Bioorganic & Medicinal Chemistry 16 (2008) 10013–10021

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

The design, synthesis, and anti-tumor mechanism study of *N*-phosphoryl amino acid modified resveratrol analogues

Huachen Liu [a,b], Aijun Dong [b], Chunmei Gao [b], Chunyan Tan [b], Hongxia Liu [b], Xuyu Zu [b], Yuyang Jiang [c,b,*]

[a] Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Ministry of Education, Department of Chemistry, Tsinghua University, Beijing 100084, China
[b] The Key Laboratory of Chemical Biology, Guangdong Province, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, China
[c] School of Medicine, Tsinghua University, Beijing 100084, China

ARTICLE INFO

Article history:
Received 20 July 2008
Revised 8 October 2008
Accepted 9 October 2008
Available online 12 October 2008

Keywords:
Resveratrol
N-Phosphoryl amino acid
Apoptosis
Mitochondrial pathway

ABSTRACT

A novel series of *trans*-*N*-phosphoryl amino acid modified resveratrol analogues were synthesized and evaluated in vitro for their cytotoxic effects against CNE-1 and CNE-2 cell lines. These analogues showed good anti-proliferative activity, among which **8d**, **8e**, **8j**, and **9d** displayed much stronger inhibition effect than resveratrol and **8d** showed the most potent activity with IC_{50} value at 3.45 ± 0.82 μM. The anti-tumor effects of **8d**, **8e**, **8j**, and **9d** were due to the induction of apoptosis, confirmed by the DNA fragmentation and flow cytometry analysis using PI (propidium iodide) staining and Annexin-V-FITC/PI staining assay. The PI staining assay also showed that **8d**, **8e**, **8j**, and **9d** caused cell cycles arrest at G_0–G_1 phase which finally led to cell apoptosis. Further mechanism study on compound **8d** against CNE-2 cells has shown the PARP cleavage, which is a hallmark of caspase-3 activation, as well as the activation of caspase-9, and the intracellular ROS generation. These results all suggest that **8d** induced a mitochondrial-dependent apoptosis pathway.

1. Introduction

The stilbene moiety has been found commonly in natural products with therapeutically properties. Resveratrol **1** (*trans*-3,5,4′-trihydroxystilbene, Fig. 1), which contains stilbene moiety, is widely present in nature, specifically in food plants and medicinal plants.[1–5] It has shown a number of therapeutic potential including anti-inflammatory, cardiovascular diseases, neurodegeneration, chemoprevention of cancer, and other biological activity.[6–9] In particular, resveratrol showed cancer chemoprevention activity in assays representing anti-initiation, anti-promotion, anti-progression activity, and inhibiting the development of preneoplastic lesions and tumorigenesis.[10] Further evidence has shown that resveratrol is able to inhibit cell growth and induce apoptosis in various human cancer cell lines.[11–15] Therefore, resveratrol might be considered as a lead compound for the preparation of analogues with enhanced bioactivity. In fact, a number of resveratrol analogues have been synthesized and the structure–activity relationship (SAR) has been analyzed.[11,14,16–21] However, minor attention was devoted to the lipophilic analogues. pterostilbene **2** (3,5-dimethoxy-4′-hydroxystilbene, Fig. 1) is a natural methoxylated analogue of resveratrol, which has demonstrated the cancer chemopreventive activity similar to that of resveratrol.[22–24] Compound **3** (4′-amino-3,5-dimethoxystilbene, Fig. 1), an analogue of compound **2**, has been reported to be more potent anti-proliferative.[17] The methoxylation of hydroxyl group may enhance lipophilicity, and thus improve cellular uptake.[12,25]

On the other hand, phosphorus plays a crucial role in metabolism and *N*-phosphoryl is considered to be a high biological activity group.[26] Our previous study showed that *N*-phosphopeptides **4** (Fig. 1) and some *N*-nitrogen heterocycle compounds can induce cancer cell apoptosis.[27–29] Being intrigued by our previous research, a series of *N*-phosphoryl amino acids modified resveratrol ana-

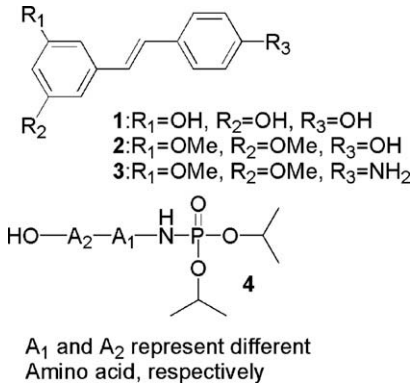


Figure 1. Resveratrol (**1**), stilbene-related proapoptotic compounds (**2**–**3**), and *N*-phosphopeptides (**4**).

* Corresponding author. Tel./fax: +86 755 2603 2094.
E-mail address: jiangyy@sz.tsinghua.edu.cn (Y. Jiang).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.10.022

logues were synthesized and their biological activities were evaluated against several human tumor cancer lines in the present study.

2. Result and discussion

2.1. Chemistry

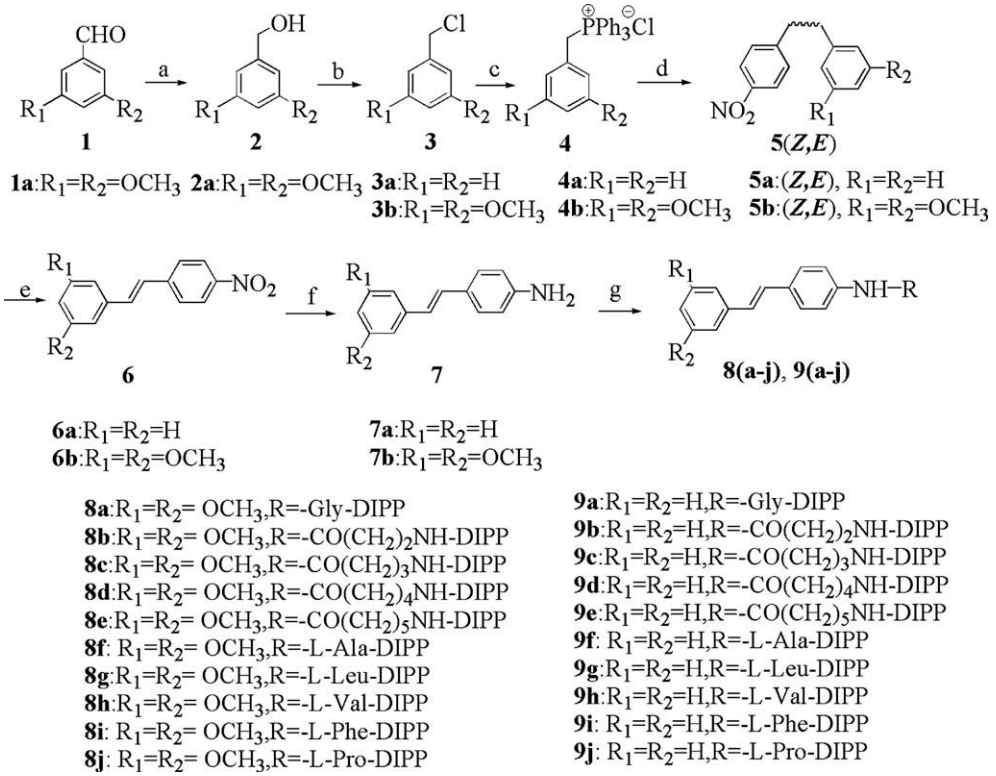
The *N*-phosphoryl amino acid modified resveratrol analogues **8–9** were synthesized as depicted in Scheme 1. Compound **2a** was prepared by reducing **1a** with $NaBH_4$ in high yields, which was then converted to **3b** in the presence of $P(Ph)_3$ and CCl_4 under the reflux condition overnight. Subsequent compound **5**, a mixture of *E* and *Z* isomers, were obtained through Wittig reaction.[30] These *E/Z* mixtures were efficiently converted to *trans*-stilbene isomer derivatives **6** with the catalytic amount of I_2 in $CHCl_3$. Nitro-derivatives **6** were then reduced by zinc dust in acetic acid to give the proper amino derivatives **7** in satisfactory yields. Final compounds 8a–**j** and **9a–j** were obtained in high yields through the reaction of the appropriate *N*-phosphoryl amino acids with **7a** and **7b**, respectively, using proper amount of HOBt and DCC in anhydrous THF solution under nitrogen.

