	—	50.4 ± 4.9	—	—	—	32.4 ± 1.6	143 ± 9.13	—
42	—	82.2 ± 4.8	—	—	—	0.3 ± 0.01	—	—
43	130 ± 8.7	—	—	—	—	—	—	—
44	133 ± 9.6	—	155 ± 11.7	—	—	—	—	—
45	—	—	—	—	—	56.1 ± 3.3	—	—
46	—	—	—	—	—	56.8 ± 4.1	—	—
47	—	30.3 ± 2.3	—	—	—	—	—	117 ± 7.1
48	—	92.6 ± 6.5	—	—	—	—	—	145 ± 6.7
49	—	8.1 ± 0.5	195 ± 10.3	89.6 ± 6.3	160 ± 8.8	99.4 ± 5.8	175 ± 8.1	108 ± 5.8
50	—	72.1 ± 5.2	—	—	172 ± 9.1	79.1 ± 5.1	190 ± 9.0	124 ± 8.3
51	—	97.4 ± 7.1	187 ± 10.6	170 ± 9.7	155 ± 6.3	121 ± 8.3	131 ± 8.4	69.4 ± 4.0
59	97.8 ± 7.6	—	—	—	—	—	—	—
60	—	73.9 ± 6.6	—	—	—	—	—	72.4 ± 5.3
61–**63**	—	—	—	—	—	—	—	—
CAPE	14.5 ± 1.2	9.8 ± 0.9	8.8 ± 0.7	54 ± 2.6	91 ± 4.3	46 ± 1.6	42.6 ± 0.9	9.9 ± 0.7
DDP	5.6 ± 0.2	6.4 ± 0.4	5.0 ± 0.3	6.7 ± 0.2	4.0 ± 0.2	11.6 ± 0.8	7.6 ± 0.5	15.6 ± 0.8

[a] Key to cell lines: BEL7404, human hepatocellular carcinoma line; HL-60, human promyelocytic leukemia cell line; HeLa, human cervical carcinoma cell line; CNE, nasopharyngeal carcinoma cell line; PC-3, human prostate cancer cell line; K562, human erythroleukemia cells; Eca109, human esophageal carcinoma cell line; LS174T, human colon carcinoma cells.

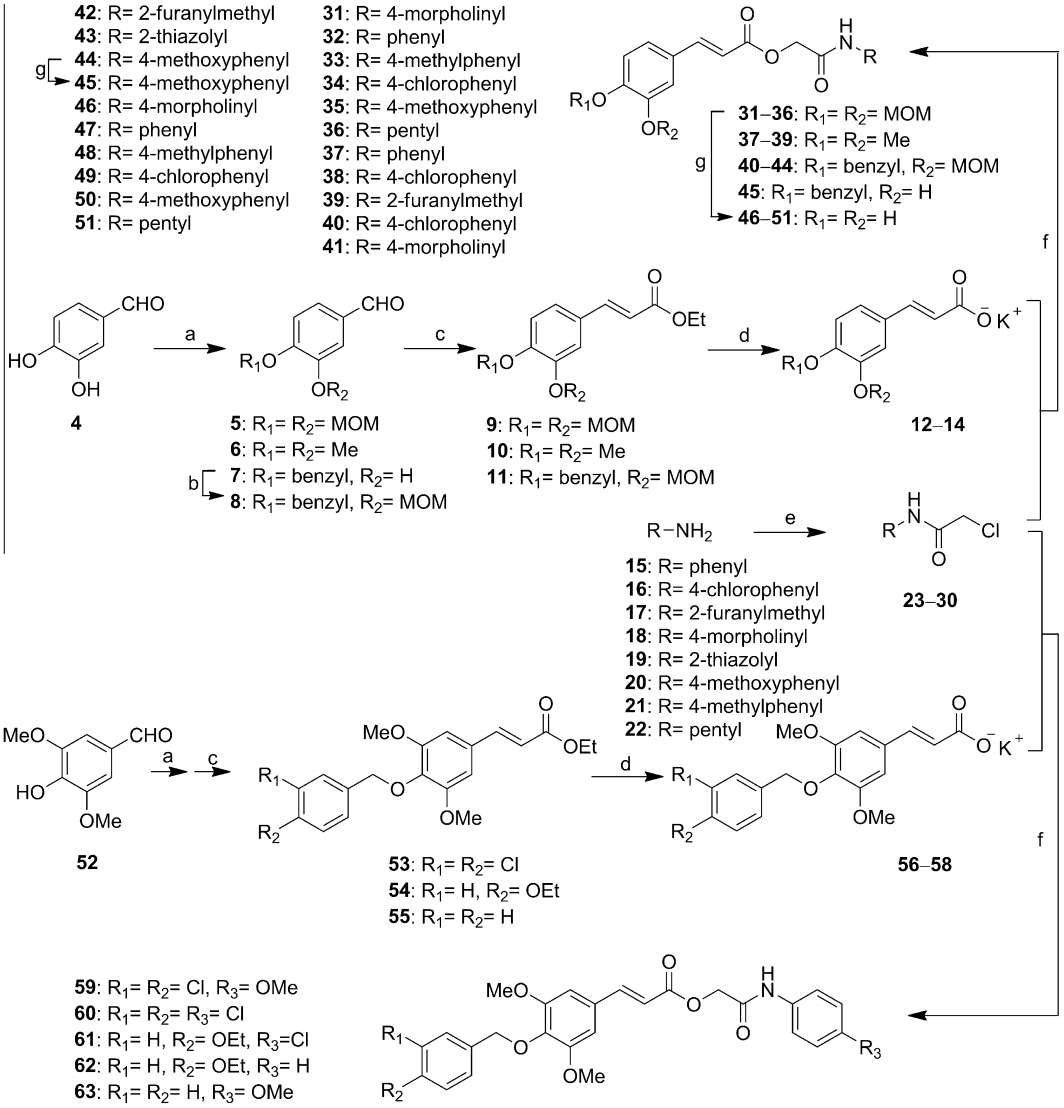
[b] IC_{50} values greater than 200 μM were considered as inactive and omitted here.

Table 2
Inhibitory results of compounds **73**–**84**, **88**, and **91** to 12 human cancer cell lines[a]

