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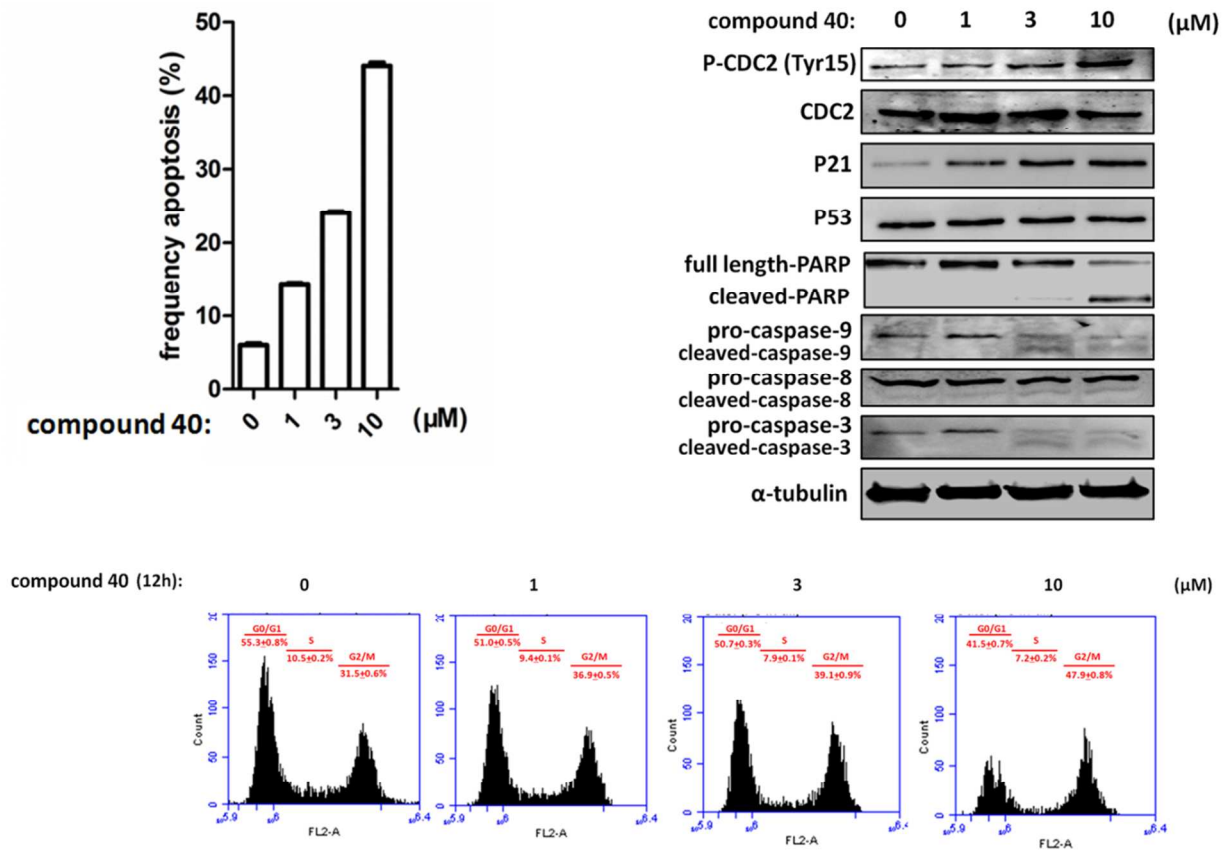

In this present study, we designed and synthesized 19 novel shikonin derivatives **(31-49)** as potent anticancer agents . All the compounds showed dramatically anticancer activities against HeLa, HepG2 and MCF-7 cells. Among which compound **40** showed the best anticancer activities against HeLa cell line with the IC_{50} of 1.26 μM. Furthermore the flowcytometry results demonstrated that compound **40** could obviously induces apoptosis in a dose and time dependent manner and also cause cell cycle arrest at G2/M phase. Western blot results shows the cleavage of PARP and upstream caspase-3were increased, and further caspase -9 was activated by cleavage but not the caspase- 8. Western blot also indicated that compound **40** could induce caspase-9 involved apoptosis and G2/M phase cell cycle arrest via P21, p – CDC2 (Tyr15) pathway independent of P53.

Design, Synthesis and Mechanism of Novel Shikonin Derivatives as Potent Anticancer Agents

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Abstract:

In this present study, a series of novel shikonin derivatives (**30-49**) was designed and synthesized and their antiproliferative activities were evaluated against five different cancer cell lines including HeLa, HepG2, MCF-7, BGC and A549. Some of the compounds shows strong antiproliferative effects against HeLa, HepG2 and MCF-7 with IC_{50} values ranging from 1.26 to 18.50 μM and shows lower side effects towards normal cell lines as compared to shikonin. Compared to other compounds and shikonin itself, compound **40** displayed the much stronger anti-proliferative effects against various cancer cell lines. Furthermore the flowcytometry results demonstrated that compound **40** could obviously induces apoptosis in a dose and time dependent manner and also cause cell cycle arrested at G2/M phase. For further investigation of aforementioned mechanisms, we performed western blot, and we found that cleavage of PARP and upstream caspase-3were increased, and further caspase-9 was activated by cleavage but not the caspase-8. The aforementioned results also indicated that compound **40** could induce caspase-9 involved apoptosis and G2/M phase cell cycle arrested via P21, p – CDC2 (Tyr15) pathway independent of P53.

Key words: Shikonin derivatives; Anticancer; Apoptosis, Caspase-3, Caspase-9, PARP.

1. Introduction

Cancer is considered to be one of the main health threats throughout the world; it has been ranked first in carnage the peoples due to severely causing emerging factors. Various advancements have been achieved recently in the chemotherapeutic management of some patients, yet the continued promise to the tedious task of discovering new anticancer agents remains critically important.[1-6] A variety of anti-cancer drugs have been developed and applied to cure the cancer patients, but numerous drugs have not succeeded to show desirable results due to problem of drug tolerance by cancer. The conventional anti-cancer drugs frequently results in apoptosis, though the cancer cells were sensitive to apoptotic initiation at an early stage but sooner or later exhibits resistance to it because of deregulation of the apoptotic machinery' which is indicated by the overexpression of anti-apoptosis proteins and also signaling defects in apoptosis.[7,8]

Besides, due to the high impact of cancer on human health, apoptosis plays a significant role in cell death mechanisms and largely takes place in various cancer cells as well.[9-11] Whereas mitochondrial apoptotic pathway contains caspase-9 which is an essential member of caspase family protein ,[12] once the mitochondrial apoptotic pathway is activated, the cytochrome c releases from the mitochondrial interrmembrane space and interacts with dATP and APAF1 to develop composite receptor.[12, 13] Moreover, the receptor also helps to recruit and turn on caspase-

9 to induce the activation of downstream proteases (for example, caspase-3 and caspase-7).[14] PARP and Caspase-3 are the vital performers of apoptosis that restrictively cleave almost all or part of key proteins, then cleaved caspase-3 and cleaved PARP come in their activation form after being cut . Therefore, after the increased level of all cleaved caspases and PARP, they induces apoptosis of cancer cells.[15, 16]

Several new drugs have been obtained from natural products and have been active against a wide variety of diseases including cancer. Numerous plants from Boraginaceae family have been used as anti-inflammatory, anti-arthritis and antimicrobial agents in Eurasia.[17-21] Shikonin and its derivatives, which primarily occur in *Lithospermum erythrorhizon*, have been arousing great interest as the hallmark molecules responsible for their significant and fascinating anti-tumor activities by different mechanisms.[22,23] The specific protocols of the cancer research that related to shikonin and alkannin have been focused on induction of cell apoptosis [24,25] and necroptosis,[26,27] DNA topoisomerases, inhibition of angiogenesis, [28-29] and protein tyrosine kinases,[30,31] etc. However, as a potential anti-cancer drug, shikonin itself is poorly soluble and believed to exert strong cytotoxic effects on normal cells.[32] Hence, large numbers of researchers are dedicated to synthesize and prepare some new and effective shikonin derivatives.