2.2. Anti-proliferative activity of synthesized compounds and structure–activity relationship

The newly synthesized compounds **8a–j** and **9a–j** were initially evaluated for anti-proliferation activity against human nasopharyngeal carcinoma cell lines CNE-1 and CNE-2 by the MTT assay. Resveratrol was used as a reference, which has IC_{50} values at 67.62 and 52.28 μM against CNE-1 and CNE-2 cells, respectively. As shown in Table 1, most of the compounds displayed good anti-proliferative activity superior to resveratrol, except **8h, 9h**, and **9i**. Several structure–activity relationship was obtained as the following: (1) the methoxy groups at 3- and 5-positions of benzene ring played an important role on inhibition activity, suggested by the stronger inhibition effect of most methoxylated derivatives **8** compared to corresponding non-methoxylated derivatives **9**. (2) Most compounds in **8a–j** and **9a–j** which contain *N*-phosphoryl amino acid moiety showed better inhibition effect than **7a** and **7b**, which have the same core structure with amine group only. Combined with the fact that the best *N*-phosphoryl amino acid (*N*-diisopropyl-phosphoryl-phenylalanine) itself has IC_{50} at about 21.34 μM (see Supporting information), our results indicate the necessity of both moities, the *N*-phosphoryl amino acid moiety and the resveratrol analogues, in order to improve the activity. (3) The cytotoxicity of both series of compounds **8a–e** and **9a–e** is relevant to the length of the alkyl chain on the *N*-phosphoryl amino acid moiety. Specifically, the activity decreased as the number of methylene groups in the chain increased from 1 to 3, suggested by the IC_{50} values of **8a–c** and **9a–c**. Furthermore, **8d–e** and **9d–e**, which contains 4 and 5 methylene groups in the chain, respectively, showed increased activity. Especially, compound **8d** and **9d** showed the most anti-proliferative potent. (4) Compounds **8f–h** and **9f–h** have different substituent groups on α-carbon of amino acid. Our results showed that the activity of **8f–h** and **9f–h** decreased as the steric effect increased from methyl, isobutyl, and to isopropyl. This comparison clearly demonstrates that the steric effect of the amino acid may affect the anti-proliferative potency.

The inhibition of CNE-2 cell growth induced by**8d, 8e, 8j, and 9d** at different time intervals is shown in Figure 2. Our results suggest that not only a dose-dependent but also a time-dependent manner is involved in the anti-proliferative effects of those compounds.

Two other tumor cells HeLa and K562 were chosen for further evaluate the anti-proliferation effect of highly active compounds **8d, 8e, 8j**, and **9d**. Results are shown in Table 2. The fact that the inhibitory effect of**8d, 8e, 8j, and 9d** in HeLa and K562 was approximately equivalent to that in CNE suggested that these four compounds could be valuable in cancer treatment.



Scheme 1. Reagents and conditions: (a) $NaBH_4$, CH_3OH, rt, 2 h; (b) CCl_4, PPh_3, reflux,12 h; (c) PPh_3, CH_2Cl_2, reflux, 24 h; (d) CH_2Cl_2, NaH, 4-nitrobenzaldehyde, 0 °C, 24 h; (e) I_2, $CHCl_3$, reflux, 24 h; (f) Zn, CH_3COOH, rt, 2 h; (g) HOBt, DCC, THF, rt, 24 h.

Table 1
IC_{50} of the synthesized compounds of **8**(**a**–**j**), **9**(**a**–**j**), **7a**, and **7b** against of the nasopharyngeal carcinoma (CNE-1 and CNE-2) cell lines IC_{50} (μM)[a]

Compound	CNE-1 IC_{50} (μM)	CNE-2 IC_{50} (μM)	Compound	CNE-1 IC_{50} (μM)	CNE-2 IC_{50} (μM)
Res.	67.62 ± 4.29	52.28 ± 4.90			
7a	54.49 ± 2.32	98.67 ± 4.54	**7b**	40.89 ± 2.58	65.21 ± 3.15
8a	8.91 ± 1.18	7.12 ± 0.77	**9a**	7.50 ± 0.98	7.01 ± 1.22
8b	10.07 ± 1.22	8.19 ± 1.33	**9b**	29.78 ± 2.77	6.81 ± 0.98
8c	12.33 ± 1.37	15.15 ± 2.01	**9c**	9.028 ± 1.11	6.47 ± 1.14
8d	5.67 ± 1.15	3.45 ± 0.82	**9d**	6.36 ± 1.08	5.19 ± 1.02
8e	6.37 ± 1.31	5.06 ± 1.21	**9e**	8.58 ± 0.95	24.32 ± 2.59
8f	29.17 ± 4.61	11.72 ± 1.69	**9f**	25.37 ± 3.81	12.78 ± 1.76
8g	55.27 ± 8.94	14.34 ± 1.77	**9g**	54.26 ± 4.23	65.63 ± 7.59
8h	116.10 ± 11.84	77.39 ± 6.87	**9h**	49.28 ± 4.78	128.44 ± 14.19
8i	43.42 ± 5.51	9.83 ± 1.58	**9i**	83.74 ± 8.61	92.06 ± 6.29
8j	8.11 ± 0.84	9.86 ± 1.67	**9j**	10.08 ± 1.24	12.19 ± 1.33

[a] Results were obtained after 72 h of treatment and means ± SEM of three experiments. Differences were considered significant at $P < 0.05$.

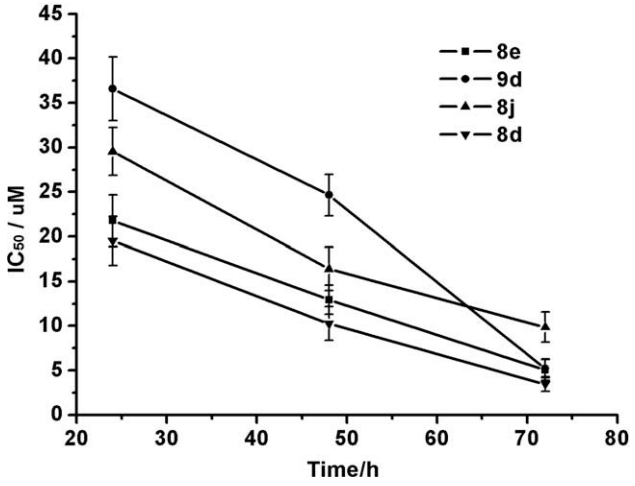


Figure 2. Time dependence of IC_{50} values of compound **8d**, **8e**, **8j**, and **9d** against CNE-2 cell lines. Cells were treated with the corresponding compound at 24, 48, and 72 h.

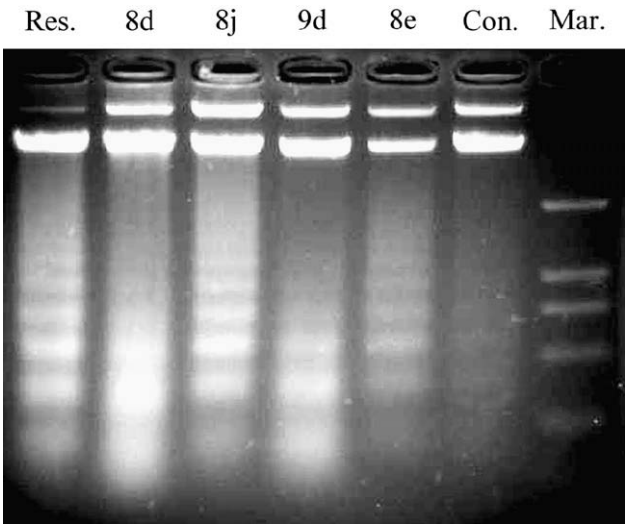


Figure 3. Apoptosis was evaluated by the induction of DNA fragmentation. CNE-2 cells were treated with compound **8d**, **8e**, **8j**, and **9d** and resveratrol at 50 μM for 24 h. Untreated CNE-2 cells were used as control.

Table 2
IC_{50} values of the synthesized compounds of **8d**, **8e**, **8j**, and **9d** against HeLa and K562 cell lines[a]

	8d	**8e**	**8j**	**9d**
HeLa	5.62 ± 0.95	6.41 ± 1.45	9.53 ± 2.12	9.75 ± 1.20
K562	5.32 ± 1.02	6.89 ± 1.63	9.15 ± 1.51	9.58 ± 1.62

[a] Results were obtained after 72 h of treatment and means ± SEM of three experiments. Differences were considered significant at $P < 0.05$.