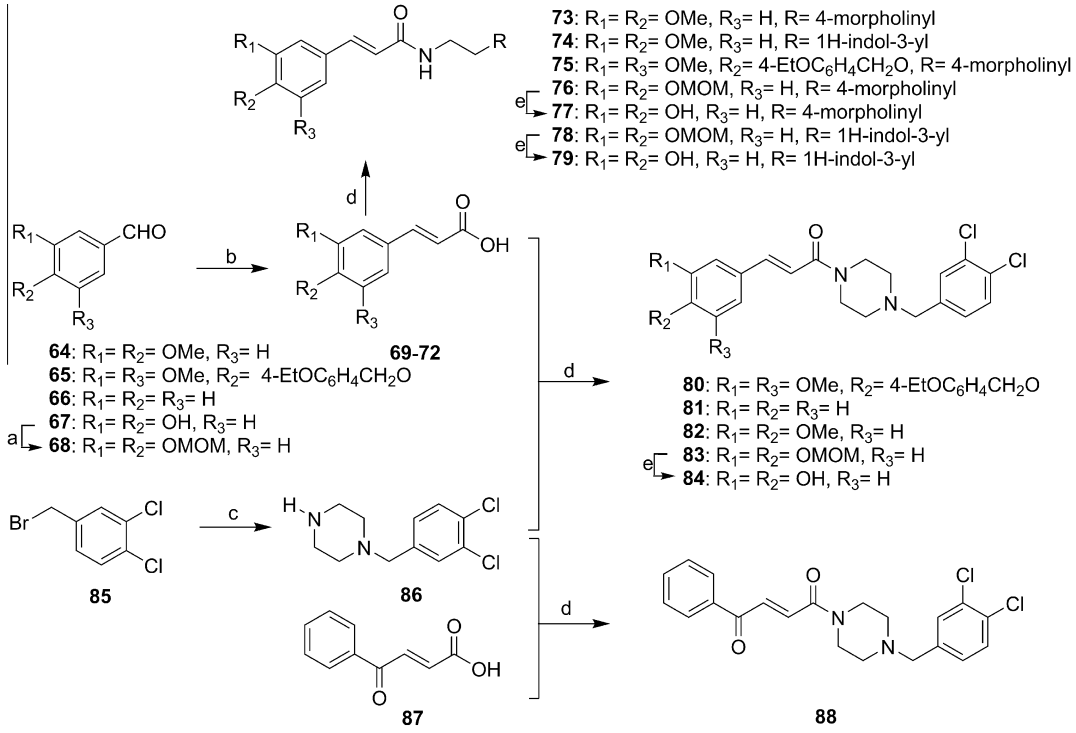
Compound	IC_{50} (μM)											
	KB	A549	BEL7404	HL-60	HeLa	CNE	PC-3	K562	BGC-823	Eca109	HO8910	LS174T
73–**77**	—[b]	—	—	—	—	—	—	—	—	—	—	—
78	—	—	—	—	—	—	157 ± 8.4	195 ± 10.3	143 ± 11.0	154 ± 9.8	—	165 ± 8.3
79	85.7 ± 5.1	—	—	124 ± 7.9	2.7 ± 0.2	—	—	—	—	—	—	8.4 ± 0.3
80	0.2 ± 0.01	63.4 ± 2.5	2.0 ± 0.1	—	—	—	—	1.7 ± 0.1	—	1.1 ± 0.06	—	—
81	20.7 ± 1.6	173 ± 8.8	44.8 ± 2.6	82.6 ± 3.2	73.9 ± 3.5	—	12.8 ± 1.1	14.1 ± 0.4	31.2 ± 2.8	17.0 ± 1.1	45.4 ± 2.5	34.1 ± 1.7
82	69.2 ± 2.8	—	85.7 ± 3.2	140 ± 5.8	76.6 ± 5.8	—	—	70.8 ± 3.9	—	—	121 ± 6.6	—
83	3.7 ± 0.02	107 ± 7.3	—	31.0 ± 1.3	29.1 ± 1.7	—	7.4 ± 0.4	19.3 ± 0.8	64.4 ± 5.9	7.4 ± 0.4	69.7 ± 3.0	139 ± 10.1
84	—	—	—	165 ± 7.8	—	—	—	—	—	—	—	50.4 ± 2.3
88	103 ± 6.4	139 ± 6.4	67.1 ± 3.6	99.4 ± 4.7	84.8 ± 4.5	—	—	—	78.8 ± 4.4	103 ± 7.0	—	67.2 ± 3.6
91	20.1 ± 1.2	72.6 ± 3.3	21.2 ± 1.0	39.3 ± 2.1	73.1 ± 2.9	43.7 ± 3.6	40.9 ± 2.8	0.9 ± 0.04	46.0 ± 3.0	41.4 ± 2.6	190 ± 11.1	20.1 ± 1.0
CAPE	45.2 ± 2.3	26.8 ± 1.6	14.5 ± 1.2	9.8 ± 0.9	8.8 ± 0.7	54.2 ± 2.6	91.4 ± 4.3	46.1 ± 1.6	19.3 ± 1.3	42.6 ± 0.9	25.5 ± 1.0	9.9 ± 0.7
DDP	4.9 ± 0.3	7.5 ± 0.4	5.6 ± 0.2	6.4 ± 0.4	5.0 ± 0.3	6.7 ± 0.2	4.0 ± 0.2	11.6 ± 0.8	5.0 ± 0.4	7.6 ± 0.5	6.0 ± 0.2	15.6 ± 0.8

[a] Key to cell lines: KB, human oral epithelial cell line; A549, human lung adenocarcinoma cell line; BGC-823, human gastric cancer cell line; HO8910, human ovarian cancer line; the rest human tumor cell lines seen from Table 1.
[b] IC_{50} values greater than 200 μM were considered as inactive and omitted here.

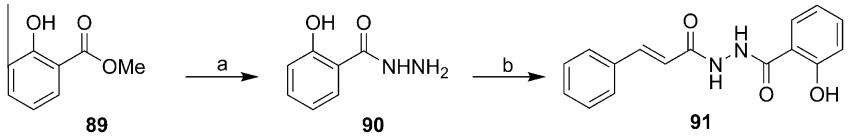
The aza-caffeic acid derivatives (**73**–**76**, **78**) with a seven bond linker B length were achieved by coupling dicyclohexylcarbodiimide (DCC) activated cinnamic acids (**69**–**72**) with commercially available corresponding substituted ethylamines in the yields of 46–86% (Scheme 2).[17] Target compounds **77** and **79** with two free hydroxy groups were obtained by hydrolyzation of the corresponding



Scheme 1. Synthesis of aza-caffeic acid derivatives **37**–**51** and **59**–**63**. Reagents and conditions: (a) for **5**: MOMCl, K_2CO_3, acetone, reflux, 4 h, 86%; for **6**–**7**: RBr, K_2CO_3, acetone, reflux, 3 h; (b) MOMCl, K_2CO_3, acetone, reflux, 4 h, 89%; (c) $Ph_3PCH{=}CO_2Et$, benzene, reflux, 2 h; (d) KOH, EtOH, H_2O, reflux, 3 h; (e) $ClCH_2COCl$, K_2CO_3, CH_2Cl_2, reflux, 4 h; (f) KI, DMF, reflux, 4 h; (g) HCl, MeOH, 50 °C, 1 h.



Scheme 2. Synthesis of aza-caffeic acid derivatives **73**–**84** and **88**. Reagents and conditions: (a) MOMCl, K_2CO_3, acetone, reflux, 4 h, 86%; (b) malonic acid, pyridine, reflux, 3 h; (c) K_2CO_3, piperazine, acetone, reflux, 2 h, 92%; (d) DCC, $CHCl_3$, 45 °C, 1 h; $RCH_2CH_2NH_2$, reflux, 5 h; (e) HCl, MeOH, 50 °C, 1 h.



Scheme 3. Synthesis of aza-caffeic acid derivative **91**. Reagents and conditions: (a) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 82%; (b) *trans*-cinnamic acid, $SOCl_2$, $CHCl_3$, 0–76 °C, 86%.

76 and **78**.[16] Compounds **69**–**72** were prepared by Knoevenagel condensation of malonic acid and the aldehyde group of corresponding intermediates (**64**–**66**, **68**) which were synthesized from protocatechualdehyde and syringaldehyde as described previously.[9,17] In order to introduce two nitrogens with a nine bond linker B length, piperazine, which was coupled with 3, and 4-dichlorobenzyl bromide (**85**), was utilized to construct **80**–**83**, followed by hydrolyzation of **83** to afford **84**.[9,16] 3,4-Dichlorobenzyl piperazine (**86**) was also used to form **88**, which has one more carbonyl as a prolonged conjugated system, by coupling with benzoylacrylic acid (**87**).[17]

In order to obtain significant insight into the impact of activity on molecular diversity, compound **91**, possessing two connected nitrogens in a seven bond linker length, was synthesized via a two step reaction starting from salicylic acid methyl ester (**89**, Scheme 3).[18] The structures of these compounds were confirmed both by mass spectrometry and ^{1}H NMR spectral data.

2.2. Biological activity tests

In order to perform the biological activity tests, caffeic phenyl ethyl ester (CAPE) and cisplatin (CDDP) were chosen as positive controls. Compounds **37**–**51**, **59**–**63**, and **73**–**84**, **88**, **91** were evaluated against 8 and 12 cancer cell lines, respectively, by the colorimeter assay MTT.[19–21] Results showing the concentrations required to inhibit cell growth by 50% (IC_{50} values) are presented in Tables 1 and 2.

Analysis of the MTT assay results, suggest that analogues of series 3 with a piperazine linker were generally more potent than those from series 1 and 2. The bioassay results also suggest that HL-60 and K562 cell lines and, in particular K562, are much more sensitive to the synthesized aza-caffeic acid derivatives. Four compounds (**39**, **42**, **80**, **91**) showed significant cytotoxicity, with IC_{50} values in the micromolar range against the K562 cell line, 10 and 50 times higher than the control CDDP and CAPE values, respectively.

In series 2, compounds **73**–**77** were found to have no cytotoxic activity towards all 12 cell lines tested, while compounds **78** and **79** were selectively cytotoxic against a number of cancer cell lines. These results suggest that series 2 compound cytotoxicity may be contained within the electron rich aromatic system of region C, as opposed to the electron poor heterocycle like morpholinyl. Comparing compounds **79**, **74**, and **78**, it indicates that various series 2 substituents of region A are crucial for activity: compound **79**, possessing two free hydroxy groups, showed selective and significant cytotoxicity, particularly with respect to HeLa and LS174T cell lines, with IC_{50} values of 2.7 and 8.4 μM, respectively.