During the past decades, number of shikonin derivatives have been synthesized and prepared and studied for their anticancer activities. Many shikonin derivatives with hydroxyl group on the

side chain modifications have been synthesized and evaluated for their anti-tumor effects on various cancer cell lines. Most of the derivatives showed better cytotoxicity than the lead compound shikonin and meanwhile the mechanisms of action were also studied. [33-34]

Moreover, Acetylshikonin, isovalerylshikonin and SH-7 exhibited obvious inhibitory actions on topoisomerase I, stronger than their mother compound shikonin.[35-37] Shikonin glycosyl derivatives were also reported to show similar or stronger cytotoxicity than mother compound shikonin.[38] To reduce the toxicity and side effects of shikonin, we synthesized some related derivatives of shikonin in our previous studies by modification of its structure, and found that the toxicity of shikonin was greatly reduced after ester modifications.[39-44] In this present study we also designed to augment the shikonin with some anticancer components to make it more effective against different cancer cell lines. The shikonin and decorating parts were conjugated by introducing amino acids as bridges. Different steric configurative compounds can be synthesized by using different chiral amino acids as bridges, and these new compounds could also act with differently mechanism against cancer cells. Based on the results of pre-experiments, a series of novel shikonin derivatives was synthesized as potent anticancer agents by using the alanine and phenylalanine as bridges.

2. Result and discussion

2.1 Chemistry

With the aim to obtaining a series of shikonin derivatives containing two functional structures, we designed some twin medicines that introduced cantharidin, norcantharidin and their analogous to shikonin skeleton. We finally conjugated norcantharidin or adjacent dicarboxylic acid to the skeleton of shikonin by using alanine, phenylalanine or norcantharidin as bridges. **(Scheme 1-4)**. However, after several attempts we could not link cantharidin to shikonin and this was probably due to cantharidin's amino acid conformation or electron donating effect of methyl that prevented the linkage. Among the obtained compounds, the yield of compounds **34, 35, 36** was >97% (highest), yield of compounds **44, 45, 46** was <65% (lower), while the compounds **41, 42, 43** had lowest yield i.e., <30% due to their amino acid conformation that resulted lowest yield. It is found that all compounds displayed different dimensional conformations; the carboxyl of **11** is completely naked after derivatization, which is good for the next esterification reaction. Nevertheless, the esterification reaction of **12** is blocked owing to its carboxyl group that is partially covered by phthalicanhydride. No significant effect was found on anti-proliferation activity due to chiral configuration of amino acids in the bridges; however derivatives in which phenylalanine was used as bridge moieties showed better anti-proliferation activities than the alanine.

(Scheme 1-4)

2.2 Biological activities

2.2.1 In vitro antitumor and cytotoxicity evaluation

Antitumor activities of all compounds against different cancer cell lines i.e., HeLa (human cervix cell line), $HepG_2$ (human liver cell line), MCF-7 (humane breast cell line), A549 (human lungs cell line) and BGC (human gastric cell line) were determined using the MTT assay. Each of these cell lines were incubated with five different concentrations (0, 0.3 μM, 1 μM, 3 μM, 10 μM) of all synthesized compounds for 24 hours and subsequently the IC_{50} (half maximal inhibitory concentration) values were calculated as shown in **(Table 1)**.

(Table 1)

From IC_{50} values it was found that after modification, not all the obtained compounds showed higher IC_{50} against five cell lines when compared with shikonin. And interestingly all the compounds lost their anti-proliferative activities against A549 and anti-proliferative activities were much lower than the shikonin against BGC. This is probably due to our modification that improved the selectivity and reduced the cytotoxicity. To determine the reduced cytotoxicity and clinically safe use of compounds MTT assay against L02 (human normal liver cell line) was performed and results (**Table 1**) showed that all compounds have no effects against L02.Some of the compounds showed strong effects against HeLa, HepG2 and MCF-7 cell lines with IC_{50} values ranging from 1.26μM to 18.50μM. Furthermore, the compound **40** showed best anti-proliferative activities against HeLa cells with the lowest IC_{50} value (1.26 μM) compared to

128 shikonin (3.11 μM) itself and was selected for further experiments.

129 **2.2.2 Apoptosis is induced in HeLa cells in dose and time dependent manner**

130 Annexin V and PI staining can distinguish the living cells (annexin V-/PI-), as early
131 apoptotic cells (annexin V+/PI-) and late apoptotic cells (annexin V+/PI+). To validate whether
132 compound **40** could cause the growth inhibition of HeLa cells by in vitro apoptosis, annexin V-
133 FITC/PI double staining assay was performed. After treating HeLa cells with different
134 concentrations (0, 1 μM, 3 μM, 10 μM) of compounds **40** for 24 hours, HeLa cells showed
135 considerable sensitivity to compounds **40** in dose-dependent manner. For highest concentration
136 (10 μM), apoptotic rates reached upto 43.95% **(Fig.1A, B)**, thus suggesting that these compounds
137 can induce the apoptosis in vitro by targeting the cancer cells. Meanwhile, the time dependent
138 assay results also indicated that when HeLa cells were treated with compound **40** in a time
139 dependent manner, the percentage of apoptotic cells were increased, compared with the mock
140 group, as shown in **(Fig.2A,B)**. In conclusion, it is obvious from the aforementioned results that
141 compound **40** could induce apoptosis in Hela cells in dose and time dependent manner.

142 **(Fig.1A, B)**

143 **(Fig.2A, B)**.

2.2.3 Cell cycle arrest in HeLa cells in dose dependent manner

To gain better understanding on the potency of compound **40**, we further explored the effect of compound **40** on the cell cycle to ascertain that the cells are blocked in mitosis. HeLa cells were treated with different concentration (0 μM, 1 μM, 3 μM, 10 μM) of compound **40** for 12hours. The results demonstrated that the treatment of HeLa cells with compound **40** led to an obvious G2/M arrest in a concentration dependent manner as shown in (**Fig.3**), Incubation of cells with 3μM of compound **40** caused 39.1% of cells arrested at G2/M phase as compare to control. When the concentration of compound **40** increased upto 10 μM, 47.9% of cells were arrested in G2/M phase. In summary, effective doses of compound **40** seem to cause an arrest of cells in G2/M phase, which leads to a significant increase in the number of apoptotic cells ultimately.

Western blot analysis

In order to investigate the process of apoptosis, we further performed the western blot to detect the expression of some related proteins in the apoptosis related pathway. From the western blot results, we found that cleavage of PARP and upstream caspase-3 were increased, indicating that compound **40** could induce caspase activation in apoptosis. Further determining the protein levels of caspase-8 and caspase-9, we found that caspase-9 other than caspase-8 was activated by cleavage. Thus, caspase-9 but not caspase-8 was involved in the apoptosis induced by compound **40**. We also found the level of P21 which is the downstream target of P53, was upregulated, and

enhanced the phosphorylation of its downstream target CDC2. However, no obvious change was found for P53 as shown in (Fig.4). Based on the above cell cycle results, we concluded P21 was activated independent of P53 and the downstream CDC2 was phosphorylated, which contributed to the G2/M arrest in Hela cells as described in pathway. (Fig.5)

(Fig.4)

(Fig.5)

3. Conclusion

In our present study we synthesized a series of novel shikonin derivatives **(30-49).** Selectivity and cytotoxicity assays were performed against five cancer cell lines along with one normal cell line. Some of the compounds showed strong effects against HeLa, HepG2 and MCF-7 with IC_{50}values ranging from 1.26 μM to 18.50 μM. Among them, compound **40** displayed the much stronger anti-proliferative effects against various cancer cell lines. Detailed apoptotic mechanism studies with compound **40** suggested that through the cell cycle and apoptosis analysis, compound **40** showed the best antiproliferation activities and exhibited strong ability to inhibit the proliferation of HeLa cancer cells by inducing a high levels of apoptosis in a dose and time dependent manner, also causes HeLa cells arrested in G2/M phase. Western blot results also

indicated that compound **40** could induce caspase-9 involved apoptosis and G2/M phase cell cycle arrest via P21, p – CDC2 (Tyr15) pathway independent of P53.