2.3. Mechanism of the anti-proliferative activity

It was reported that resveratrol can induce apoptosis in many tumor cell lines.[12] We therefore speculate that these novel resveratrol analogues may also have similar effects. On the basis of the cytotoxicity study, four compounds, **8d, 8e, 8j**, and **9d**, were selected for further examinations to determine whether the cytotoxicity is mediated by the induction of apoptosis. Apoptosis is regulated by genetic mechanisms and is principally characterized by morphological and biochemical changes in the nucleus, including chromatin condensation and internucleosomal DNA fragmentation. As we expected, the exposure of CNE-2 cells to compound **8d, 8e, 8j**, and **9d** for 24 h resulted in the appearance of DNA ladders on the agarose gels, which is a hallmark of apoptosis, as shown in Figure 3. Another typical feature of cells undergoing apoptosis is the occurrence of a sub-G0–G1 cell population by flow cytometry analysis of DNA-content histogram after propidium iodide (PI) staining. As shown in Figure 4, cells treated with 50 μM **8d**, **8e**, **8j**, and **9d** for 24 h resulted in the obvious sub-G_0–G_1 peak, ranged from 32.15% to 91.59%. From Figure 4 we also find that cell cycle was arrested in G_0–G_1 phase in this condition.

In addition, the Annexin-V/PI binding assay was used to further confirm the drug induced apoptosis effect. Annexin-V conjugated with the fluorochrome FITC serves as a marker for apoptotic cells because it has a strong binding affinity to phosphatidylserine (PS), which re-distributes from the inner to the outer layer of the plasma membrane in apoptotic cells. Figure 5 shows the histogram with dual parameters of Annexin-V-FITC and PI, among which the upper right represents the late stage apoptotic cells (Annexin positive and PI positive), and the lower right represents the early stage apoptotic cell population (Annexin positive and PI negative). As the concentration of **8d** increased from 5 to 50 μM, the Annexin-V positive/PI negative cells increased from 6.45% to 21.24%, and then decreased back to 18.78%, whereas the double positive cells increased from 9.86% to 66.79%, suggesting that more and more apoptotic cells progressed from the early stage to the late stage resulting in either death or secondary necrosis under the effect of **8d** at higher concentrations. This confirms again that **8d** induced the cell apoptosis of CNE-2 cells.

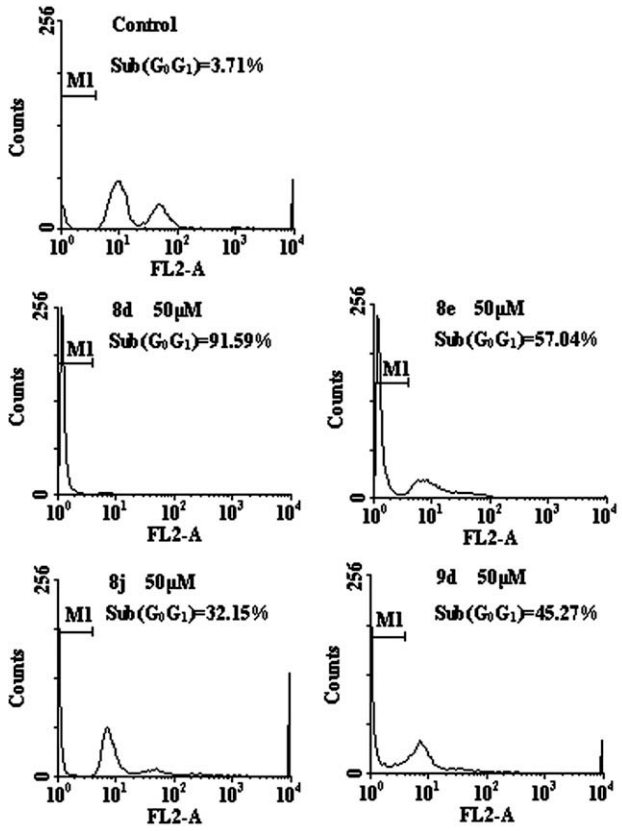


Figure 4. Flow cytometry analysis of cell membrane integrity (PI staining) and cell cycle arrest. CNE-2 cells were treated with compound **8d**, **8e**, **8j**, and **9d** at 50 μM for 24 h. The percentage of apoptotic events accumulating under the marker in the Sub-G_0–G_1 phase and a decrease of cells in both S and G_2/M phases, especially in G_2/M of the cell cycle was analyzed using CellQuest software (Becton–Dickinson).

2.4. Apoptosis pathway study

2.4.1. Activation of caspase-3 activity

Apoptosis is mainly brought about by activation of caspases, a family of cystein proteases capable of cleaving essential cellular substrates after aspartic residues. Caspase-3 is the key apoptotic executive protein which could be activated by both death-receptor pathway and mitochondrial pathway. We tested the induction of poly-(ADP-ribose)-polymerase (PARP) cleavage by the four analogues (**8d, 8e, 8j**, and **9d**). The 89 kDa band, which is an evidence of PARP cleavage, was observed. Since PARP is a substrate for caspase-3, the apoptotic effect of compounds might be caspase-mediated (Fig. 6).[31,32]

2.4.2. Activation of caspase-8 or caspase-9?

To gain insights into the pathway by which caspase-3 was activated, we investigated the activity of caspase-8 and caspase-9. As known, caspase-8 is involved in the death-receptor pathway, while caspase-9 mediates the mitochondrial pathway.[33] Once activated, both caspase-8 and caspase-9 can then activate downstream caspase-3, which finally causes cell apoptosis. Figure 7 demonstrated that the activity of caspase-9 increased dramatically in the **8d**-treated CNE-2 cells after different length of incubation times ranging from 0 to 24 h. While on the contrary, the activity of caspase-8 had no obvious change. This comparison suggests that compound **8d** had no significant effect on the activation of caspase-8. Therefore, compound **8d** induces CNE-2 cell apoptosis through mitochondria pathway instead of the death-receptor pathway.

2.4.3. Compound induces the transient generation of ROS

In a mitochondrial-dependent apoptosis, mitochondria undergo what is referred to as the mitochondrial permeability transition (MPT), which has several consequences including the collapse of $\Delta\Psi_m$, Ca^{2+} release, and the generation of reactive oxygen species (ROS). To further confirm that the mitochondria pathway was activated in the compound **8d** induced apoptotic process, we traced the ROS level in the treated CNE-2 cells. As shown in Figure 8, compound **8d** rapidly induced the generation of ROS and the percentage of cells with higher fluorescence reached a maximum at 4 h and then declined, reached the minimum value at 19 h determined by DCFH-DA fluorescence.[34]

3. Conclusion

In conclusions, combination of resveratrol and *N*-phosphoryl amino acids offered the discovery of a series of potent growth inhibitors of CNE-1 and CNE-2 cell lines. Compound **8d** has the most potent inhibitory activity, which is 15-fold higher than that of resveratrol. Compounds **8d, 8e, 8j, and 9d** induced cell cycle G_0/G_1 arrest and apoptosis in a dose- and time-dependent manner. Compound **8d** induced apoptosis was associated with caspase-9 activation, indicating that the mitochondrial pathway was involved in the apoptosis pathway, which was further confirmed by the generation of ROS. Taking into consideration that these novel compounds possess anti-tumor activity, they may become candidates of effective chemopreventive agents for cancer chemotherapy.

4. Experimental

4.1. Chemistry

^{1}H NMR and ^{13}C NMR were recorded in the indicated deuterated solvent at 300 or 500 MHz on Bruker NMR spectrometers. Chemical shifts are expressed in ppm (δ) and coupling constants *J* in Hertz (Hz). High-resolution mass spectra were obtained with Waters micromass Q-Tof Premier mass spectrometer. Melting points were determined by the open capillary method on a SGW X-4 electrothermal melting apparatus and are uncorrected. Reaction progress was monitored using analytical thin-layer chromatography (TLC) on pre-coated silica gel plates and the spots were detected under UV light (254 nm). All other reagents and analytical grade solvents were bought from commercial sources and used without further purification unless indicated. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride (CaH_2) and tetrahydrofuran (THF) was distilled from sodium immediately prior to use.