The bioassay results of **81**–**84** demonstrated the crucial effect of double substituents of region A on activity as follows: **83** (OMOM) ⩾ **81** (H) > **82** (OMe) > **84** (OH), this trend is dissimilar to that observed for analogues of series 2 (**74**, **78**, and **79**). Compared to **81**, compound **88**, which possesses 10 linker B bonds including an additional conjugated carbonyl, lost cytotoxicity against PC-3, K562, and HO8910 cell lines and showed weaker activity to the remaining cell lines; this confirms that the most

favorable linker B length is nine bonds. Also of note is the finding that hydrazide **91**, possessing a seven bond linker B length, exhibited cytotoxicity against all 12 of the cancer cell lines, the best with an IC_{50} value of 0.9 μM being observed for the K562 cell. Among these aza-caffeic acid derivatives, compound **80** of series 3 was found to be the most potent and selective cytotoxic agent, showing IC_{50} values of 0.2, 2.0, 1.7, and 1.1 μM against KB, BEL7404, K562, and Eca109 cell lines, respectively, higher than those observed for the two control compounds CAPE and CDDP.

In order to determine the possible mechanism involved in the cytotoxicity, the K562 cell line treated with compound **80** was chosen for further biological analysis. The results show a concentration- and time-dependency for both the cell viability and apoptosis rate by MTT assay and flow cytometric analysis, respectively (Fig. 3A and B). With treatment of compound **80** at 8.0 μM for 72 h, the K562 cell viability was reduced to 37.9 ± 1.4% of the control (Fig. 3A) and 20.4% of the cells were detected to be apoptotic cells (Fig. 3B). The apoptotic cells in both early and late stage were detected by 48 h treatment of compound **80** while only apoptosis in late stage was observed at 72 h with double positive dyeing of Annexin V and PI. Simultaneously, the apoptotic protein caspase-3 expression level was also up-regulated detected by western blot after 48 h treatment with compound **80** (Fig. 3C). These results indicate that compound **80** suppresses K562 cell viability by, at least in part inducing cell apoptosis.

Bcl-2 and Bax are suggested to be key Bcl-2 family members in determining K562 cell survival.[22] The Bax/Bcl-2 ratio is an index suggesting cell apoptosis tendency. Bcl-2 family proteins are upstream regulators responsible for controlling mitochondria-dependent cell apoptosis. Both Bax and Bcl-2 protein expression was detected after 48 h incubation with the compounds. As results shown in Figure 3D, 48 h treatment with compound **80** dramatically increased the Bax/Bcl-2 ratio in a concentration dependent manner, indicating that mitochondria dysfunction was involved in compound **80** induced cell apoptosis.

3. Conclusions

Three series of 34 aza-caffeic acid derivatives, including compounds **88** and **91** were synthesized. Compounds **37**–**51**, **59**–**63** and **73**–**84**, **88**, **91** were used to screen 8 or 12 cultured human cancer cell lines, respectively. It was found that HL-60 and K562 cell lines, in particular K562, were especially sensitive to the synthesized compounds. Four compounds (**39**, **42**, **80**, and **91**) showed significant cytotoxicity, with IC_{50} values on K562 cell line in the micromolar range, 10 and 50 times better than the control compounds CDDP and CAPE, respectively. The aza-caffeic acid derivatives with nine bond linkers show better cytotoxicity to the cancer cell lines than the other series of compounds, which indicates that the linker with nine bonds might be the best linker length in aza-caffeic acid derivatives against the investigated cancer cell lines. Yet this observation still needs to be confirmed by testing N-containing caffeic acid derivatives with different linker lengths. The structure–cytotoxicity relationship of these analogues based on three structural variations of region A, B, and C was discussed. This extensive SAR analysis will guide us in the design of optimized aza-caffeic derivatives. Among these aza-caffeic acid derivatives compound **80** is the most potent and selective cytotoxic agent against KB, BEL7404, K562, and Eca109 cell lines with IC_{50} values of 0.2, 2.0, 1.7, and 1.1 μM, respectively, which is stronger than CAPE and CDDP. Based on a series of biological studies, compound **80** demonstrates a role in mitochondria-dependent

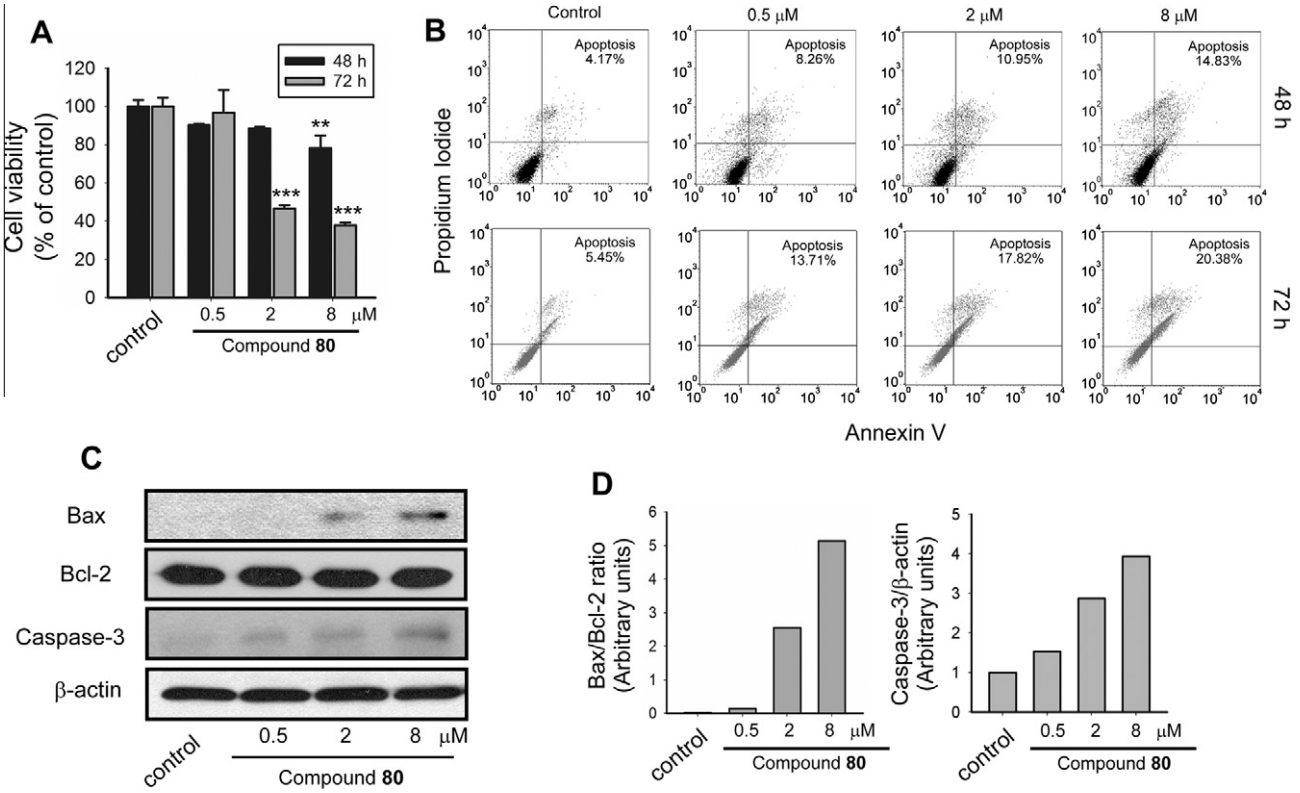


Figure 3. Apoptosis involved in compound **80** cytotoxicity on K562 cells. K562 cells were exposed to indicate concentrations of compound **80** for 48 or 72 h. (A) Cell viability was determined by MTT assay with 48 and 72 h compound **80** incubation, respectively. (B) Cell apoptosis rate following Annexin V/propidium iodide staining was quantified by flow cytometric analysis after 72 h compound **80** exposure. Cell apoptosis rate in each group was marked. (C) Western blot analysis was carried out to determine caspase-3, Bax, and Bcl-2 expression. The treatment duration was indicated. (D) Bands were semi-quantified by densitometric measurement. Data were expressed as mean ± S.D. from at least three independent experiments. $^{**}P$ <0.01, $^{***}P$ <0.001 versus control.

apoptosis activity by suppressing K562 cell proliferation in a concentration- and time-dependent manner. These results shed light on the possibility of developing compound **80** as a lead compound for a promising antitumor agent especially for leukemia.