4. Materials and methods

4.1 Chemicals

All chemicals (reagent grade) were purchased from J&K Chemical Ltd., and Nanjing Chemical Reagent Co. Ltd. (China). All the ^{1}H NMR spectra were recorded on a Bruker DRX 500 spectrometer in $CDCl_3$,. TLC was carried out on glass-backed silica gel sheets (silica gel 60Å GF254). The ESI-MS spectra were obtained on a Mariner Biospectrometry Workstation (ESI-TOF) mass spectrometer. Chemical shifts (δ) for ^{1}H NMR spectra were reported in ppm (δ). Melting points (uncorrected) were measured on a XT4 MP Apparatus (Taike Corp., Beijing, China).

4.1.1 General synthesis procedure of compound 8-17 and 21-29

A mixture of compound **4-7** (50 mmol), amino acid (50 mmol) were dissolved in acetic acid and stirred on 120°Cover night. Reaction mixture was poured into ice water and white precipitate was filtered and dried under vacuum to obtain the compound **8-17** and **21-29** (Scheme 1, 2).

4.1.2 General synthesis procedure of compound 31-49

Compounds **8-17** and **21-29** were dissolved in 16 mL of dichloromethane and 0.072 g (0.354 mmol) of *N, N'*-dicyclohexylcarbodiimide (DCC) was added into reaction system. The reaction

196 mixture was stirred under nitrogen atmosphere in ice bath for 15 min. 0.004 g (0.044 mmol) of 4-
197 dimethylaminopyridine (DMAP) was added, stirred in the ice bath and continued for further 15
198 min. Then 0.050 g of Shikonin (0.175mmol) was added to the reaction mixture and stirred in the
199 ice bath for 12 hours to afford the target compounds **31-49** (Scheme 3, 4).

200 **4.1.2.1** (2R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-
201 ((4R,7S)-1,3-dioxohexahydro-1H-4,7-epoxyisoindol-2(3H)-yl)-3-phenylpropanoate(**31**)

202 Red powder, 76% yield. Mp: 69.5-71.4°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.592 (s, 1H);
203 12.477 (s, 1H); 7.286-7.136 (m, 8H); 7.033 (s, 1H); 6.133 (t, 1H, J_1 = 6.5 Hz, J_2 = 5 Hz); 5.148-
204 5.066 (m, 1H); 5.024-4.991 (m, 2H); 4.898-4.843 (m, 1H); 3.504-3.308 (m, 3H); 2.833-2.715 (m,
205 2H); 2.607-2.560 (m, 1H); 2.501-2.440 (m, 1H); 1.975-1.518 (m, 4H); 1.369-1.095 (m, 4H). ESI-
206 MS: Calcd for $C_{33}H_{31}NO_9$ ([M-H]$^-$) 584.20, found 584.1025. Anal. Calcd for $C_{33}H_{31}NO_9$: C,
207 67.68; H, 5.34; N, 2.39; O, 24.59. Found: C, 67.71; H, 5.38; N, 2.41; O, 24.61.

208 **4.1.2.2** (2S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-
209 ((4R,7S)-1,3-dioxohexahydro-1H-4,7-epoxyisoindol-2(3H)-yl)-3-phenylpropanoate(**32**)

210 Red powder, 70% yield. Mp: 77.3-79.2°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.591 (s, 1H);
211 12.476 (s, 1H); 7.286-7.136 (m, 8H); 7.034 (s, 1H); 6.135 (t, 1H, J_1 = 4.5 Hz, J_2 = 7.5 Hz); 5.104
212 (t, 1H, J_1 = 6.5 Hz, J_2 = 6.5 Hz); 5.025-4.991 (m, 2H); 4.909-4.851 (m, 1H); 3.504-3.456 (m, 2H);
213 3.360-3.309 (m, 1H); 2.834-2.793 (m, 1H); 2.731-2.702 (m, 1H); 2.611-2.537 (m, 1H); 2.502-

2.430 (m, 1H); 1.899-1.772 (m, 2H); 1.975-1.518 (m, 3H); 1.369-1.095 (m, 3H). ESI-MS: Calcd for $C_{33}H_{31}NO_9$ ([M-H]$^-$) 584.20, found 584.2288. Anal. Calcd for $C_{33}H_{31}NO_9$: C, 67.68; H, 5.34; N, 2.39; O, 24.59. Found: C, 67.70; H, 5.36; N, 2.40; O, 24.62.

4.1.2.3 1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-((4R,7S)-1,3-dioxohexahydro-1H-4,7-epoxyisoindol-2(3H)-yl)-3-phenylpropanoate(**33**)

Red powder, 70% yield. Mp: 68.7-70.5°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.591 (s, 1H); 12.476 (s, 1H); 7.286-7.136 (m, 8H); 7.034 (s, 1H); 6.135 (t, 1H, J_1 = 4.5 Hz, J_2 = 7.5 Hz); 5.104 (t, 1H, J_1 = 6.5 Hz, J_2 = 6.5 Hz); 5.025-4.991 (m, 2H); 4.909-4.851 (m, 1H); 3.504-3.456 (m, 2H); 3.360-3.309 (m, 1H); 2.834-2.793 (m, 1H); 2.731-2.702 (m, 1H); 2.611-2.537 (m, 1H); 2.502-2.430 (m, 1H); 1.899-1.772 (m, 2H); 1.975-1.518 (m, 3H); 1.369-1.095 (m, 3H). ESI-MS: Calcd for $C_{33}H_{31}NO_9$ ([M-H]$^-$) 584.20, found 584.2055. Anal. Calcd for $C_{33}H_{31}NO_9$: C, 67.68; H, 5.34; N, 2.39; O, 24.59. Found: C, 67.69; H, 5.40; N, 2.43; O, 24.64.

4.1.2.4 (2R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoate(**34)**

Red powder, 98% yield. Mp: 70.5-71.8°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.566(s, 1H); 12.443 (s, 1H); 7.849-7.736 (m, 4H); 7.224-7.196 (m, 7H); 6.948 (s, 1H, naphthoquinone-H); 6.108 (t, 1H, J_1 = 5 Hz, J_2 = 6 Hz); 5.266-5.233 (m, 1H); 5.019 (t,1H, J_1 = 7.5 Hz, J_2 = 5.5 Hz); 3.640-3.572 (m, 2H); 2.640-2.610 (m, 1H); 2.499-2.440 (m, 1H); 1.615-1.585 (m, 3H); 1.483-

1.465 (m, 3H). ESI-MS: Calcd for $C_{33}H_{27}NO_8$ ([M-H]$^-$) 564.17, found 564.2332. Anal. Calcd for $C_{33}H_{27}NO_8$: C, 70.08; H, 4.81; N, 2.48; O, 22.63. Found: C, 70.13; H, 4.95; N, 2.71; O, 22.72.