4.1.1. Synthesis of 3,5-dimethoxylbenzyl alcohol (2)

The $NaBH_4$ (35 mmol) of methanol solution was dropped into a solution of 10 mmol of 3,5-dimethoxybenzaldehyde at room temperature, keeping the dropped in an appropriative velocity. The mixture was stirred for 2 h and evaporated methanol under vacuum, then adding water, extracted with 20 ml EtOAc twice, the organic layer was dried over anhydrous $NaSO_4$, and evaporated under vacuum obtaining the compound **2**. Yield 94%, mp: 47–48 °C, ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.80 (s, 6H), 4.52 (s, 2H), 6.41 (m, 1H), 6.54 (m, 2H).

4.1.2. Synthesis of 3,5-dimethoxylbenzyl chloride (3)

A solution of 3,5-dimethoxylbenzyl alcohol (10 mmol) and PPh_3 (11 mmol) in CCl_4 (50 ml) was stirred at reflux condition. After 12 h the solvent of CCl_4 was evaporated under vacuum, and the mixture was filtered through silica gel/Celite to afford 3,5-dimethoxylbenzyl chloride as a white solid. (92%, mp: 46–48 °C), ^{1}H

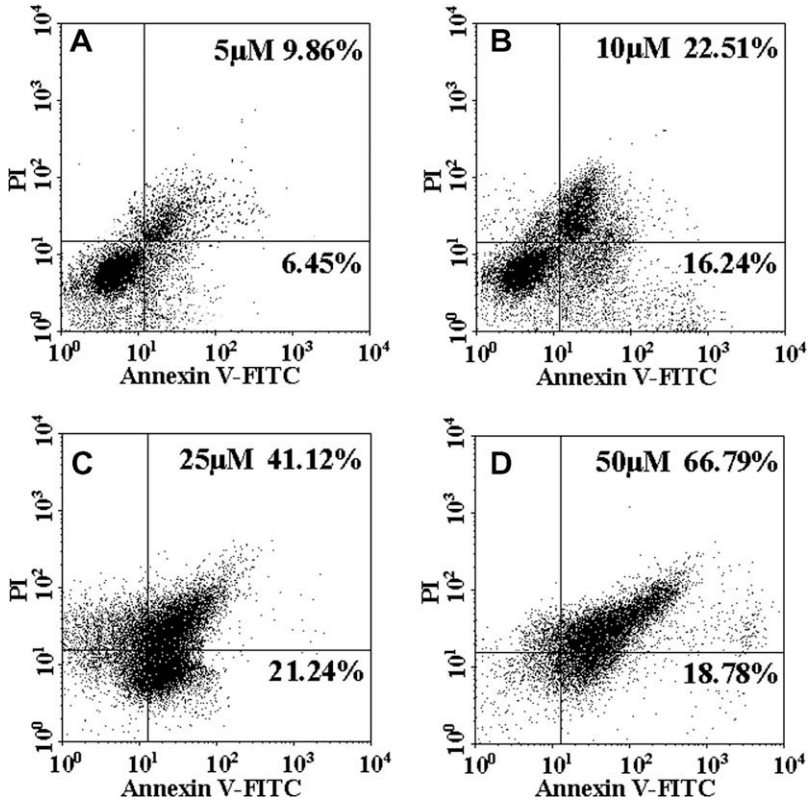


Figure 5. Flow cytometric analysis of phosphoatidylserine externalization (Annexin-V binding) and cell membrane integrity (PI staining). CNE-2 cells were treated with compound **8d** at 5, 10, 25, and 50 μM for 24 h.

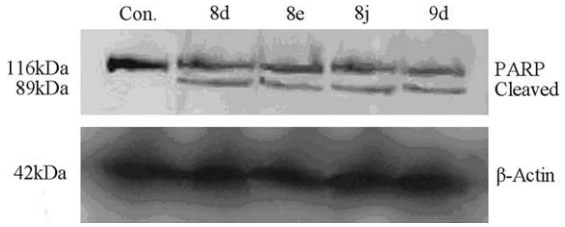


Figure 6. The proapoptotic effect was determined by the PARP cleavage assay, evaluated as appearance of the 89 kDa band in CNE-2 cells treated with **8d**, **8e**, **8j**, and **9d** at 50 μM for 24 h.

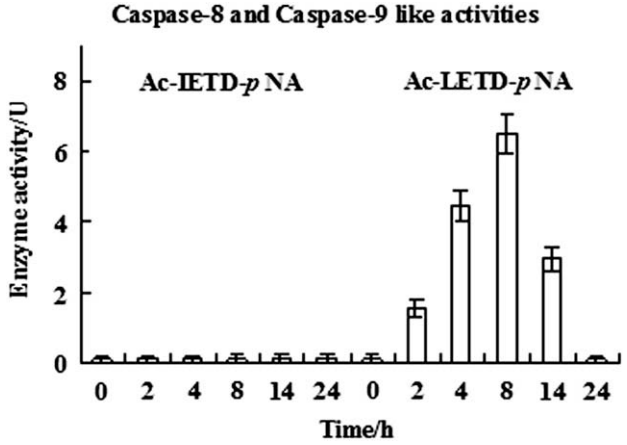


Figure 7. Caspase-8 and capase-9 activities were detected by Ac-IETD-*p*NA and Ac-LEHD-*p*NA at 0, 2, 4, 8, 14, and 24 h of compound **8d** at 50 μM, respectively. Results are mean ± SD (n = 3).

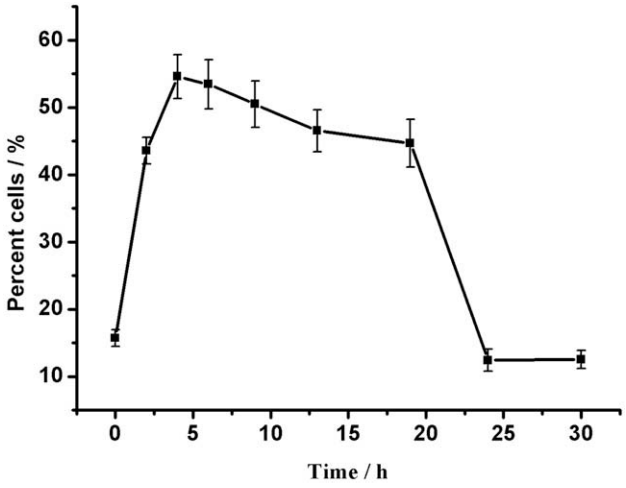


Figure 8. The ROS generation determined in terms of DCFH-DA fluorescence by flow cytometric analysis. CNE-2 cells was treated with compound **8d** at 50 μM for the indicated times.

NMR ($CDCl_3$, 500 MHz): δ 3.80 (s, 6H), 4.52 (s, 2H), 6.41 (m, 1H), 6.54 (m, 2H).

4.1.3. Synthesis of (*E*/*Z*)-4′-nitrostilbene derivatives (5)

The appropriate benzyl chloride (10 mmol) was dissolved in CH_2Cl_2 (50 ml) and then PPh_3 (13 mmol) was added. The mixture was stirred for 24 h at room temperature and then cooled in ice-water bath, and then 4-nitrobenzaldehyde (10 mmol) was added. The solution of NaH in CH_2Cl_2 was dropped with appropriated velocity into the reaction system under a nitrogen

atmosphere. After 24 h, water was added, and the product was isolated by extraction with CH_2Cl_2. The organic phase was washed with saturated brine and dried over anhydrous $NaSO_4$. The solvent was removed under reduced pressure to obtain **5** (*E*/*Z*) as a mixture of isomers which was determined by ^1H NMR signals.

4.1.4. Transform 5 (*E*/*Z*) to *trans*-4-nitrostilbene (6a) or *trans*-3,5-dimethoxyl-4′-nitrostilbene (6b)

A solution of **5** (*E*/*Z*) (10 mmol) in $CHCl_3$ (60 ml) with the catalytic amount of I_2 was stirred and reflowed for 12 h, the catalytic I_2 of system reaction was reduced with saturated sodium hydrogen sulfite, and continued stirring for 2 h. The production was extracted with 30 ml $CHCl_3$ twice. The organic phase was washed with saturated brine and dried over $NaSO_4$ and evaporated under vacuum to obtain compound **6a–b**. Compound **6a**: 95%, mp: 157–159 °C. ^1H NMR (DMSO, 500 MHz,): δ 6.55 (m, 2H), 6.88 (d, 1H, J_{13} = 16.40 Hz), 7.05 (d, 1H, J_{13} = 16.40 Hz), 7.17 (m, 1H), 7.26 (m, 2H), 7.31 (m, 2H), 7.48 (m, 2H). Compound **6b**: 96%, mp: 135–137 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.85 (s, 6H), 6.46 (m, 1H), 6.70 (m, 2H), 7.11 (d, 1H, J_{13} = 16.20 Hz), 7.21 (d, 1 H, J_{13} = 16.20 Hz), 7.63 (d, 2H, J_{13} = 8.70 Hz), 8.23 (d, 2H, J_{13} = 9.00 Hz).