4. Experimental section

4.1. Materials

Melting points were measured on a Perkin-Taike X-4 apparatus and then corrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian INOVA 400 spectrometer with TMS as an internal standard and $CDCl_3$ as solvent. ESI-MS data were recorded on a Bruker Esquire 3000+ spectrometer and EI-MS was performed on a Varian MAT-95 MS instrument. TLC was performed on Silica Gel (GF_{254}). Column chromatography was carried out on silica gel H (10–40 μm). All of the Silica Gel GF_{254} and Silica Gel H were purchased from Qingdao Marine Chemical Factory, China.

4.2. Synthesis

4.2.1. General procedure for the preparation of intermediates

4.2.1.1. General procedure for the preparation of substituted cinnamic acid potassium salts 12–14 and 56–58. A solution of KOH (1 M, 4.3 mL) was added to ethanol (20 mL) dissolved substituted cinnamic acid ethyl ester derivatives **9**–**11** and **53**–**55** (4.0 mmol), which were prepared as described previously using protocatechualdehyde and syringaldehyde as starting material.[9,17] The mixture was refluxed for 3 h and then cooled to room temperature. The solvent was removed and the concentrate was dried overnight by vacuum drying oven to obtain **12**–**14** and **56–58** as intermediates for the synthesis of **31**–**44** and **59**–**63**, respectively.

4.2.1.2. General procedure for the preparation of N-substituted-2-chloro-acetamides 23–30. K_2CO_3 (25 mmol) was added to a solution of substituted amines (10 mmol) in CH_2Cl_2 (60 mL) and the suspension was stirred for 30 min at room temperature. Chloroacetyl chloride (0.88 mL, 11 mmol) was slowly added to the mixture and was refluxed for 4 h after 10 min stirring. After cooling to room temperature the filtrate was concentrated to obtain the residue which was recrystallized in hexane to afford **23**–**30** (69–91%).

4.2.1.3. General procedure for the preparation of substituted cinnamic acids 69–72. Compounds **64**–**66** and **68** were synthesized as described previously.[9,17] Malonic acid (2.1 g, 20 mmol) and two drops of piperidine were added to corresponding aldehydes **64**–**66** and **68** (16 mmol) in pyridine (20 mL). The mixture was refluxed for 3–4 h and evaporated to remove pyridine. The residue was suspended in H_2O (30 mL) and extracted with EtOAc (2 × 50 mL) which was further purified by column chromatography (pet. ether/EtOAc/HCOOH 4:1:0.05 to 3:1:0.05) to afford **69**–**72** (71–92%).

4.2.2. General procedure for the preparation of N-substituted-3-phenyl-2-oxo-2-amino-2-propenoic acid ethyl ester derivatives 31–44, 59–63

KI (91 mg, 0.55 mmol) was added to N-substituted-2-chloroacetamides **23**–**30** (0.50 mmol) in dry DMF (10 mL), and the suspension stirred at 40 °C for 20 min. Dry potassium salts **12**–**14** and **56**–**58** (0.58 mmol) prepared as described in Section 4.2.1.1 was added to the suspension and refluxed for 4 h followed by evaporation under reduced pressure to remove DMF. The residue was extracted with EtOAc (2 × 20 mL) and the organic layer washed to neutral by water (2 × 15 mL) and brine (2 × 10 mL) followed by overnight drying over Na_2SO_4. The filtrate was concentrated and purified by column chromatography (pet. ether/EtOAc 7:1 to 2:1) to afford **31**–**44** and **59**–**63** (56–87%).

4.2.2.1. (2*E*)-3-(3,4-Dimethoxyphenyl)-2-oxo-2-(phenylamino)-2-propenoic acid ethyl ester (37). Yield: 76%; yellow powder; mp 165–166 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.42; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (1H, br s), 7.78 (1H, d, *J* = 16.0 Hz), 7.57 (2H, d, *J* = 8.0 Hz), 7.36 (3H, m), 7.17 (1H, d, *J* = 7.2 Hz), 7.10 (1H, s), 6.91 (1H, d, *J* = 8.0 Hz), 6.43 (1H, d, *J* = 15.6 Hz), 4.84 (2H, s), 3.95 (6H, s); ESI-MS *m/z* $[M+H]^+$ 342.

4.2.2.2. (2*E*)-3-(3,4-Dimethoxyphenyl)-2-oxo-2-(4-chlorophenylamino)-2-propenoic acid ethyl ester (38). Yield: 80%; yellow powder; mp 167–169 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.44; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (1H, br s), 7.78 (1H, d, *J* = 16.0 Hz), δ 7.53 (2H, d, *J* = 8.8 Hz), 7.32 (2H, d, *J* = 8.4 Hz), 7.18 (1H, d, *J* = 8.4 Hz), 7.09 (1H, s), 6.91 (1H, d, *J* = 8.4 Hz), 6.43 (1H, d, *J* = 16.0 Hz), 5.94 (2H, s), 3.95 (6H, s); ESI-MS *m/z* $[M+H]^+$ 376.

4.2.2.3. (2*E*)-3-(3,4-Dimethoxyphenyl)-2-oxo-2-[(2-furanylmethyl)amino]-2-propenoic acid ethyl ester (39). Yield: 79%; yellow powder; mp 145–146 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.40; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (1H, d, *J* = 16.0 Hz), 7.50 (1H, d, *J* = 0.8 Hz), 7.14 (1H, d, *J* = 8.4 Hz), 7.08 (1H, s), 6.91 (1H, d, *J* = 8.4 Hz), 6.52 (1H, br s), 6.40 (1H, d, *J* = 16.0 Hz), 6.33 (1H, dd, *J* = 2.8, 0.8 Hz), 6.28 (1H, dd, *J* = 2.8, 1.8 Hz), 5.94 (2H, s), 4.53 (2H, d, *J* = 6.0 Hz), 3.95 (6H, s); ESI-MS *m/z* $[M+H]^+$ 346.

4.2.2.4. (2*E*)-3-(3-Methoxymethoxy-4-phenylmethoxy)-2-oxo-2-[(4-chlorophenyl)amino]-2-propenoic acid ethyl ester (40). Yield: 87%; white powder; mp 132–133 °C; R_f (pet. ether/EtOAc 3:1) 0.22; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (1H, br s), 7.74 (1H, d, *J* = 16.0 Hz), 7.53 (2H, d), 7.44–7.31 (8H, m), 7.15 (1H, d, *J* = 8.0 Hz), 6.93 (1H, d, *J* = 8.8 Hz), 6.41 (1H, d, *J* = 15.6 Hz), 5.28 (2H, s), 5.22 (2H, s), 4.82 (2H, s), 3.55 (3H, s); ESI-MS *m/z* $[M+H]^+$ 482.

4.2.2.5. (2*E*)-3-(3-Methoxymethoxy-4-phenylmethoxy)-2-oxo-2-[(4-morpholinyl)amino]-2-propenoic acid ethyl ester (41). Yield: 61%; white powder; mp 117–119 °C; R_f (pet. ether/EtOAc 3:1) 0.26; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (1H, br s), 7.64 (1H, d, *J* = 16.0 Hz), 7.49 (2H, d, *J* = 6.8 Hz), 7.43–7.35 (3H, m), 7.39 (1H, s), 7.25 (1H, d, *J* = 8.4 Hz), 7.10 (1H, d, *J* = 8.8 Hz), 6.40 (1H, d, *J* = 16.0 Hz), 5.26 (2H, s), 5.20 (2H, s), 4.04 (2H, s), 3.81 (4H, m), 3.46 (3H, s), 2.85 (4H, m); ESI-MS *m/z* $[M+H]^+$ 457.

4.2.2.6. (2*E*)-3-(3-Methoxymethoxy-4-phenylmethoxy)-2-oxo-2-[(2-furanylmethyl)amino]-2-propenoic acid ethyl ester (42). Yield: 60%; white powder; mp 120–122 °C; R_f (pet. ether/EtOAc 3:1) 0.23; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67 (1H, d, *J* = 15.6 Hz), 7.44–7.31 (7H, m), 7.13 (1H, d, *J* = 8.4 Hz), 6.92 (1H, d, *J* = 8.0 Hz), 6.52 (1H, br s), 6.35 (1H, d, *J* = 16.0 Hz), 6.33 (1H, d, *J* = 2.0 Hz), 6.28 (1H, d, *J* = 2.8 Hz), 5.27 (2H, s), 5.21 (2H, s), 4.72 (2H, s), 4.53 (2H, d, *J* = 6.0 Hz), 3.54 (3H, s); ESI-MS *m/z* $[M+H]^+$ 452.