4.1.2.5 (2S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoate(**35**)

Red powder, 97% yield. Mp: 60.9-62.6°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.565 (s, 1H); 12.443 (s, 1H); 7.849-7.833 (m, 2H); 7.751-7.735 (m, 2H); 7.240-7.168 (m, 7H); 6.949 (s, 1H); 6.109 (t, 1H, J_1 = 5.5 Hz, J_2 = 5.5 Hz); 5.268-5.236 (m, 1H); 5.021 (t, 1H, J_1 = 7 Hz, J_2 = 6 Hz); 3.653-3.546 (m, 2H); 2.641-2.611 (m, 1H); 2.499-2.441 (m, 1H); 1.615-1.585 (m, 3H); 1.484-1.465 (m, 3H). ESI-MS: Calcd for $C_{33}H_{27}NO_8$ ([M-H]$^-$) 564.17, found 564.2804. Anal. Calcd for $C_{33}H_{27}NO_8$: C, 70.08; H, 4.81; N, 2.48; O, 22.63. Found: C, 70.21; H, 4.86; N, 2.51; O, 22.73.

4.1.2.6 1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoate(**36**)

Red powder, 98% yield. Mp: 61.6-63.8°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.565 (s, 1H); 12.442 (s, 1H); 7.839-7.810 (m, 2H); 7.745-7.713 (m, 2H); 7.210-7.194 (m, 7H); 6.949 (s, 1H); 6.108 (t, 1H, J_1 = 5.5 Hz, J_2 = 5.5 Hz); 5.266-5.237 (m, 1H); 5.022 (t,1H, J_1 = 7 Hz, J_2 = 6 Hz); 3.641-3.573 (m, 2H); 2.640-2.612 (m, 1H); 2.497-2.442 (m, 1H); 1.614-1.586 (m, 3H); 1.483-1.465 (m, 3H). ESI-MS: Calcd for $C_{33}H_{27}NO_8$ ([M-H]$^-$) 564.17, found 564.2018. Anal. Calcd for

$C_{33}H_{27}NO_8$: C, 70.08; H, 4.81; N, 2.48; O, 22.63. Found: C, 70.23; H, 4.91; N, 2.62; O, 22.69.

4.1.2.7 (2R)-(R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3- en-1-yl-2-(1,3-dioxohexahydro-1H-isoindol-2(3H)-yl)-3- phenylpropanoate(**37**)

Red powder, 64% yield. Mp: 72.1-73.6 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.601 (s, 1H); 12.455 (s, 1H); 7.315-7.168 (m, 6H); 7.022 (s, 1H); 6.104 (t, 1H, J_1 = 5 Hz, J_2 = 7 Hz); 5.160-5.091 (m, 3H); 3.903 (d, 2H, J = 15 Hz); 3.778 (t, 3H, J_1 = 7 Hz, J_2 = 8.5 Hz); 3.532-3.509 (m, 3H); 2.943 (t, 3H, J_1 = 8 Hz, J_2 = 7 Hz); 2.782 (t, 3H, J_1 = 4 Hz, J_2 = 3.5 Hz); 1.715 (s, 3H); 1.586 (s, 3H). ESI-MS: Calcd for $C_{33}H_{33}NO_8$ ([M-H]$^-$) 570.62, found 570.631. Anal. Calcd for $C_{33}H_{33}NO_8$: C, 69.34; H, 5.82; N, 2.45; O, 22.39. Found: C, 69.47; H, 5.96; N, 2.51; O, 22.42.

4.1.2.8 *(*R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl-2-(1,3-dioxohexahydro-1H-isoindol-2(3H)-yl)-3-phenylpropanoate(**38**)

Red powder, 64% yield. Mp: 72.1-73.6°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.596 (s, 1H); 12.452 (s, 1H); 7.286-7.180 (m, 6H); 7.020 (s, 1H); 6.103 (t, 1H, J_1 = 5 Hz, J_2 = 6.5 Hz); 5.161-5.091 (m, 3H); 3.903 (d, 2H, J =15 Hz); 3.778 (t, 3H, J_1 = 7 Hz, J_2 = 8.5Hz); 3.568-3.508 (m, 3H); 2.943 (t,3H, J_1 = 8 Hz, J_2 = 7 Hz); 2.782 (t, 3H, J_1 = 4 Hz, J_2 = 3.5 Hz); 1.716 (s, 3H); 1.585 (s, 3H). ESI-MS: Calcd for $C_{33}H_{33}NO_8$ ([M-H]$^-$) 570.62, found 570.631. Anal. Calcd for $C_{33}H_{33}NO_8$: C, 69.34; H, 5.82; N, 2.45; O, 22.39. Found: C, 69.45; H, 5.93; N, 2.58; O, 22.46.

4.1.2.9 (2R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-

((3aR,4S,7S,7aS)-1,3-dioxo-3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindol-2(3H)-yl)-3-phenylpropanoate(**39**)

Red powder, 64% yield. Mp: 60.5-62.6°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.594 (s, 1H); 12.448 (s, 1H); 7.286-7.256 (m, 2H); 7.223-7.173 (m, 5H); 6.960 (s, 1H); 6.286-6.220 (m, 2H); 6.121 (t, 1H, J_1 = 6 Hz, J_2 = 5.5 Hz); 5.140-5.077 (m, 2H); 3.554-3.438 (m, 2H); 3.232 (s, 1H); 3.168 (s, 1H); 2.676-2.592 (m, 2H); 2.536-2.466 (m, 2H); 1.715-1.582 (m, 8H). ESI-MS: Calcd for $C_{34}H_{31}NO_8$ ([M-H]$^-$) 580.20, found 580.2168. Anal. Calcd for $C_{34}H_{31}NO_8$: C, 70.21; H, 5.37; N, 2.41; O, 22.01. Found: C, 70.34; H, 5.51; N, 2.55; O, 22.31.

4.1.2.10 1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-((3aR,4S,7S,7aS)-1,3-dioxo-3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindol-2(3H)-yl)-3-phenylpropanoate(**40**)

Red powder, 67% yield. Mp: 51.6-53.7°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.598 (s, 1H); 12.451 (s, 1H); 7.287-7.176 (m, 7H); 6.963 (s, 1H); 6.289-6.223 (m, 2H); 6.125 (t, 1H, J_1 = 4.5 Hz, J_2 = 6 Hz); 5.140-5.079 (m, 2H); 3.556-3.439 (m,2H); 3.253 (s, 1H); 3.173 (s, 1H); 2.689-2.595 (m, 2H); 2.538-2.470 (m, 2H); 1.718-1.584 (m, 8H). ^{13}C NMR (300 MHz, $CDCl_3$) δ: 185.37, 183.12, 175.23, 172.57, 161.21, 160.14, 150.24, 149.22, 139.93, 139.56, 139.15, 135.68, 134.33, 133.64, 132.26, 125.28, 118.37, 74.38, 62.86, 55.34, 48.80, 47.65, 16.89, 36.97, 31.23, 20.21, 19.87. ESI-MS: Calcd for $C_{34}H_{31}NO_8$ ([M-H]$^-$) 580.20, found 580.2368. Anal. Calcd for

285 $C_{34}H_{31}NO_8$: C, 70.21; H, 5.37; N, 2.41; O, 22.01. Found: C, 70.32; H, 5.41; N, 2.51; O, 22.31.