4.1.5. Synthesis of *trans*-4-aminostilbene and *trans*-3,5-dimethoxyl-4′-aminostilbene (7a–b)

To a stirred solution of **6a–b** (10 mmol) in acetic acid was added zinc dust (40 mmol). After 2 h, the solution was filtered under vacuum through silica gel and the filtrate was concentrated under vacuum. The product was separated by silica gel/Celite to obtain compounds **7a–b**. Compound **7a**: 74%, ^1H NMR ($CDCl_3$, 300 MHz,): δ 3.82 (s, 6H), 6.36 (m, 1H), 6.66 (m, 4H), 6.85 (d, 1H, J_{13} = 16.20 Hz), 7.00 (d, 1H, J_{13} = 16.20 Hz), 7.33 (m, 2H). **7b**: 73.4%, mp: 88–91 °C. ^1H NMR ($CDCl_3$, 300 MHz,): δ 3.75 (s, 2H), 6.68 (d, 2H), 6.92 (d, 1H, J_{13} = 16.20 Hz), 7.03 (d, 1H, J_{13} = 16.20 Hz), 7.21 (m, 1H), 7.32 (m, 2H), 7.34 (m, 2H), 7.47 (m, 2H).

4.1.6. General procedure for synthesis of compound 8a–j and 9a–j

The appropriate *N*-phosphoryl amino acids (1.05 mmol), the compounds **7a–b** (1 mmol) and HOBt (1.05 mmol) in anhydrous tetrahydrofuran was stirred at under ice-water bath in nitrogen atmosphere, and then the solution of DCC (1.05 mmol) in THF (20 ml) was dropped with a appropriated velocity. The temperature of system reaction was slowly heightened to room temperature. After 24 h, the deposition was filtered and the filtrate was concentrated under vacuum. The residue was washed with saturated $NaHCO_3$ and stirred. The aqueous solution was twice extracted with 30 ml $CHCl_3$. The combined organic phases were washed with saturated $NaHCO_3$, 10% citric acid solution and saturated brine by turns twice, dried over anhydrous $NaSO_4$, and evaporated under vacuum. The products were separated by silica gel/Celite to obtain compound **8a–j** and **9a–j** (eluent, chloroform/methanol).

4.1.6.1. Compound 8a: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Gly)amido-*E*-stilbene. Yield 71%; mp: 118–120 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 6.646. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.35 (d, 12H, J_{13} = 6.20 Hz), 3.46 (m, 1H), 3.75 (d, 2H, J_{13} = 13.10 Hz), 3.83 (s, 6H), 4.66 (m, 2H), 6.39 (m, 1H), 6.65 (m, 2H), 6.97 (d, 1H, J_{13} = 16.25 Hz), 7.05 (d, 1H, J_{13} = 16.25 Hz), 7.47 (d, 2H, J_{13} = 8.50 Hz), 7.58 (d, 2H, J_{13} = 8.50 Hz), 8.95 (s,1H); ^{13}C NMR ($CDCl_3$, 125 MHz,): δ 23.77, 45.77, 55.35, 71.96, 71.92, 99.98, 104.55, 119.96, 127.16, 133.25, 137.51, 139.45, 161.03, 127.89, 128.57, 168.53. HRMS: calcd for $[C_{24}H_{34}N_2O_6P]^+$: 477.2155; found: 477.2168.

4.1.6.2. Compound 8b: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-β-alanine)amido-*E*-stilbene. Yield: 66%; mp: 153–154 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.070. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.29 (m, 12H), 2.04 (m, 1H), 2.63 (m, 2H), 3.31 (m, 2H), 3.82 (s, 6H), 4.59 (m, 2H), 6.38 (m, 1H), 6.65 (m, 2H), 6.95 (d, 1H, J_{13} = 16.50 Hz), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.43 (d, 2H, J_{13} = 8.50 Hz), 7.60 (d, 2H, J_{13} = 8.50 Hz), 8.85 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.77, 23.81, 37.99, 38.93, 55.34, 71.27, 71.32, 99.98, 104.54, 119.90, 127.07, 132.98, 137.98, 139.50, 161.03, 127.72, 128.65, 169.97. HRMS: calcd for $[C_{25}H_{36}N_2O_6P]^+$: 491.2311; found: 491.2331.

4.1.6.3. Compound 8c: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-4-aminobutyric)amido-*E*-stilbene. Yield: 70%; mp: 109–111 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.40. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.30 (m, 12H), 1.92 (m, 2H), 2.54 (m, 2H), 2.99 (m, 2H), 3.82 (s, 6H), 4.59 (m, 2H), 6.38 (m, 1H), 6.65 (m, 2H), 6.95 (d, 1H, J_{13} = 16.50 Hz), 7.04 (d, 1H, J_{13} = 16.50 Hz), 7.43 (d, 2H, J_{13} = 8.50 Hz), 7.66 (d, 2H, J_{13} = 8.50 Hz), 9.44 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.78, 23.81, 28.10, 33.98, 39.81, 55.36, 71.30, 71.34, 99.95, 104.50, 119.90, 127.00, 132.72, 138.44, 139.57, 161.03, 127.50, 128.78, 171.59. HRMS: calcd for $[C_{26}H_{38}N_2O_6P]^+$: 505.2468; found: 505.2476.

4.1.6.4. Compound 8d: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-5-aminovaleric)amido-*E*-stilbene. Yield 63%; oil; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.32. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.31 (m,12H), 1.57 (m, 2H), 1.84 (m, 2H), 2.40 (m, 2H), 2.61 (m, 1H), 3.00 (m, 2H), 3.82 (s, 6H), 4.58 (m, 2H), 6.38 (m, 1H), 6.65 (d, 2H), 6.95 (d, 1H, J_{13} = 16.50 Hz), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.44 (d, 2H, J_{13} = 8.50 Hz),7.60 (d, 2H, J_{13} = 8.50 Hz), 8.51 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 22.51, 23.78, 23.81, 30.88, 36.67, 40.44, 55.33, 70.96, 71.00, 99.86, 104.43, 119.90, 127.01, 132.60, 138.41, 139.50, 160.96, 127.44, 128.70, 171.92. HRMS: calcd for $[C_{27}H_{40}N_2O_6P]^+$: 519.2426; found: 519.2635.

4.1.6.5. Compound 8e: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-6-aminocaproic)amido-*E*-stilbene. Yield 61%; mp: 80–82 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.59. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.30 (m, 12H), 1.40 (m, 2H), 1.53 (m, 2H), 1.74 (m, 2H), 2.38 (m, 2H), 2.63 (m, 1H), 2.90 (m, 2H),3.82 (s, 6H), 4.57 (m, 2H), 6.38 (m, 1H), 6.65 (m, 2H), 6.95 (d, 1H, J_{13} = 16.50 Hz), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.43 (d, 2H, J_{13} = 8.50 Hz), 7.59 (d, 2H, J_{13} = 8.50 Hz), 8.32 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 23.81, 23.86, 25.08, 26.08, 31.25, 37.31, 41.17, 55.36, 70.78, 70.86, 99.86, 104.41, 119.87, 127.08, 132.76, 138.09, 139.45, 160.95, 127.55, 128.63, 171.61. HRMS: calcd for $[C_{28}H_{42}N_2O_6P]^+$: 533.2781; found: 533.2770.

4.1.6.6. Compound 8f: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Ala)amido-*E*-stilbene. Yield 73%; mp: 135–137 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 5.86. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.33 (m, 12H), 1.49 (d, 3H, J_{13} = 6.80 Hz), 3.66 (m, 1H), 3.82 (s, 6H), 4.02 (m, 1H), 4.61 (m, 2H), 6.38 (m, 1H), 6.65 (m, 2H), 6.96 (d, 1H, J_{13} = 16.50 Hz), 7.04 (d, 1H, J_{13} = 16.50 Hz), 7.45 (d, 2H, J_{13} = 8.50 Hz), 7.63 (d, 2H, J_{13} = 8.50 Hz), 9.43 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.10, 23.74, 23.79, 52.13, 55.38, 71.65, 71.78, 99.86, 104.41, 119.81, 127.08, 132.89, 138.04, 139.45, 160.95, 127.62, 128.63, 172.13. HRMS: calcd for $[C_{25}H_{36}N_2O_6P]^+$: 491.2311; found: 491.2295.