4.2.2.7. (2*E*)-3-(3-Methoxymethoxy-4-phenylmethoxy)-2-oxo-2-(2-thiazolylamino)-2-propen-oic acid ethyl ester (43). Yield: 63%; white powder; mp 133–134 °C; R_f (pet. ether/EtOAc 3:1) 0.26; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (1H, d, *J* = 16.0 Hz), 7.48–7.31 (7H, m), 7.15 (1H, d, *J* = 8.4 Hz), 7.04 (1H, s), 6.90 (1H, d, *J* = 8.4 Hz), 6.45 (1H, d, *J* = 15.6 Hz), 5.25 (2H, s), 5.18 (2H, s), 4.95 (2H, s), 3.52 (3H, s); ESI-MS *m/z* $[M+H]^+$ 455.

4.2.2.8. (2*E*)-3-(3-Methoxymethoxy-4-phenylmethoxy)-2-oxo-2-[(4-methoxyphenyl)amino]-2-propenoic acid ethyl ester (44). Yield: 80%; white powder; mp 129–130 °C; R_f (pet. ether/EtOAc 3:1) 0.24; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.18 (1H, br s), 7.67

(1H, d, J = 16.4 Hz), 7.56–7.30 (9H, m), 7.14 (1H, d, J = 8.0 Hz), 6.86 (2H, d, J = 8.8 Hz), 6.49 (1H, d, J = 16.0 Hz), 5.25 (2H, s), 5.21 (2H, s), 4.74 (2H, s), 3.74 (3H, s), 3.46 (3H, s); ESI-MS m/z $[M+H]^+$ 478.

4.2.2.9. (2*E*)-3-(3-Hydroxy-4-phenylmethoxy)-2-oxo-2-[(4-methoxyphenyl)amino]-2-propen-oic acid ethyl ester (45). Hydrochloric acid (1 M, 0.3 mL) was added to **44** (124 mg, 0.26 mmol) in methanol (5 mL) and stirred at 55 °C for 30 min. After cooling to room temperature it was concentrated and the residue purified by column chromatography with CH_2Cl_2/MeOH (70:1) as eluting system to get **45** (82 mg). Yield: 73%; yellow powder; mp 173–174 °C; R_f (pet. ether/EtOAc 4:3) 0.36; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.12 (1H, br s), 8.08 (1H, br s), 7.64 (1H, d, J = 15.6 Hz), 7.55 (2H, d, J = 8.8 Hz), 7.47 (2H, d, J = 8.8 Hz), 7.40–7.31 (3H, m), 7.22 (1H, s), 7.14–7.07 (2H, m), 6.85 (2H, d, J = 9.2 Hz), 6.45 (1H, d, J = 15.6 Hz), 5.21 (2H, s), 4.73 (2H, s), 3.74 (3H, s); ESI-MS m/z $[M+H]^+$ 434.

4.2.2.10. (2*E*)-3-(3,4-Dihydroxy)-2-oxo-2-[(4-morpholinyl)amino]-2-propenoic acid ethyl ester (46). HCl (1 M, 0.6 mL) was added to **31** (102 mg, 0.25 mmol) in methanol (5 mL) and stirred at 55 °C for 30 min followed by evaporation to remove methanol. The residue was purified by column chromatography (CH_2Cl_2/MeOH 55:1) to get **46** (45 mg). Yield: 56%; white powder; mp 145–146 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.33; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67 (1H, d, J = 16.0 Hz), 7.11 (1H, s), 7.06 (1H, d, J = 8.4 Hz), 6.83 (1H, d, J = 8.4 Hz), 6.39 (1H, d, J = 16.4 Hz), 5.05 (1H, br s), 4.64 (2H, s), 3.80 (4H, m), 2.86 (4H, m); ESI-MS m/z $[M+H]^+$ 323.

4.2.2.11. (2*E*)-3-(3,4-Dihydroxy)-2-oxo-2-(4-phenylamino)-2-propenoic acid ethyl ester (47). This compound was prepared in the same way as **46** from **32**. Yield: 74%; yellow powder; mp 165–166 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.42; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.29 (1H, br s), 8.60 (1H, br s), 8.31 (1H, br s), 7.65 (2H, d, J = 7.2 Hz), 7.31–7.27 (2H, m), 7.15 (1H, s), 7.08–7.04 (3H, m), 6.87 (1H, d, J = 7.2 Hz), 6.39 (1H, d, J = 16.0 Hz), 4.76 (2H, s); ESI-MS m/z $[M+H]^+$ 314.

4.2.2.12. (2*E*)-3-(3,4-Dihydroxy)-2-oxo-2-[(4-methylphenyl)-amino]-2-propenoic acid ethyl ester (48). This compound was prepared in the same way as **46** from **33**. Yield: 75%; yellow powder; mp 202–203 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.44; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.11 (1H, br s), 8.51 (1H, br s, OH-7), 8.23 (1H, br s), 7.52 (1H, d, J = 16.0 Hz), 7.46 (2H, d, J = 7.6 Hz), 7.11 (1H, s), 7.04–6.99 (3H, m), 6.80 (1H, d, J = 7.6 Hz), 6.27 (1H, d, J = 15.6 Hz), 4.67 (2H, s), 2.18 (3H, s); ESI-MS m/z $[M+H]^+$ 329.

4.2.2.13. (2*E*)-3-(3,4-Dihydroxy)-2-oxo-2-[(4-chlorophenyl)amino]-2-propenoic acid ethyl ester (49). This compound was prepared in the same way as **46** from **34**. Yield: 78%; yellow powder; mp 167–169 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.44; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 7.67 (2H, d, J = 8.8 Hz), 7.52 (1H, d, J = 16.0 Hz), 7.25 (2H, d, J = 8.4 Hz), 7.08 (1H, s), 7.00 (1H, d, J = 8.0 Hz), 6.80 (1H, d, J = 8.4 Hz), 6.31 (1H, d, J = 15.6 Hz), 4.69 (2H, s); ESI-MS m/z $[M+H]^+$ 348.

4.2.2.14. (2*E*)-3-(3,4-Dihydroxy)-2-oxo-2-[(4-methoxyphenyl)-amino]-2-propenoic acid ethyl ester (50). This compound was prepared in the same way as **46** from **35**. Yield: 70%; yellow powder; mp 202–203 °C; R_f (CH_2Cl_2/MeOH 20:1) 0.36; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.13 (1H, br s), 8.59 (1H, br s), 8.34 (1H, br s), 7.61 (1H, d, J = 16.0 Hz), 7.55 (2H, d, J = 8.8 Hz), 7.17 (1H, s), 7.06 (1H, d, J = 8.4 Hz), 6.78 (3H, m), 6.34 (1H, d, J = 16.0 Hz), 4.72 (2H, s), 3.74 (3H, s); ESI-MS m/z $[M+H]^+$ 344.

4.2.2.15. (2*E*)-3-(3,4-Dihydroxy)-2-oxo-2-(4-pentylamino)-2-propenoic acid ethyl ester (51). This compound was prepared in the same way as **46** from **36**. Yield: 71%; white powder; mp 168–169 °C; R_f (CH_2CH_2/MeOH 20:1) 0.46; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 8.61 (1H, br s), 8.37 (1H, br s), 7.58 (1H, d, J = 16.0 Hz), 7.43 (1H, br s), 7.14 (1H, s), 7.03 (1H, d, J = 6.8 Hz), 6.86 (1H, d, J = 8.4 Hz), 4.58 (2H, s), 3.21 (2H, m), 1.47 (2H, m), 1.28 (4H, m), 0.87 (3H, t, J = 6.8 Hz); ESI-MS m/z $[M+H]^+$ 308.