286 **4.1.2.11** (2R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-

287 en-1-yl-2-((4R,7S)-1,3-dioxohexahydro-1H-4,7-epoxyisoindol-2(3H)-yl)propanoate(**41**)

288 Red powder, 27% yield. Mp: 63.2-64.8°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.601(s, 1H);

289 12.484 (s, 1H); 7.028 (s, 2H); 7.049 (s, 1H); 6.151-6.063(m, 1H); 6.035 (t, 1H, J_1 = 7.5 Hz, J_2 =

290 11Hz); 5.168-5.063 (m, 1H); 5.024-4.991 (m, 2H); 4.898-4.843 (m, 1H); 3.504-3.308 (m, 3H);

291 2.833-2.715 (m, 2H); 2.607-2.560 (m, 1H); 2.501-2.440 (m, 1H); 1.975-1.518 (m, 3H);

292 1.568~1.412 (m, 3H);1.387-1.196 (m, 3H).ESI-MS: Calcd for $C_{27}H_{27}NO_9$ ([M-H]$^-$) 508.50,

293 found 508.5245. Anal. Calcd for $C_{27}H_{27}NO_9$: C, 63.65; H, 5.34; N, 2.75; O, 28.26. Found: C,

294 63.72; H, 5.43; N, 2.81; O, 28.37.

295 **4.1.2.12**(2S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-

296 ((4R,7S)-1,3-dioxohexahydro-1H-4,7-epoxyisoindol-2(3H)-yl)propanoate(**42**)

297 Red powder, 25% yield. Mp: 62.7-63.8°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.601(s, 1H);

298 12.484 (s, 1H); 7.028 (s, 2H); 7.049 (s, 1H); 6.151~6.063(m, 1H); 6.035 (t, 1H, J_1 = 7.5 Hz, J_2 =

299 11Hz); 5.168-5.063 (m, 1H); 5.024-4.991 (m, 2H); 4.898-4.843 (m, 1H); 3.504-3.308 (m, 3H);

300 2.833-2.715 (m, 2H); 2.607-2.560 (m, 1H); 2.501-2.440 (m, 1H); 1.975-1.518 (m, 3H); 1.568-

301 1.412 (m, 3H);1.387-1.196 (m, 3H).ESI-MS: Calcd for $C_{27}H_{27}NO_9$ ([M-H]$^-$) 508.50, found

302 508.5245. Anal. Calcd for $C_{27}H_{27}NO_9$: C, 63.65; H, 5.34; N, 2.75; O, 28.26. Found: C, 63.72; H,

303 5.43; N, 2.81; O, 28.37.

304 **4.1.2.13**1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-

305 ((4R, 7S)-1,3-dioxohexahydro-1H-4,7-epoxyisoindol-2(3H)-yl)propanoate(**43**)

306 Red powder, 30% yield. Mp: 71.8-72.7°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.601(s, 1H);

307 12.484 (s, 1H); 7.028 (s, 2H); 7.049 (s, 1H); 6.151-6.063(m, 1H); 6.035 (t, 1H, J_1 = 7.5 Hz, J_2 =

308 11Hz); 5.168-5.063 (m, 1H); 5.024-4.991 (m, 2H); 4.898-4.843 (m, 1H); 3.504-3.308 (m, 3H);

309 2.833-2.715 (m, 2H); 2.607-2.560 (m, 1H); 2.501-2.440 (m, 1H); 1.975-1.518 (m, 3H);

310 1.568~1.412 (m, 3H);1.387-1.196 (m, 3H).ESI-MS: Calcd for $C_{27}H_{27}NO_9$ ([M-H]$^-$) 508.50, found

311 508.5245. Anal. Calcd for $C_{27}H_{27}NO_9$: C, 63.65; H, 5.34; N, 2.75; O, 28.26. Found: C, 63.72; H,

312 5.43; N, 2.81; O, 28.37.

313 **4.1.2.14**(R)-(R)-2-(3-hydroxybenzoyl)-6-methylhepta-1,5-dien-3-yl2-(1,3-dioxoisoindolin-2-

314 yl)propanoate (**44**)

315 Red powder, 60% yield. Mp: 76.5-77.3°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.569 (s, 1H);

316 12.444 (s, 1H); 7.952-7.923 (m, 2H); 7.821-7.794 (m, 2H); 7.204 (s, 2H); 6.950 (s, 1H); 6.080 (t,

317 1H, J_1 = 9 Hz, J_2 = 11 Hz); 5.117-4.978 (m, 2H); 2.677-2.585 (m, 1H); 2.518-2.419 (m, 1H);

318 1.795-1.755 (m, 3H); 1.629 (s, 3H); 1.485 (s, 3H). ESI-MS: Calcd for $C_{26}H_{25}NO_6$ ([M-H]$^-$)

319 446.17, found 446.1760. Anal. Calcd for $C_{26}H_{25}NO_6$: C,69.79; H, 5.63; N, 3.13; O, 21.45. Found:

320 C, 69.36; H, 5.88; N, 3.09; O, 21.32

4.1.2.15(S)-(R)-2-(3-hydroxybenzoyl)-6-methylhepta-1,5-dien-3-yl2-(1,3-dioxoisoindolin-2-yl)propanoate (**45**)

Red powder, 55% yield. Mp: 55.7-57.5°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.581 (s, 1H); 12.476 (s, 1H); 7.929-7.901 (m, 2H); 7.822-7.770 (m, 2H); 7.208 (s, 2H); 7.013 (s, 1H); 6.078 (t, 1H, J_1 = 9 Hz, J_2 = 10.5 Hz); 5.117-4.975 (m, 2H); 2.634-2.568 (m, 1H); 2.516-2.441 (m, 1H); 1.795-1.741 (m, 3H); 1.648-1.565 (m, 3H); 1.491 (s, 3H). ^{13}C NMR (300 MHz, $CDCl_3$) δ: 186.3, 184.8, 173.9, 171.5, 166.5, 165.8, 151.8, 140.2, 130.8, 130.1, 125.1, 122.5, 110.6, 73.8, 60.5, 31.9, 25.8, 19.1, 12.4. ESI-MS: Calcd for $C_{26}H_{25}NO_6$ ([M-H]$^-$) 446.17, found 446.1728. Anal. Calcd for $C_{26}H_{25}NO_6$: C, 69.79; H, 5.63; N, 3.13; O, 21.45. Found: C, 69.76; H, 5.52; N, 3.09; O, 21.57.

4.1.2.16(R)-2-(3-hydroxybenzoyl)-6-methylhepta-1,5-dien-3-yl2-(1,3-dioxoisoindolin-2-yl)propanoate1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-(1,3-dioxoisoindolin-2-yl)propanoate(**46**)

Red powder, 65% yield. Mp: 43.2-44.8°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.572 (s, 1H); 12.466 (s, 1H); 7.954-7.952 (m, 2H); 7.824-7.795 (m, 2H); 7.207 (s, 2H); 6.950 (s, 1H); 6.081 (t, 1H, J_1 = 7.5 Hz, J_2 = 11 Hz); 5.118-5.069 (m, 2H); 2.652-2.609 (m, 1H); 2.510-2.445 (m, 1H); 1.796-1.631 (m, 9H). ESI-MS: Calcd for $C_{26}H_{25}NO_6$ ([M-H]$^-$) 446.17, found 446.1728. Anal. Calcd for $C_{26}H_{25}NO_6$: C, 69.79; H, 5.63; N, 3.13; O, 21.45. Found: C, 69.66; H, 5.52; N, 3.24; O,

339 21.40.

340 **4.1.2.17**(2R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-

341 yl-2-(1,3-dioxohexahydro-1H-isoindol-2(3H)-yl)propanoate(**47**)

342 Red oil, 90% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.588 (s, 1H); 12.455 (s, 1H); 7.207 (s,

343 2H); 7.027 (s, 1H); 6.070 (t, 1H, J_1 = 11.5 Hz, J_2 = 6.5 Hz); 5.094 (t, 1H, J_1 = 11.5 Hz, J_2 = 12

344 Hz); 4.919-4.846 (m, 1H); 2.962-2.905 (m, 3H); 2.694-2.607 (m, 1H); 2.570-2.472 (m, 1H);

345 1.861-1.481 (m, 16H). ESI-MS: Calcd for $C_{27}H_{29}NO_8$ ([M-H]$^-$) 494.19, found 494.2081. Anal.