4.1.6.7. Compound 8g: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Leu)amido-*E*-stilbene. Yield 69%; mp: 169–173 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 6.27. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.97 (d, 6H, J_{13} = 6.25 Hz), 1.30 (m, 12H), 1.57 (m, 1H), 1.80 (m, 2H), 3.34 (m,1H), 3.83 (s, 6H), 3.87 (m, 1H), 4.60

(m, 2H), 6.39 (m, 1H), 6.65 (m, 2H), 6.96 (d, 1H, J_{13} = 16.50 Hz), 7.04 (d, 1H, J_{13} = 16.50 Hz), 7.45 (d, 2H, J_{13} = 8.50 Hz), 7.62 (d, 2H, J_{13} = 8.50 Hz), 9.24 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.99, 23.03, 23.73, 23.77, 24.52, 43.79, 55.17, 55.35, 71.71, 71.79, 99.85, 104.40, 119.88, 126.99, 132.76, 138.24, 139.48, 160.96, 127.51, 128.70, 172.574. HRMS: calcd for $[C_{28}H_{42}N_2O_6P]^+$: 533.2781; found: 533.2795.

4.1.6.8. Compound 8h: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Val)amido-*E*-stilbene. Yield 71%; mp: 170–172 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 6.18. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.99 (d, 3H, J_{13} = 6.80 Hz), 1.30 (m, 12H), 2.28 (m, 1H), 3.50 (m, 1H), 3.72 (m, 1H), 3.83 (s, 6H), 4.61 (m, 2H), 6.39 (m, 1H), 6.65 (m, 2H), 6.96 (d, 1H, J_{13} = 16.50 Hz), 7.04 (d, 1H, J_{13} = 16.50 Hz), 7.45 (d, 2H, J_{13} = 8.50 Hz), 7.63 (d, 2H, J_{13} = 8.50 Hz), 9.17 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 19.82, 21.36, 25.64, 25.71, 33.79, 57.26, 64.06, 73.46, 73.58, 101.88, 106.44, 121.87, 128.9, 134.87, 139.88, 141.44, 162.95, 129.58, 130.62, 173.34. HRMS: calcd for $[C_{27}H_{40}N_2O_6P]^+$: 519.2624; found: 519.2617.

4.1.6.9. Compound 8i: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Phe)amido-*E*-stilbene. Yield 75%; 165–167 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.26 (m, 12H), 3.14 (m, 2H), 3.47 (m, 1H), 3.82 (s, 6H), 4.11 (m, 1H), 4.49 (m, 2H), 6.41 (m, 1H), 6.66 (m, 2H), 6.97 (d, 1H, J_{13} = 16.50 Hz), 7.04 (d, 1H, J_{13} = 16.50 Hz), 7.25 (m, 5H), 7.45 (d, 2H, J_{13} = 8.50 Hz), 7.51 (d, 2H, J_{13} = 8.50 Hz), 8.63 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 23.72, 23.78, 39.97, 57.70, 71.77, 71.85, 99.88, 104.44, 120.00, 126.98, 127.07, 128.48, 129.68, 133.12, 136.69, 137.54, 139.43, 160.97, 127.75, 128.60, 170.95. HRMS: calcd for $[C_{31}H_{40}N_2O_6P]^+$: 567.2624; found: 567.2622.

4.1.6.10. Compound 8j: 3,5-dimethoxyl-4′-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Pro)amido-*E*-stilbene. Yield 73%; oil; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.82. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.32 (m, 12H), 1.95 (m, 4H), 2.53 (m, 1H), 3.24 (m, 2H), 3.83 (s, 6H), 4.27 (m, 1H), 4.62 (m, 2H), 6.39 (m, 1H), 6.65 (m, 2H), 6.96 (d, 1H, J_{13} = 16.50 Hz), 7.04 (d, 1H, J_{13} = 16.50 Hz), 7.46 (d, 2H, J_{13} = 8.50 Hz), 7.59 (d, 2H, J_{13} = 8.50 Hz), 9.60 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 23.92, 23.98, 25.58, 29.00, 47.81, 61.73, 55.37, 71.78, 71.86, 99.87, 104.39, 119.45, 127.18, 132.79, 137.98, 139.51, 160.95, 27.54, 128.67, 170.81. HRMS: calcd for $[C_{27}H_{38}N_2O_6P]^+$: 517.2468; found: 517.2477.

4.1.6.11. Compound 9a: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Gly)amido-*E*-stilbene. Yield 61.8%; mp: 139–141 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 5.91. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.33 (m, 12H), 3.77 (m, 2H), 3.90 (m, 1H), 4.64 (m, 2H), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.06 (d, 1H, J_{13} = 16.50 Hz), 7.24 (m, 1H), 7.34 (m, 2H),7.46 (m, 2H), 7.48 (m, 2H), 7.59 (m, 2H), 9.19 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.77, 23.81, 45.80, 71.90, 71.94, 119.83, 127.47, 127.94, 128.08, 128.65, 133.48, 137.39, 137.44, 126.40, 127.08, 170.29. HRMS: calcd for $[C_{22}H_{30}N_2O_4P]^+$: 417.1943; found: 417.1951.

4.1.6.12. Compound 9b: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-β-alanine)amido-*E*-stilbene. Yield 57.6%; mp: 172–174 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 6.70. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30 (m, 12H), 2.63 (m, 2H), 3.32 (m, 2H), 4.60 (m, 2H), 7.02 (d, 1H, J_{13} = 16.50 Hz), 7.07 (d, 1H, J_{13} = 16.50 Hz), 7.22 (m, 1H), 7.35 (m, 2H), 7.45 (m, 2H), 7.49 (m, 2H), 7.60 (m, 2H), 8.61 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.80, 23.84, 38.05, 38.72, 71.17, 71.22, 120.02, 127.42, 127.63, 128.16, 128.66, 133.13, 137.45, 138.13, 126.37, 126.96, 170.29. HRMS: calcd for $[C_{23}H_{32}N_2O_4P]^+$: 431.2100; found: 431.2107.

4.1.6.13. Compound 9c: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-4-aminobutyric)amido-*E*-stilbene. Yield 63%; mp: 115–118 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.22. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.31 (m, 12H), 1.91 (m, 2H), 2.54 (m, 2H), 3.01 (m, 2H), 4.60 (m, 2H), 7.02 (d, 1H, J_{13} = 16.50 Hz), 7.07 (d, 1H, J_{13} = 16.50 Hz), 7.34 (m, 2H), 7.45 (m, 2H), 7.49 (m, 2H), 7.67 (m, 2H), 9.31 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.76, 23.78, 27.87, 33.97, 39.98, 71.10, 71.14, 120.01, 127.36, 127.52, 128.26, 128.62, 132.89, 137.51, 138.43, 126.35, 126.89, 171.71. HRMS: calcd for $[C_{24}H_{34}N_2O_4P]^+$: 445.2256; found: 445.2261.

4.1.6.14. Compound 9d: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-5-aminovaleric)amido-*E*-stilbene. Yield 62%; mp: 122–125 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.969. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.31 (m, 12H), 1.57 (m, 2H), 1.85 (m, 2H), 2.40 (m, 2H), 3.00 (m, 2H), 4.58 (m, 2H), 7.02 (d, 1H, J_{13} = 16.50 Hz), 7.06 (d, 1H, J_{13} = 16.50 Hz), 7.25 (m, 1H), 7.34 (m, 2H), 7.45 (d, 2H), 7.49 (m, 2H), 7.61 (m, 2H), 8.43 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.53, 23.78, 23.84, 30.79, 36.72, 40.32, 71.01, 71.09, 119.86, 127.40, 127.46, 128.17, 128.66, 132.80, 137.44, 138.29, 126.35, 126.96, 171.93. HRMS: calcd for $[C_{25}H_{36}N_2O_4P]^+$: 459.2413; found: 459.2420.