4.2.2.16. (2*E*)-3-[3,5-Dimethoxy-4-(3,4-dichlorophenylmethoxy)]-2-oxo-2-[(4-methoxy-phenyl)amino]-2-propenoic acid ethyl ester (59). Yield: 72%; white powder; mp 151–153 °C; R_f (hexane/EtoAc 3:2) 0.36; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.20 (1H, br s), 7.77 (1H, d, J = 1.6 Hz), 7.70 (1H, d, J = 16.0 Hz), 7.58–7.56 (3H, m, J = 8.8 Hz), 7.48 (1H, dd, J = 8.0, 1.6 Hz), 7.10 (2H, s), 6.88 (2H, d, J = 8.4 Hz), 6.40 (1H, d, J = 16.0 Hz), 5.06 (2H, s), 4.78 (2H, s), 3.92 (6H, s), 3.76 (3H, s); ESI-MS m/z $[M+H]^+$ 546.

4.2.2.17. (2*E*)-3-[3,5-Dimethoxy-4-(3,4-dichlorophenylmethoxy)]-2-oxo-2-[(4-cholorphenyl)-amino]-2-propenoic acid ethyl ester (60). Yield: 70%; yellow powder; mp 164–166 °C; R_f (hexane/EtoAc 5:2) 0.30; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.44 (1H, br s), 7.77 (1H, d, J = 2.0 Hz), 7.70 (2H, d, J = 8.8 Hz), 7.68 (1H, d, J = 16.0 Hz), 7.56 (1H, d, J = 8.0 Hz), 7.47 (1H, d, J = 8.0 Hz), 7.34 (2H, m), 7.10 (2H, s), 6.63 (1H, d, J = 16.0 Hz), 5.06 (2H, s), 4.81 (2H, s), 3.92 (6H, s); ESI-MS m/z $[M+H]^+$ 550.

4.2.2.18. (2*E*)-3-[3,5-Dimethoxy-4-(4-ethoxyphenylmethoxy)]-2-oxo-2-[(4-cholorphenyl)-amino]-2-propenoic acid ethyl ester (61). Yield: 67%; white powder; mp 180–182 °C; R_f (hexane/EtoAc 5:2) 0.21; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.49 (1H, br s), 7.70 (2H, d, J = 8.8 Hz), 7.69 (1H, d, J = 16.0 Hz), 7.40 (2H, d, J = 8.0 Hz), 7.34 (2H, m), 7.07 (2H, s), 6.88 (2H, d, J = 7.6 Hz), 6.61 (1H, d, J = 16.0 Hz), 4.96 (2H, s), 4.80 (2H, s), 4.04 (2H, q, J = 7.2 Hz), 3.90 (6H, s), 1.35 (3H, t, J = 7.2 Hz); ESI-MS m/z $[M+H]^+$ 526.

4.2.2.19. (2*E*)-3-[3,5-Dimethoxy-4-(4-ethoxyphenylmethoxy)]-2-oxo-2-(phenylamino)-2-propenoic acid ethyl ester (62). Yield: 70%; white powder; mp 133–134 °C; R_f (hexane/EtoAc 3:2) 0.40; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.33 (1H, br s), 7.70 (1H, d, J = 16.0 Hz), 7.67 (2H, m), 7.40 (2H, d, J = 8.0 Hz), 7.32 (2H, dd, J = 7.6, 7.6 Hz), 7.09 (1H, m), 7.08 (2H, s), 6.88 (2H, d, J = 7.6 Hz), 6.63 (1H, d, J = 16.0 Hz), 4.95 (2H, s), 4.80 (2H, s), 4.04 (2H, q, J = 6.8 Hz), 3.90 (6H, s), 1.35 (3H, t, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.6, 166.3, 159.7, 154.8, 154.0 146.6, 140.1, 139.4, 130.8, 130.6, 129.5, 124.6, 120.4, 117.2, 114.7, 106.6, 74.8, 63.8, 63.5, 56.5, 15.0; ESI-MS m/z $[M+H]^+$ 492.

4.2.2.20. (2*E*)-3-[3,5-Dimethoxy-4-(phenylmethoxy)]-2-oxo-2-[(4-methoxyphenyl)amino]-2-propenoic acid ethyl ester (63). Yield: 68%; white powder; mp 155–156 °C; R_f (hexane/EtoAc 3:2) 0.33; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 9.20 (1H, br s), 7.70 (1H, d, J = 16.0 Hz), 7.57 (2H, d, J = 8.8 Hz), 7.52 (2H, d, J = 7.6 Hz), 7.36 (2H, dd, J = 7.2, 7.2 Hz), 7.30 (1H, dd, J = 7.2, 7.2 Hz), 7.09 (2H, s), 6.88 (2H, d, J = 8.8 Hz), 6.63 (1H, d, J = 16.0 Hz), 5.03 (2H, s), 4.78 (2H, s), 3.91 (6H, s), 3.76 (3H, s); ESI-MS m/z $[M+H]^+$ 478.

4.2.3. General procedure for the preparation of N-substituted-3-phenyl-2-propenamide derivatives 73–76, 78 and 4-(3,4-dichlorophenylmethyl)-1-(phenyl-1-oxo-2-propenyl)-piperazine derivatives 80–83

DCC (512 mg, 2.0 mmol) was added to substituted cinnamic acid derivatives **69–72** (2.0 mmol) in $CHCl_3$ (15 mL) and was stirred at 45 °C for 1 h. Substituted amines (2.2 mmol) was added and the reaction mixture refluxed for 8 h. After cooling to room temperature, it was filtrated and concentrated to obtain a residue

which was further purified by column chromatography using CH_2Cl_2/MeOH (100:1 to 30:1) as the eluting system, to afford **73–76**, **78** and **80–83** (46–86%).

4.2.3.1. (2*E*)-3-(3,4-Dimethoxyphenyl)-*N*-[2-(4-morpholinyl)-ethyl]-2-propenamide (73). Yield: 67%; white powder; mp 173–174 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.49; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (1H, d, J = 15.6 Hz), 7.11 (1H, dd, J = 8.4, 1.6 Hz), 7.05 (1H, d, J = 1.6 Hz), 6.87 (1H, d, J = 8.4 Hz), 6.31 (1H, d, J = 15.6 Hz), 6.15 (1H, br s), 3.93–3.92 (6H, s), 3.75 (4H, t, J = 4.4 Hz), 3.51 (2H, q, J = 5.6 Hz), 2.56 (2H, t, J = 6.0 Hz), 2.49 (4H, m); ESI-MS m/z $[M+H]^+$ 321.

4.2.3.2. (2*E*)-3-(3,4-Dimethoxyphenyl)-*N*-[2-(1*H*-indol-3-yl)ethyl]-2-propenamide (74). Yield: 54%; yellow powder; mp 150–151 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.66; This compound was identical to that reported by Michalet et al.[12]

4.2.3.3. (2*E*)-3-[3,5-Dimethoxy-4-(4-ethoxyphenylmethoxy)]-*N*-[2-(4-morpholinyl)ethyl]-2-propenamide (75). Yield: 59%; white powder; mp 162–164 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.45; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (1H, d, J = 15.6 Hz), 7.37 (2H, d, J = 8.8 Hz), 6.85 (2H, d, J = 8.4 Hz), 6.72 (2H, s), 6.33 (1H, d, J = 15.6 Hz), 6.17 (1H, br s), 4.97 (2H, s), 4.02 (2H, q, J = 6.8 Hz), 3.85 (6H, s), 3.74 (4H, t, J = 4.4 Hz), 3.50 (2H, q, J = 6.0 Hz), 2.56 (2H, t, J = 6.0 Hz), 2.49 (4H, m), 1.41 (3H, t, J = 6.8 Hz); ESI-MS m/z $[M+H]^+$ 471.

4.2.3.4. (2*E*)-3-(3,4-Dimethoxymethoxy)-*N*-[2-(4-morpholinyl)-ethyl]-2-propenamide (76). Yield: 58%; white powder; mp 155–157 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.45; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (1H, d, J = 15.6 Hz), 7.38 (1H, d, J = 1.6 Hz), 7.16 (1H, d, J = 8.4 Hz),7.12 (1H, dd, J = 8.4, 1.6 Hz), 6.31 (1H, d, J = 15.6 Hz), 6.16 (1H, br s), 5.29–5.27 (2H, s), 3.76 (4H, t, J = 4.4 Hz), 3.55–3.52 (6H, s), 3.49 (2H, q, J = 6.0 Hz), 2.55 (2H, t, J = 6.0 Hz), 2.49 (4H, d, J = 4.4 Hz); ESI-MS m/z $[M+H]^+$ 381.