346 Calcd for $C_{27}H_{29}NO_8$: C, 65.44; H, 5.90; N, 2.83; O, 25.83. Found: C, 65.56; H, 6.08; N, 2.99; O,

347 25.97.

348 **4.1.2.18**(2S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-

349 yl-2-(1,3-dioxohexahydro-1H-isoindol-2(3H)-yl)propanoate(**48**)

350 Red oil, 87% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.594 (s, 1H); 12.447 (s, 1H); 7.203 (s,

351 2H); 7.006 (s, 1H); 6.067 (t, 1H, J_1 = 6.5 Hz, J_2 = 13.5 Hz); 5.099 (t, 1H, J_1 = 12 Hz, J_2 = 12.5

352 Hz); 4.921-4.848 (m, 1H); 2.961-2.900 (m, 3H); 2.715-2.625 (m, 1H); .559-2.461 (m, 1H);

353 1.876-1.580 (m, 16H). ESI-MS: Calcd for $C_{27}H_{29}NO_8$ ([M-H]$^-$) 494.19, found 494.1879. Anal.

354 Calcd for $C_{27}H_{29}NO_8$: C, 65.44; H, 5.90; N, 2.83; O, 25.83. Found: C, 65.59; H, 6.67; N, 2.97; O,

355 25.90.

356 **4.1.2.19**1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-3-yl)-4-methylpent-3-en-1-yl-2-

(1,3-dioxohexahydro-1H-isoindol-2(3H)-yl)propanoate(**49**)

Red oil, 93% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.599 (s, 1H); 12.453 (s, 1H); 7.206 (s, 2H); 7.012 (s, 1H); 6.071 (t, 1H, J_1 = 6.5 Hz, J_2 = 4 Hz); 5.102 (t, 1H, J_1 = 11.5 Hz, J_2 = 12.5 Hz); 4.923-4.850 (m, 1H); 2.964-2.908 (m, 3H); 2.718-2.610 (m, 1H); 2.566-2.467 (m, 1H); 1.880-1.547 (m, 16H). ESI-MS: Calcd for $C_{27}H_{29}NO_8$ ([M-H]$^-$) 494.19, found 494.2054. Anal. Calcd for $C_{27}H_{29}NO_8$: C, 65.44; H, 5.90; N, 2.83; O, 25.83. Found: C, 65.58; H, 6.01; N, 2.94; O, 25.90.

4.2. Cell culture

All cell lines were obtained from State Key Laboratory of Pharmaceutical Biotechnology(Nanjing University) and maintained in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

4.3. Cell Viability Assay (MTT Assay)

Cells were planted in 96-well plates at appropriate densities to ensure exponential growth throughout the experimental period (2.0×10^3 cells per well), and then allowed to adhere for 6 hours. Cells were then treated for 20 hours with four serial concentrations (0, 1 μM, 3 μM, 10 μM) of each compound. Shikonin and norcantharidin were used as positive controls. After 20 hours incubation, 20 μL MTT solution was added in each well to a final concentration of 4 mg/mL. Plates were then incubated for further 4 hours, after incubation all the plates were

centrifuged (1500 rpm, 10 min) and then the entire medium was removed. 150 μL of DMSO was added to each well for coloration. The plates were shaken vigorously to ensure complete solubilization for 10 min at room temperature Optometric density (OD) was read on a microplate reader ELx800 (BioTek, Highland Park, Winooski, VT, USA) at the wavelength of 570 nm, and subsequently the data was analyzed using Origin7.5.

4.4. Analysis for Apoptosis by Flow Cytometry.

Apoptosis was detected using an Apoptosis Detection Kit (invitrogen, Eugene, Oregon, USA). Briefly, cells were plated in 6 well plates (5.0×10^4 cells/well) and incubated at 37 ^{o}C for 12 hours. Exponentially growing cells were then incubated with compounds **40** of different concentrations (0 μM, 1 μM, 3 μM, 10 μM). Following 24 hours treatment, cells were collected and washed twice with PBS and subsequently washed once with 1 × binding buffer and then stained with 5μL of Annexin V-FITC and 2.5μL of PI (5 μg/mL) in 1 × binding buffer for 30 min at room temperature in the dark. Apoptotic cells were quantified using a FACScan cytofluorometer(PT. MadagasiBrosa Inc. Jl. BatangHari No.73, Propinsi Sumatera Utara, Indonesia). Statistical analysis was performed using WINMDI software version 2.8 (The Scripps Research Institute (TSRI), San Diego, CA, USA).

4.5. Analysis for cell cycle by Flow cytometry

HepG2 cells were plated in 6-well plates (5.0×10^3 cells per well) and incubated at 37 ^{o}C for 24 hours. Exponentially growing cells were then incubated with the compound **40** at different concentrations (0 mM, 1 mM, 3 mM, and 10 mM). After 12 hours, untreated cells (control) or cells treated with compound **40** were centrifuged at 1500 rpm at 4 ^{o}C for 10 min, and then fixed in 70% ethanol at 4 ^{o}C for at least 12 hours and subsequently resuspended in phosphate buffered saline (PBS) containing 0.1 mg/ mL RNase A and 5μg /mL propidium iodide (PI). The cellular DNA content, for cell cycle distribution analysis was measured by flow cytometry using a Becton_Dickinson FACScan cytoflouorometer, plotting at least 10,000 events per sample. The percentage of cells in the G0/G1, S and G2/M phases of the cell cycle were determined using the Verity Software BD Accuri C6 software.

4.6. Western blot analysis

Cells were rinsed with PBS and lysed in cold RIPA buffer (10 Mm Tris-HCl, 1 mM EDTA, 1% SDS, 1 mM DTT, 0.1 mM PMSF, protease inhibitors, 1% Nonidet P-40, pH 8.0). Lysates were centrifuged at 12,000 g for 10 min at 4°C to remove cell debris and protein content was analyzed by a Micro BCA Protein Assay kit (Pierce). Aliquots of proteins (40~60 μg) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to polyvinylidenedifluoride (PVDF) membranes. Membranes were blocked with 5% nonfat dry milk or BSA in TBST (TBS plus 0.1% Tween 20) for 1 hour. Blots were then

probed with primary antibodies against PARP, caspase-3, caspase-8, caspase-9, P53, P21, CDC2 , phosphor-CDC2(Tyr 15) and α-tubulin, incubated at 4°C overnight, followed by HRP-conjugated secondary antibodies and protein expression was detected with an enhanced chemiluminescent reagent (Cell Signaling Technology). The PARP antibody was purchased from Oncogene Company, and other antibodies were purchased from Cell Signalling Technology Company. The autoradiographic intensity of each band was scanned.

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Figure and table captions:

Scheme. 1 Synthesis of Phenylalanine Derivatives.

Scheme. 2 Synthesis of Alanine Derivatives.

Scheme. 3 Synthesis of Phenylalanine Shikonin Ester.

Scheme. 4 Synthesis of Alanine Shikonin Ester.

Table. 1 In Vitro Anticancer Activity of Shikonin Derivatives Against Five Cancer Cell Lines.

Fig.1A+B Cellular apoptosis study of compound **40** tested on HeLa cellsin dose dependent manner(0 μM,1 μM,3 μM,10 μM). The percentage of early apoptotic cells in the lower right quadrant (annexin V-FITC-positive／PI-negative cells), as well as late apoptotic cells located in the upper right quadrant (annexin V-FITC-positive／PI-positive cells).