4.1.6.15. Compound 9e: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-6-aminocaproic)amido-*E*-stilbene. Yield 66%; mp: 127–128 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.61. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.31 (m, 12H), 1.43 (m, 2H), 1.56 (m, 2H), 1.76 (m, 2H), 2.38 (m, 2H), 2.50(m, 1H), 2.92 (m, 2H), 4.58 (m, 2H), 7.02 (d, 1H, J_{13} = 16.50 Hz), 7.07 (d, 1H, J_{13} = 16.50 Hz), 7.23 (m, 1H), 7.34 (m, 2H), 7.46 (m, 2H), 7.49 (m, 2H), 7.56 (m, 2H), 7.77 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 23.80, 23.86, 25.10, 26.09, 31.23, 37.37, 41.15, 70.81, 70.88, 119.90, 127.44, 127.60, 128.11, 128.67, 133.01, 137.41, 137.96, 126.37, 127.00, 171.56. HRMS: calcd for $[C_{26}H_{38}N_2O_4P]^+$: 473.2569; found: 473.2581.

4.1.6.16. Compound 9f: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Ala)amido-*E*-stilbene. Yield 70%; mp: 155–157 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 5.13. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.32 (m, 12H), 1.50 (d, 3H, J_{13} = 6.85 Hz), 3.87 (m, 1H), 4.05 (m, 1H), 4.61 (m, 2H), 7.02 (d, 1H, J_{13} = 16.50 Hz), 7.08 (d, 1H, J_{13} = 16.50 Hz), 7.23 (m, 1H), 7.34 (m, 2H), 7.47 (m, 4H),7.65 (m, 2H), 9.58 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.20, 23.77, 23.80, 52.17, 71.71, 71.79, 119.85, 127.46, 127.65, 128.13, 128.69, 133.09, 137.42, 137.99, 126.39, 127.01, 172.21. HRMS: calcd for $[C_{23}H_{32}N_2O_4P]^+$: 431.2100; found: 431.2107.

4.1.6.17. Compound 9g: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Leu)amido-*E*-stilbene. Yield 76%; mp: 168–170 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 5.83. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.97 (d, 6H, J_{13} = 6.35 Hz), 1.30 (m, 12H), 1.58 (m, 1H), 1.80 (m, 2H), 3.48 (m, 1H), 3.88 (m, 1H), 4.62 (m,2H), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.07 (d, 1H, J_{13} = 16.50 Hz), 7.23 (m, 1H), 7.34 (m, 2H), 7.46 (m, 2H), 7.49 (m, 2H), 7.63 (m, 2H), 9.31 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.97, 23.04, 23.73, 23.80, 24.52, 43.59, 55.22, 71.75, 1.83, 119.86, 127.43, 127.58, 128.17, 128.68, 133.03, 137.45, 138.05, 126.37, 126.94, 172.37. HRMS: calcd for $[C_{26}H_{38}N_2O_4P]^+$: 473.2569; found: 473.2584.

4.1.6.18. Compound 9h: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Val)amido-*E*-stilbene. Yield 74%; mp: 140–142 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 6.39. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.99 (d, 3H, J_{13} = 6.80 Hz), 1.05 (d, 3H, J_{13} = 6.80 Hz), 1.31 (m, 12H), 2.29 (m, 1H), 3.46 (m, 1H), 3.68 (m, 1H), 4.61 (m, 2H), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.08 (d, 1H, J_{13} = 16.50 Hz), 7.24 (m, 1H), 7.35 (m, 2H), 7.46 (m, 2H), 7.50 (m, 2H), 7.62 (d, 2H), 9.09 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 17.87, 19.43, 23.69, 23.74, 31.89, 61.96,

71.99, 72.07, 119.85, 127.46, 127.65, 128.13, 128.69, 133.09, 137.42, 137.99, 126.39, 127.01, 174.11. HRMS: calcd for $[C_{25}H_{36}N_2O_4P]^+$: 459.2413; found: 459.2419.

4.1.6.19. Compound 9i: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Phe)amido-*E*-stilbene. Yield 73%; mp: 188–191 °C; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 5.54. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.26 (m, 12H), 3.11 (m, 2H), 3.48 (m, 1H), 4.11 (m, 1H), 4.49 (m, 2H), 7.06 (m, 2H), 7.26 (m, 6H), 7.35 (m, 2H), 7.50 (m, 6H), 8.58 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 23.73, 23.77, 39.81, 57.58, 71.70, 71.77, 120.00, 127.50, 127.84, 128.05, 128.43, 128.69, 129.59, 129.68, 133.41, 136.58, 137.302, 137.38, 126.40, 127.02, 170.80. HRMS: calcd for $[C_{29}H_{36}N_2O_4P]^+$: 507.2413; found: 507.2424.

4.1.6.20. Compound 9j: 4-(*N*-*O*,*O*′-diisopropylphosphoryl-L-Pro) amido-*E*-stilbene. Yield 78%; oil; ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.87. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.31 (m, 12H), 1.96 (m,4H), 2.52 (m, 1H), 3.25 (m, 2H), 4.27 (m, 1H), 4.63 (m, 2H), 7.03 (d, 1H, J_{13} = 16.50 Hz), 7.07 (d, 1H, J_{13} = 16.50 Hz), 7.24 (m, 1H), 7.34 (m, 2H), 7.46 (m, 2H), 7.49 (m, 2H), 7.59 (m, 2H), 9.59 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.92, 23.99, 25.59, 29.01, 47.77, 61.73, 71.75, 71.84, 119.46, 127.40, 127.56, 128.16, 128.66, 132.99, 137.47, 137.89, 126.37, 127.09, 170.80. HRMS: calcd for $[C_{25}H_{34}N_2O_4P]^+$: 457.2257; found: 457.2270.

4.2. Biology

4.2.1. Cell culture

Cell lines CNE-1 and CNE-2 were cultured in DMEM, with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin in humidified air at 37 °C with 5% CO_2.

4.2.2. Cell-growth inhibition assay

The CNE-1/CNE-2 cells were seeded in 96-well microtiter plate and treated with the synthesized compounds at different concentrations, ranging from 0.5 to 100 μM for 24, 48, and 72 h after treatment, the cells were incubated with MTT (5 mg/ml) in 15 μL for 4 h, The formazan precipitate was dissolved in 100 μL DMSO and the absorbance at 595 nm of each well was measured by Multimode Detector DTX880 (Beckman Coulter).

4.2.3. DNA fragmentation assay

DNA was prepared from the untreated and compound-treated cells by standard digestion and organic extraction. Extracted DNA was separated by electrophoresis in 2% agarose gel and visualized with ethidium bromide staining.

4.2.4. PARP cleavage assay

Cells treated for 24 h with different analogues (**8d, 8e, 8j, and 9d**) at 50 μM and without treated, were lysed for 30 min on ice lysis buffer (50 mM Tris–base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM PMSF). Cytosolic proteins were obtained by centrifugation at 15,000*g* for 15 min of extracts of cells. Proteins (25 μg) were separated on 12% SDS–polyacrylamide minigel and transferred to nitrocellulose membranes. Membranes were incubated in blocking solution (5% non-fat dry milk in 0.05% Tween 20 in 50 mM Tris, 150 mM NaCl) and anti-PARP antibody (1:1000 dilution in blocking solution). PARP, intact protein and cleaved fragment were visualized using an enhanced chemiluminescence detection system (Bio-Rad). The expression of β-actin was used as a normalization control for protein loading.

4.2.5. Flow cytometric analysis of apoptosis

Untreated and drug-treated cells were collected by centrifugation and washed twice with ice-cold PBS. The cells were fixed with 70% ethanol at 4 °C overnight, and resuspended at 5×10^5/ml cells of propidium iodide [50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40] and incubated at ice bath for 30 min the tubes were packed with tin foil at ice bath until use in flow cytometry analysis using an FACscan flow cytometry (Becton–Dickinson) with CellQuest software. Surface exposure of phosphatidylserine in apoptosis was detected by detection kit.