4.2.3.5. (2*E*)-3-(3,4-Dihydroxy)-*N*-[2-(4-morpholinyl)ethyl]-2-propenamide (77). This compound was prepared in the same way as **42** from **73**; yield: 42%; white powder; mp 101–103 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.31; ^{1}H NMR (400 MHz, CD_3OD): δ 8.42 (2H, br s), 7.96 (1H, s), 7.45 (1H, d, J = 15.2 Hz), 7.05 (1H, s), 6.95 (1H, d, J = 6.0 Hz), 6.81 (1H, d, J = 8.4 Hz), 6.42 (1H, d, J = 15.6 Hz), 3.83 (4H, m), 3.58 (2H, m), 2.86 (6H, m); ESI-MS m/z $[M+H]^+$ 293.

4.2.3.6. (2*E*)-3-(3,4-Dimethoxymethoxy)-*N* -[2-(1*H*-indol-3-yl)-ethyl]-2-propenamide (78). Yield: 48%; yellow oil; R_f (CH_2Cl_2/MeOH 12:1) 0.66; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (1H, br s), 7.65 (1H, d, J = 8.0 Hz), 7.53 (1H, d, J = 15.6 Hz), 7.41 (1H, d, J = 8.0 Hz), 7.32 (1H, s), 7.22 (1H, d, J = 7.2 Hz), 7.15–7.10 (3H, m), 7.09 (1H, br s), 6.18 (1H, d, J = 15.6 Hz), 5.37 (1H, br s), 5.26 (4H, s), 3.76 (2H, m), 3.52 (6H, s), 3.06 (2H, m); ESI-MS m/z $[M+H]^+$ 411.

4.2.3.7. (2*E*)-3-(3,4-Dihydroxy)-*N* -[2-(1*H*-indol-3-yl)ethyl]-2-propenamide (79). This compound was prepared in the same way as **42** from **75**. Yield: 54%; this compound was identical to that reported by Park and Chen.[23]

4.2.3.8. (*E*)-4-(3,4-Dichlorophenylmethyl)-1-[3-(3,5-dimethoxy-4-(4-ethoxyphenyl methoxy)-phenyl-1-oxo-2-propenyl]-piperazine (80). Yield: 57%; pale yellow oil; R_f (CH_2Cl_2/MeOH 12:1) 0.57; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (1H, d, J = 15.2 Hz3), 7.46 (1H, s), 7.40 (1H, d, J = 8.0 Hz), 7.37 (2H, d, J = 8.4 Hz), 7.18 (1H, d, J = 8.4 Hz), 6.85 (2H, d, J = 8.4 Hz), 6.74 (1H, d, J = 15.2 Hz), 6.71 (2H, s), 4.97 (2H, s), 4.03 (2H, q, J = 7.2 Hz), 3.85 (6H, s), 3.76–3.67 (4H, m), 3.49 (2H, s), 2.48 (4H, t, J = 4.4 Hz), 1.41 (3H, t, J = 7.2 Hz); ESI-MS m/z $[M+H]^+$ 585.

4.2.3.9. (*E*)-4-(3,4-Dichlorophenylmethyl)-1-[1-oxo-3-phenyl-2-propenyl]-piperazine (81). Yield: 67%; white powder; mp 114–116 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.58; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68 (1H, d, J = 15.6 Hz), 7.53 (2H, dd, J = 8.0, 2.0 Hz), 7.46 (1H, s), 7.40 (1H, d, J = 8.0 Hz), 7.38–7.36 (3H, m), 7.18 (1H, d, J = 8.4 Hz), 6.87 (1H, d, J = 15.6 Hz), 3.76–3.66 (4H, m), 3.48 (2H, s), 2.47 (4H, t, J = 4.4 Hz); ESI-MS m/z $[M+H]^+$ 375.

4.2.3.10.(*E*)-4-(3,4-Dichlorophenylmethyl)-1-[3-(3,4-dimethoxy-phenyl)-1-oxo-2-propenyl]-piperazine (82). Yield: 56%; pale yellow oil; R_f (CH_2Cl_2/MeOH 12:1) 0.58; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63 (1H, d, J = 15.2 Hz), 7.46 (1H, s), 7.40 (1H, d, J = 8.0 Hz), 7.18 (1H, d, J = 8.0 Hz), 7.11 (1H, dd, J = 8.4, 1.6 Hz), 7.03 (1H, s), 6.86 (1H, d, J = 8.4 Hz), 6.73 (1H, d, J = 15.2 Hz), 3.92–3.91 (6H, s), 3.76–3.67 (4H, m), 3.48 (2H, s), 2.47 (4H, m); ESI-MS m/z $[M+H]^+$ 435.

4.2.3.11. (*E*)-4-(3,4-Dichlorophenyl-methyl)-1-[3-(3,4-dimethoxymethoxyphenyl)-1-oxo-2-propenyl]-piperazine (83). Yield: 51%; pale yellow oil; R_f (CH_2Cl_2/MeOH 12:1) 0.57; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60 (1H, d, J = 15.6 Hz), 7.46 (1H, d, J = 1.2 Hz), 7.40 (1H, d, J = 8.4 Hz), 7.34 (1H, d, J = 1.2 Hz), 7.18 (1H, dd, J = 8.0, 2.0 Hz), 7.15 (1H, dd, J = 8.0, 2.4 Hz), 7.12 (1H, d, J = 8.4 Hz), 6.73 (1H, d, J = 15.2 Hz), 5.27 (4H, s), 3.75–3.66 (4H, m), 3.52 (6H, s), 3.49 (2H, s), 2.46 (4H, m); ESI-MS m/z $[M+H]^+$ 495.

4.2.3.12. (*E*)-4-(3,4-Dichlorophenylmethyl)-1-[3-(3,4-dihydroxy-phenyl)-1-oxo-2-propenyl]-piperazine (84). This compound was prepared in the same way as **42** from **80**; yield: 54%; white powder; mp 197–199 °C; R_f (CH_2Cl_2/MeOH 12:1) 0.39; ^{1}H NMR (400 MHz, CD_3OD): δ 7.81 (1H, br s), 7.73 (1H, d, J = 8.4 Hz), 7.56 (1H, d, J = 15.2 Hz), 7.52 (1H, d, J = 8.4 Hz), 7.11 (1H, s), 7.04 (1H, d, J = 8.0 Hz), 6.94 (1H, d, J = 15.2 Hz), 6.82 (1H, d, J = 8.0 Hz), 4.38 (4H, m), 3.48 (2H, s), 3.47 (4H, m); ESI-MS m/z $[M+H]^+$ 407.

4.2.3.13. (*E*)-1-[3-Butenyl-1,4-dioxo-4-phenyl]-4-(3,4-dichlorophenylmethyl)-piperazine (88). Yield: 46%; pale yellow oil; R_f (hexane/EtOAc 3:1) 0.38; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04 (2H, d, J = 8.0 Hz), 7.90 (1H, dd, J = 15.6 Hz), 7.63 (1H, dd, J = 7.6, 7.6 Hz), 7.51 (1H, s), 7.50 (1H, d, J = 15.6 Hz), 7.48 (2H, dd, J = 8.0, 7.6 Hz), 7.40 (1H, d, J = 8.4 Hz), 7.17 (1H, d, J = 8.4 Hz), 3.76–3.65 (4H, m), 3.48 (2H, s), 2.48 (4H, br s); ESI-MS m/z $[M+H]^+$ 403.

4.2.3.14. (*E*)-2-[1-Oxo-3-phenyl-2-propenyl]-2-hydroxy-benzoic acid hydrazide (91). Sulfoxide chloride (0.24 mL, 3.4 mmol) was added to *trans*-cinnamic acid (503 mg, 3.4 mmol) in chloroform (15 mL) containing triethylamine (0.49 mL, 3.4 mmol) at 0 °C over a 5 min period with stirring. The solution was refluxed for 2 h and slowly cooled to ambient temperature. Upon addition of salicylhydrazide (380 mg, 2.5 mmol) (prepared from salicylic acid methyl ester and $NH_2NH_2{\cdot}H_2O$) a white suspension immediately appeared.[24] After additional stirring for 30 min, the white precipitate obtained was filtered and washed with hexane (2 × 20 mL) and water (2 × 25 mL), followed by overnight drying in vacuum oven to get **91** (819 mg). Yield: 86%; this compound was identical to that reported by Moon et al.[18]

4.3. Cell culture

Various human cancer cell lines were cultured in minimum essential medium (MEM), supplemented with 10% fetal calf serum (Gibco Laboratories, Grand island, NY), 100 units/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere in 5% CO_2 at 37 °C. Cell culture media were renewed every 3 days, up to the confluence of the monolayer. Cell culture was passaged upon formation of confluent cultures, using trypsin–EDTA to detach the

cells from their culture flasks or dishes. Test compounds were stored at −70 °C and solubilized in 100% DMSO.