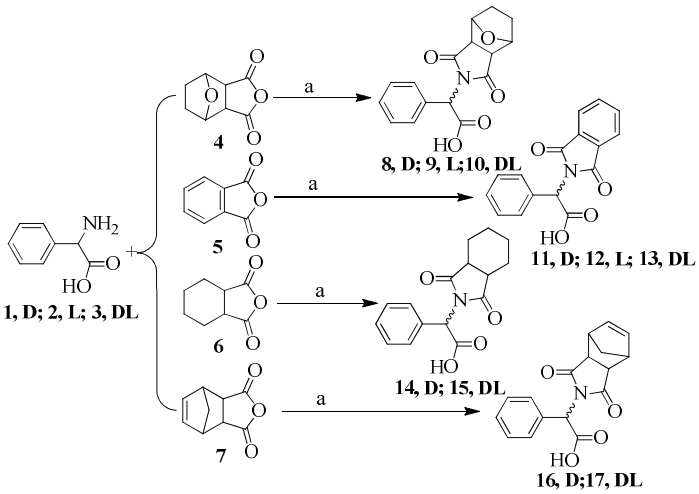
Fig.2A+B Cellular apoptosis study of compound **40** tested on HeLa cells in time dependent manner compared(0h,8h,16h and 24h) with the mock group.

Fig.3 Effect of comound **40** on the cell cycle distribution of HeLa cells in dose dependent manner (0 μM,1 μM,3 μM,10 μM).

Fig. 4 Immunodetection of apoptosis related proteins of HeLa cells treated with different concentrations (0 μM,1 μM,3 μM,10 μM) of compound **40**.

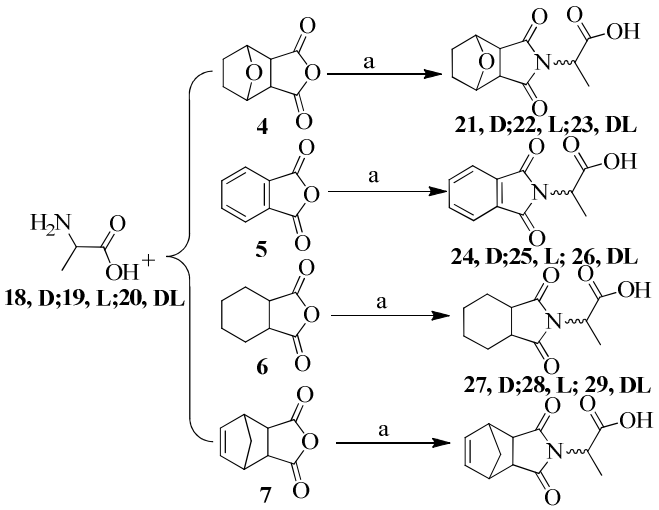
Fig.5 Compound **40** induced caspase-9 involved apoptosis and G2/M phase cell cycle arrest via P21, p – CDC2 (Tyr15) pathway independent of P53.

Scheme 1. Synthesis of Phenylalanine Derivatives



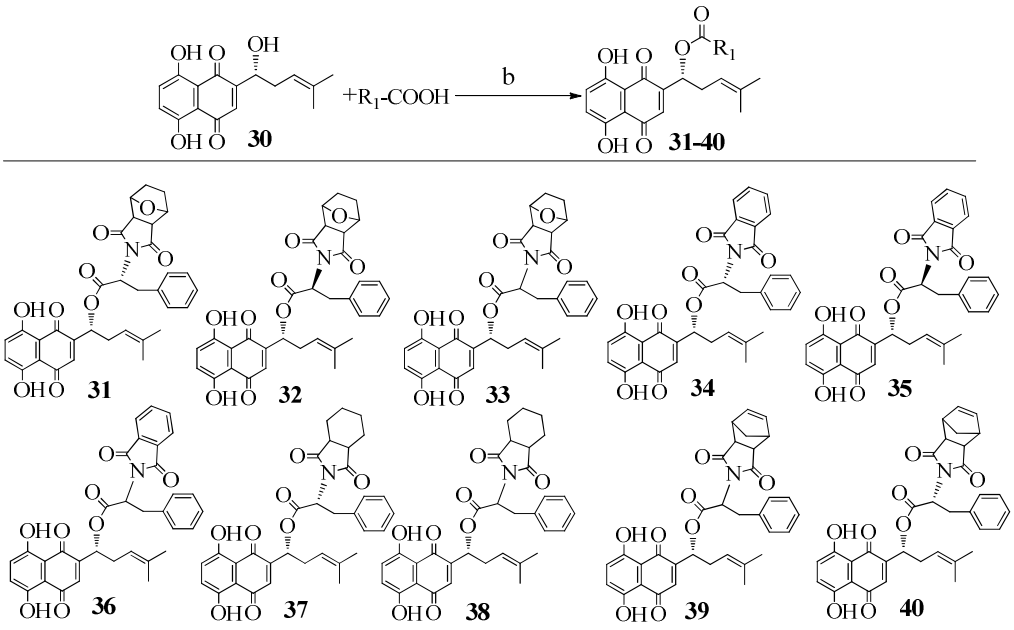
[a]Reagents and conditions: acetic acid, reflux, 12h.

Scheme 2. Synthesis of Alanine Derivatives



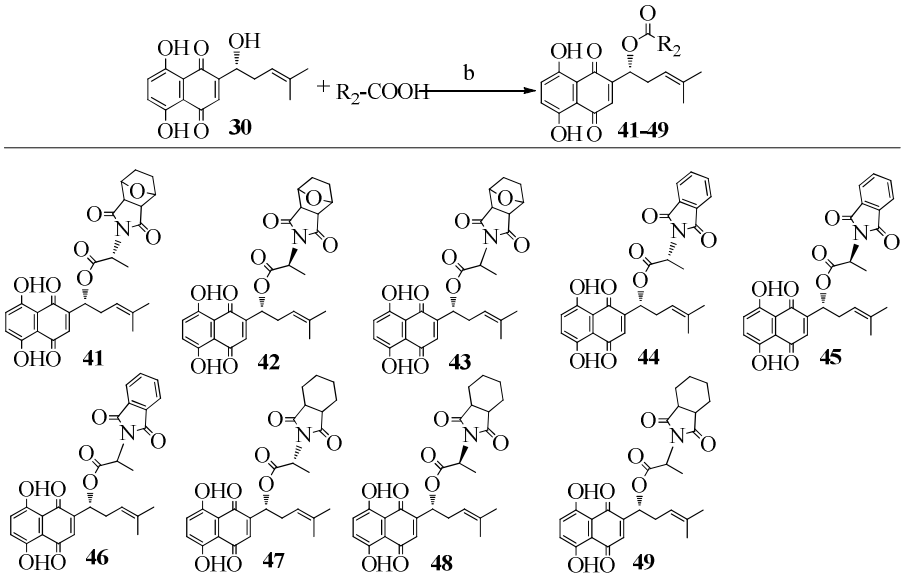
[a]Reagents and conditions: acetic acid, reflux, 12h.

Scheme 3. Synthesis of PhenylalanineShikonin Ester



[b]Reagents and conditions: DCC, DMAP, CH_2Cl_2 as solvent, ice-bath, overnight.

Scheme 4. Synthesis of Alanine Shikonin Ester



[b]Reagents and conditions: DCC, DMAP, CH_2Cl_2 as solvent, ice-bath, overnight

Table 1. In Vitro Anticancer Activity of Shikonin Derivatives against Five Cancer Cell Lines.