4.2.6. Capase-8 and caspase-9 assays

The untreated and compound-treated cells for 0, 2, 4, 8, 14, and 24 h were collected by trypsinization, rinsed twice with PBS, resuspended in lysis buffer (caspase-8 and caspase-9 activity assay kit), and incubated on ice for 15 min. The lysate was centrifuged at 4 °C for 15 min at 20,000*g* for 15 min. The supernatant was assayed for protein by the Bradford method with BSA as a standard, and 0.1 mg was used to assay caspase activity with Ac-IETD-*p*NA and Ac-LEHD-*p*NA as substrate for caspase-8 and caspase-9, respectively, Abs405 nm caused by the production of *p*NA were continuously recorded by Multimode Detector DTX880 (Beckman Coulter) after incubation for 2 h at 37 °C incubator

4.2.7. ROS generation assays

The untreated and compound-treated cells for 0, 2, 4, 6, 8, 13, 19, 24, and 30 h, were collected by trypsinization, precipitated through centrifugation at 1000*g* for 5 min, and washed twice with PBS. Intracellular ROS production was measured by using a fluorescent dye DCFH-DA, which can be converted to DCFH by esterases when the cells take it up. The ROS of cells can oxidize the DCFH to DCF which can be detected by FACS. The cells were incubated with DCFH-DA (10 μM) at 37 °C for 30 min and then analyzed with the FACS.

Acknowledgments

The authors thank the financial supports from the Ministry of Science and Technology of China (2007AA02Z160), the Chinese National Natural Science Foundation (20672068, 20872077, and 90813013), the Ministry of Education (20070003077).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.10.022.

References and notes

1. Lyons, M.; Yu, C.; Toma, R. B.; Cho, S. Y.; Reiboldt, W.; Lee, J.; Breemen, R. B. V. *J. Agric. Food Chem.* **2003**, *51*, 5867.
2. Todaro, A.; Palmeri, R.; Barbagallo, R. N.; Pifferi, P. G.; Spagna, G. *Food Chem.* **2008**, *107*, 1570.
3. Burns, J.; Yokota, T.; Ashihara, H.; Lean, M. J.; Crozier, A. *J. Agric. Food Chem.* **2002**, *50*, 3337.
4. Sanders, T. H.; McMichael, R. W., Jr.; Hendrix, K. W. *J. Agric. Food Chem.* **2000**, *48*, 1243.
5. Ibern-Gomez, M.; Roig-Perez, S.; Lamuela-Raventos, R. M.; Torre-Boronat, M. C. d. l. *J. Agric. Food Chem.* **2000**, *48*, 6352.
6. Baur, J. A.; Pearson, K. J.; Price, N. L.; Jamieson, H. A.; Lerin, C.; Kalra, A.; Prabhu, V. V.; Allard, J. S.; Lopez-Lluch, G.; Lewis, K.; Pistell, P. J.; Poosala, S.; Becker, K. G.; Boss, O.; Gwinn, D.; Wang, M.; Ramaswamy, S.; Fishbein, K. W.; Spencer, R. G.; Lakatta, E. G.; Couteur, D. L.; Shaw, R. J.; Navas, P.; Puigserver, P.; Ingram, D. K.; Cabo, R. d.; Sinclair, D. A. *Nature* **2006**, *444*, 337.
7. Frekmont, L. *Life Sci.* **2000**, *66*, 663.
8. Bureau, G.; Longpre, F.; Martinoli, M.-G. *J. Neurosci. Res.* **2008**, *86*, 403.
9. Lagouge, M.; Argmann, C.; Gerhart-Hines, Z.; Meziane, H.; Lerin, C.; Daussin, F.; Messadeq, N.; Milne, J.; Lambert, P.; Elliott, P.; Geny, B.; Laakso, M.; Puigserver, P.; Auwerx, J. *Cell* **2006**, *127*, 1109.
10. Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218.
11. Minutolo, F.; Sala, G.; Bagnacani, A.; Bertini, S.; Carboni, I.; Placanica, G.; Prota, G.; Rapposelli, S.; Sacchi, N.; Macchia, M.; Ghidoni, R. *J. Med. Chem.* **2005**, *48*, 6783.

12. Cardilea, V.; Lombardoa, L.; Spataforab, C.; Tringali, C. *Bioorg. Chem.* **2005**, *33*, 22.
13. Kuo, P.-L.; Chiang, L.-C.; Lin, C.-C. *Life Sci.* **2002**, *72*, 23.
14. Pettit, G. R.; Grealish, M. P.; Jung, M. K.; Hamel, E.; Pettit, R. K.; Chapuis, J.-C.; Schmidt, J. M. *J. Med. Chem.* **2002**, *45*, 2534.
15. Park, C.-S.; Lee, Y.-C.; Kim, J.-D.; Kim, H.-M.; Kim, C.-H. *Vasc. Pharmacol.* **2004**, *40*, 279.
16. Stivala, L. A.; Savio, M.; Carafoli, F.; Perucca, P.; Bianchi, L.; Maga, G.; Forti, L.; Pagnoni, U. M.; Albinii, A.; Prosperi, E.; Vannini, V. *J. Biol. Chem.* **2001**, *276*, 22586.
17. Roberti, M.; Pizzirani, D.; Simoni, D.; Rondanin, R.; Baruchello, R.; Bonora, C.; Buscemi, F.; Grimaudo, S.; Tolomeo, M. *J. Med. Chem.* **2003**, *46*, 3546.
18. Lion, C. J.; Matthews, C. S.; Stevens, M. F. G.; Westwell, A. D. *J. Med. Chem.* **2005**, *48*, 1292.
19. Reddy, M. V. R.; Mallireddigari, M. R.; Cosenza, S. C.; Pallela, V. R.; Iqbal, N. M.; Robell, K. A.; Kang, A. D.; Reddy, E. P. *J. Med. Chem.* **2008**, *51*, 86.
20. Gao, M.; Wang, M.; Miller, K. D.; Sledge, G. W.; Hutchins, G. D.; Zheng, Q.-H. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5767.
21. Vilar, S.; Quezada, E.; Santana, L.; Uriarte, E.; Yanez, M.; Fraiz, N.; Alcaide, C.; Cano, E.; Orallo, F. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 257.
22. Pan, M. H.; Chang, Y. H.; Brdmaev, V.; Nagabhushanam, K.; Ho, C.-T. *J. Agric. Food Chem.* **2007**, *55*, 7777.
23. Rimando, A. M.; Cuendet, M.; Desmarchlier, C.; Mehta, R. G.; Pezzuto, J. M.; Duke, S. O. *J. Agric. Food Chem.* **2002**, *50*, 3453.
24. Tolomeo, M.; Grimaudo, S.; Cristina, A. D.; Roberti, M.; Pizzirani, D.; Meli, M.; Dusonchet, L.; Gebbia, N.; Abbadessa, V.; Crosta, L.; Barucchello, R.; Grisolia, G.; Invidiata, F.; Simoni, D. *Int. J. Biochem. Cell B* **2005**, *37*, 1709.
25. Zhang, W.; Go, M. L. *Eur. J. Med. Chem.* **2007**, *42*, 841.
26. Galardy, R. E.; Grobelny, D. *J. Med. Chem.* **1985**, *28*, 1422.
27. Niu, Y. L.; Du, W.; Jiang, Y. Y.; Zhao, Y. F. *Phosphorus, Sulfur Silicon Relat. Elem.* **2002**, *177*, 2083.
28. Yang, J.; Jiang, Y. Y.; Liu, F.; Zhao, Y. F. *Protein Peptide Lett.* **2006**, *13*, 129.
29. Ding, G. Y.; Liu, F.; Yang, T.; Jiang, Y. Y.; Fu, H.; Zhao, Y. F. *Bioorg. Med. Chem.* **2006**, *14*, 3766.
30. Pinney, K. G.; Mejia, M. P.; Villalobos, V. M.; Rosenquist, B. E.; Pettit, G. R.; Verdier-Pinard, P.; Hamel, E. *Bioorg. Med. Chem.* **2000**, *8*, 2417.
31. Minutolo, F.; Sala, G.; Bagnacani, A.; Bertini, S.; Carboni, I.; Placanica, G.; Prota, G.; Rapposelli, S.; Sacchi, N.; Macchia, M.; Ghidoni, R. *J. Med. Chem.* **2005**, *48*, 6783.
32. Kothakota, S.; Azuma, T.; Reihard, C.; Klippel, A.; Tang, J.; Chu, K.; McGarry, T. J.; Kirschner, M. W.; Koths, K.; Kwiatkowski, D. J. *Science* **1997**, *278*, 294.
33. Fesik, S. W. *Cell* **2000**, *103*, 273.
34. Simoni, D.; Tolomeo, M. *Curr. Pharm. Des.* **2001**, *7*, 1823.