4.4. MTT assay for cell viability

Exponentially growing cells were seeded in quadruplicate into 96-well flat-bottomed plates at a concentration of 5×10^3 cells per well. After 24 h incubation, the compounds studied were added to the wells. After 72 h, 10 μL of MTT solution (5 mg/mL in phosphate buffered solution) was added to the culture medium and incubated at 37 °C for a further 4 h. After removing unconverted MTT, 200 μL of DMSO was added to each well and the plates shaken to dissolve the reduced MTT crystals (formazan); the optical density was measured on a microplate reader at a wavelength of 570 nm. The average 50% inhibitory concentration (IC_{50}) was determined graphically from the dose–response curves.

4.5. Detection of apoptosis by flow cytometric analysis

To determine the cell apoptosis, fluorescent probes Annexin V/propidium iodide (PI, BioVision, CA, USA) were used. After 48 and 72 h treatment with indicated concentrations of compound **80**, K562 cells were collected by centrifuging. Cell pellets were resuspended in pre-warmed D-Hank's buffer. Cell suspension was stained by Annexin V/PI staining kit following manufacturer's instructions. Flow cytometry was performed on FACScan (BD Biosciences, San Jose, CA, USA).

4.6. Western blot

For Bax/Bcl-2 and caspase-3 detection, K562 cells were treated with indicated concentrations of compound **80** for 48 h. Cells were then collected by centrifugation. Cell pellets were lysed in a lysis buffer (50 mmol/L Tris–HCl, pH 7.5; 150 mmol/L NaCl; 20 mmol/L EGTA; 1% Triton X-100; 0.5% sodium deoxycholate; 1 mmol/L DTT; 20 mmol/L NaF; 1 mmol/L sodium vanadate; 1 mmol/L PMSF; 10 μg/mL leupeptin; 30 μg/mL aprotinin). Protein concentration was detected by Lowry's assay (Bio-Rad).

SDS–PAGE and Western blot were performed by standard protocols. Briefly, 40 μg of total protein was separated by SDS–PAGE followed by protein transfer to a PVDF membrane and blocked in 5% fat-free milk in Tris-buffered saline (TBS). Primary antibodies were added in TBS with 5% fat-free milk, respectively. Anti-caspase-3 (1:1000; Cell Signaling Technology, Inc. Danvers, MA, USA), anti-Bax, anti-Bcl-2, (1:800; Millipore, MA, USA) anti-β-actin, (1:1000; Santa Cruz Biotechnology, CA, USA), were added and incubated over night at 4 °C. The blots were rinsed and incubated with the secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase conjugated; Santa Cruz Biotechnology) in TBS with 5% fat-free milk (1:5000). Then the blots were exposed to a chemiluminescent detection system using the Super Signal West Pico Substrate (Pierce, Rockford, IL, USA) and exposed to film. Digital images were quantified using densitometric measurement by Quantity-One software. β-actin was detected as housekeeping protein.

4.7. Statistical analysis

All data were expressed as mean ± S.D. from at least three independent experiments. Differences between groups were examined for statistical significance using one-way ANOVA analysis with SPSS 10.0 for WINDOWS. In all cases, $P < 0.05$ was considered significant.

Acknowledgments

This study was supported by the National Natural Sciences Foundation of China (No. 20802066), the China Postdoctoral Science Foundation (No. 20080441268), National Science & Technology Major Project "Key New Drug Creation and Manufacturing Program" of China (No. 2009ZX09501-010) and the Department of Education of Zhejiang Province Foundation (No. Y200805312). We thank Dr. Jiali Luo and Mr. Xiaoren Wang at the R&D center of Zhejiang Hisun group for measuring of the mass data.

References and notes

1. Papay, V.; Toth, L.; Soltesz, M.; Nagy, E.; Litkei, G. *Stud. Org. Chem.* **1986**, *23*, 233.
2. Kishimoto, N.; Kakino, Y.; Iwai, K.; Mochida, K.; Fujita, T. *Biocontrol Sci.* **2005**, *10*, 155.
3. Grunberger, D.; Banerjee, R.; Eisinger, K.; Oltz, E. M.; Efros, L.; Caldwell, M.; Estevez, V.; Nakanishi, K. *Experientia* **1988**, *44*, 230.
4. Fache, F.; Suzan, N.; Piva, O. *Tetrahedron* **2005**, *61*, 5261.
5. Nagaoka, T.; Banskota, A. H.; Tezuka, Y.; Saiki, I.; Kadota, S. *Bioorg. Med. Chem.* **2002**, *10*, 3351.
6. Wang, X.; Stavchansky, S.; Bowman, P. D.; Kerwin, S. M. *Bioorg. Med. Chem.* **2006**, *14*, 4879.
7. Lin, L.; Shi, Q.; Nyarko, A. K.; Bastow, K. F.; Wu, C.; Su, C.; Shih, C. C.; Lee, K. *J. Med. Chem.* **2006**, *49*, 3963.
8. Ahn, B.; Sok, S. *Curr. Pharm. Des.* **1996**, *2*, 247.
9. Zou, H. B.; Dong, S. Y.; Zhou, C. X.; Hu, L. H.; Wu, Y. H.; Li, H. B.; Gong, J. X.; Sun, L. L.; Wu, X. M.; Bai, H.; Fan, B. T.; Hao, X. J.; Stöckigt, J.; Zhao, Y. *Bioorg. Med. Chem.* **2006**, *14*, 2060.
10. Rajan, P.; Vedernikova, I.; Cos, P.; Berghe, D. V.; Augustyns, K.; Haemers, A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 215.
11. Okombi, S.; Rival, D.; Bonnet, S.; Mariotte, A.; Perrier, E.; Boumendjel, A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2252.
12. Michalet, S.; Cartier, G.; David, B.; Mariotte, A.; Dijoux-franca, M.; Kaatz, G. W.; Stavri, M.; Gibbons, S. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1755.
13. Nesterenko, V.; Putt, K. S.; Hergenrother, P. J. *J. Am. Chem. Soc.* **2003**, *125*, 14672.
14. Clark, J. H.; Miller, J. M. *Tetrahedron Lett.* **1977**, *7*, 599.
15. Baraldi, P. G.; Preti, D.; Tabrizi, M. A.; Fruttarolo, F.; Saponaro, G.; Baraldi, S.; Romagnoli, R.; Moorman, A. R.; Gessi, S.; Varani, K.; Borea, P. A. *Bioorg. Med. Chem.* **2007**, *15*, 2514.
16. Roelens, F.; Heldring, N.; Dhooge, W.; Bengtsson, M.; Comhaire, F.; Gustafsson, J.; Treuter, E.; De Keukeleire, D. *J. Med. Chem.* **2006**, *49*, 7357.
17. Hu, L. H.; Zou, H. B.; Gong, J. X.; Li, H. B.; Yang, L. X.; Cheng, W.; Zhou, C. X.; Bai, H.; Guéritte, F.; Zhao, Y. *J. Nat. Prod.* **2005**, *68*, 342.
18. Moon, D.; Lee, K.; John, R. P.; Kim, G. H.; Suh, B. J.; Lah, M. S. *Inorg. Chem.* **2006**, *45*, 7991.
19. Horowitz, D.; King, A. G. *J. Immunol. Methods* **2000**, *244*, 49.
20. Putnam, K. P.; Bombick, D. W.; Doolittle, D. *J. Toxicol. In vitro* **2002**, *16*, 599.
21. Russell, C. A.; Vindelov, L. L. *J. Immunol. Methods* **1998**, *217*, 165.
22. Chen, Y. J.; Liu, W. H.; Kao, P. H.; Wang, J. J.; Chang, L. S. *Toxicon* **2010**, *55*, 1306.
23. Park, J. B.; Chen, P. *J. Chromatogr., B* **2007**, *852*, 398.
24. Macaev, F.; Rusu, G.; Pogrebnoi, S.; Gudima, A.; Stingaci, E.; Vlad, L.; Shvets, N.; Kandemirli, F.; Dimoglo, A.; Reynolds, R. *Bioorg. Med. Chem.* **2005**, *13*, 4842.