Table 1.In Vitro Anticancer Activity of Shikonin Derivatives Against Five Cancer Cell Lines.

entry	compd	IC_{50}±SD[a](μM)					
		HeLa	HepG2	MCF-7	BGC	A549	L02
1	**31**	4.88±0.87	2.33±0.43	6.06±1.49	20.53±3.76	>100	>100
2	**32**	4.51±0.66	2.48±0.38	4.58±0.34	>100	>100	>100
3	**33**	5.68±1.34	2.98±0.61	7.59±1.15	52.70±4.12	>100	>100
4	**34**	3.21±0.45	2.96±0.88	6.12±0.84	>100	>100	>100
5	**35**	2.92±0.67	1.69±0.25	3.05±0.21	>100	>100	>100
6	**36**	2.92±0.34	5.18±0.87	3.51±0.33	36.12±3.89	>100	>100
7	**37**	9.99±1.85	2.77±1.05	5.88±0.65	>100	>100	>100
8	**38**	3.75±0.44	3.65±1.30	8.47±0.53	7.63±1.63	>100	>100
9	**39**	5.91±0.98	2.21±0.32	3.43±0.21	>100	>100	>100
10	**40**	1.26±0.25	1.92±0.19	3.55±0.34	11.56±2.81	>100	>100
11	**41**	5.54±1.24	2.33±0.54	5.69±0.88	>100	>100	>100
12	**42**	7.38±2.08	2.67±0.29	9.06±1.54	>100	>100	>100
13	**43**	3.28±0.71	2.15±0.14	3.82±0.58	>100	>100	>100
14	**44**	5.54±1.90	5.14±0.91	4.24±0.82	92.85±6.73	>100	>100
15	**45**	1.93±1.36	5.35±0.28	2.55±0.35	13.34±1.78	>100	>100
16	**46**	2.96±0.98	5.11±0.47	4.30±0.51	14.67±2.05	>100	>100
17	**47**	8.53±0.67	2.10±0.13	4.19±0.32	14.94±2.41	>100	>100
18	**48**	18.50±2.71	3.16±0..45	6.27±0.69	57.54±4.67	>100	>100
19	**49**	11.39±2.28	3.57±0.19	16.08±2.09	61.25±3.65	>100	>100
20	shikonin	3.11±0.82	0.92±0.12	1.03±0.21	2.20±0.19	2.51±0.35	65.34±3.18

[a]SD: standard deviation. All experiments were independently performed at least three times.

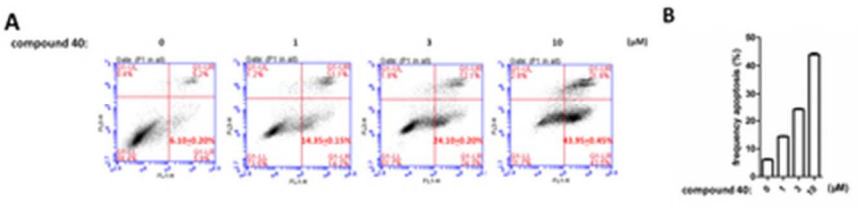


Fig.1A+B Cellular apoptosis study of compound 40 tested on HeLa cellsin dose dependent manner(0 μM,1 μM,3 μM,10 μM). The percentage of early apoptotic cells in the lower right quadrant (annexin V-FITC-positive / PI-negative cells), as well as late apoptotic cells located in the upper right quadrant (annexin V-FITC-positive / PI-positive cells).
37x10mm (300 x 300 DPI)

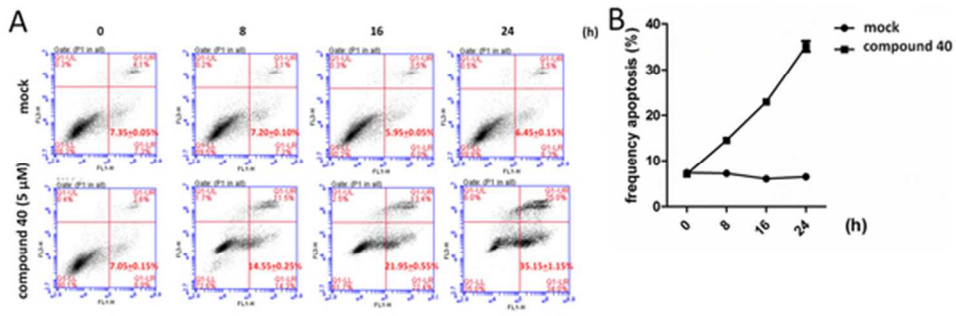


Fig.2A+B Cellular apoptosis study of compound 40 tested on HeLa cells in time dependent manner compared(0h,8h,16h and 24h) with the mock group.
53x19mm (300 x 300 DPI)

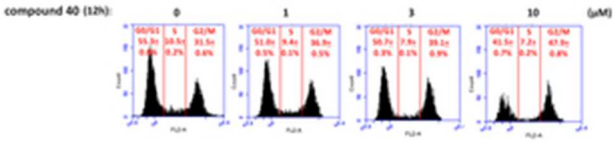
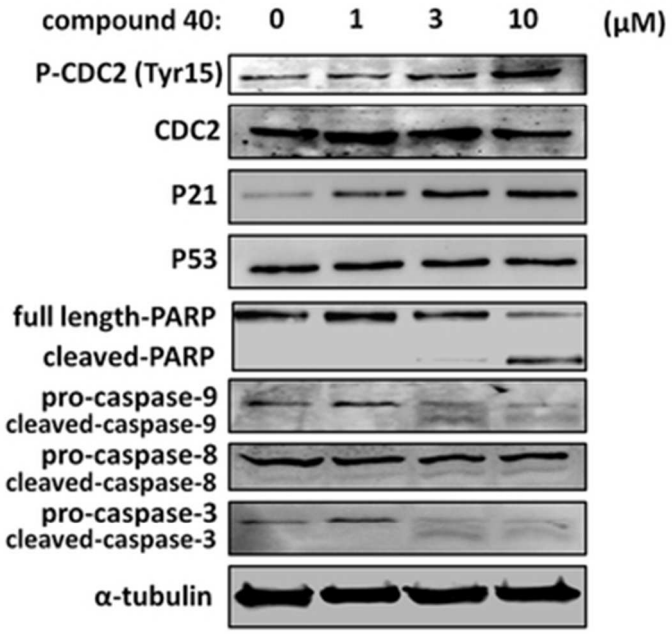
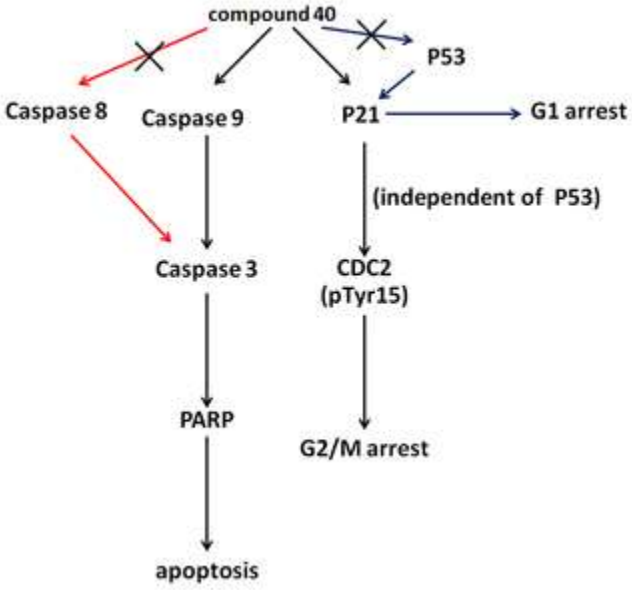


Fig.3 Effect of comound 40 on the cell cycle distribution of HeLa cells in dose dependent manner (0 μM,1 μM,3 μM,10 μM).

27x6mm (300 x 300 DPI)



30x29mm (300 x 300 DPI)



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