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Synthesis, DNA-binding abilities and anticancer activities of triazole-pyrrolo[2,1-*c*][1,4]benzodiazepines hybrid scaffolds

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ABSTRACT

We synthesized a new series of PBD-hybrid derivatives having tethered triazoles and investigated for their cytotoxicity. The studies indicated that *cis*-olefin compounds induce higher cytotoxicity with increase in the G1 cell cycle phase compared with the *trans*-compounds. Quantitative RT-PCR assay indicated that compounds (**16a**–**d**) induced G1 phase arrest through down-regulation of cyclin D1 and up-regulation of p21, p27, and p53 mRNA expressions. Compounds **16a**–**d** induced A375 early apoptosis as detected by flow cytometry after double-staining with annexin V and propidium iodide. Moreover, the Western blot analysis showed that A375 treated by compounds (**16a**–**d**) resulted in decreased levels of Bcl-2 and Bcl-xL, increased levels of Bax and Bad, and caspase/PARP degradation to identify apoptotic cells.

Pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) was isolated from *Streptomyces* species and are known for their unique characteristics to interact with DNA in minor groove.[1] However, there are only a few DNA-interactive agents that bind to DNA with high sequence selectivity. Therefore, the development of small molecular DNA-interactive agents with highly sequence-selective DNA-interactive properties is considered to be one of the most important tasks. The conjugate agents with active moieties of known antitumor and antiviral agents are being designed and synthesized to provide highly sequence-selective DNA-interactive properties and antitumor activity.[2] Based on above reason, a series of hybrid agents PBD-indole was designed and synthesized to provide highly sequence-selective DNA-interactive properties and antitumor activity.[3] Amongst them two compounds In-4C-PBD[3a] and In-6C-PBD[3b] showed prominent selectivity against cancer cell lines (Fig. 1A).

Both of these two compounds have great significance in developing potent anti-tumor agent that could be potentially applied for both inhibiting melanoma cells and preventing melanoma metastasis.[3a,b] Moreover, the enediyne contains either DNA intercalating groups[4] or DNA minor groove binding functions[5]

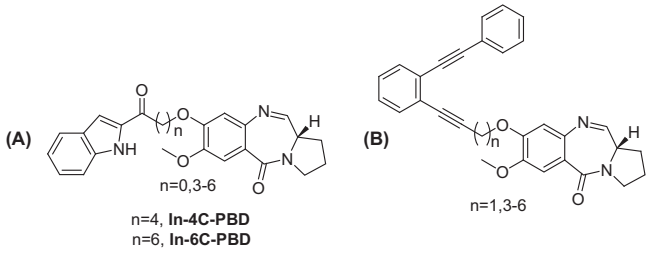

Figure 1. PBD–indole and PBD–enediyne hybrid agent.

encouraged us to design and synthesize a diversity of PBD–enediyne conjugate agents,[6] which also shown good selectivity activity against various cancer cell lines (Fig. 1B).

A vast number of pharmaceutical drugs and natural products have been engineered to synthesize the triazole systems with substituent at the 1- and 4-positions.[7] Most of them showed antimicrobial,[8] anti-inflammatory,[9] anticonvulsant,[10] and anticancer activities.[11] In addition, there are numerous reports available on the biological applications of such ring systems[12] and descriptions of their application in material chemistry.[13] In the past few years, the triazole moiety incorporated into natural and synthetic anticancer agents such as 1,2,3-triazole-linked pyrrolobenzodiazepine[14a] and 3-phenylpyrazolopyrimidine-1,2,3-triazole[14b] shows

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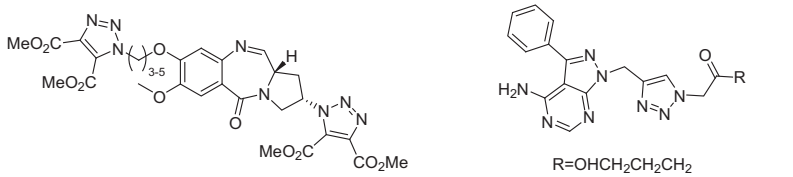


Figure 2. The antitumor agents of triazoles incorporated derivatives.

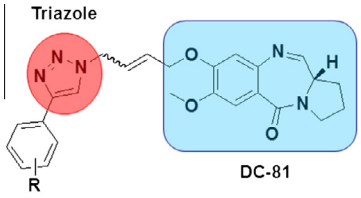


Figure 3. PBD–triazole derivatives.

potent cytotoxicity (Fig. 2). Herein, we report a new series of PBD-triazole analogues via CuAAC reaction (Fig. 3), and verify whether hybrid agents induced antiproliferation, leading to cell growth cycle perturbation, induced G1 phase arrest through down-regulation of cyclin D1 and up-regulation of p21, p27, and p53 mRNA expressions and subsequent apoptotic cell death.

The synthesis of **2** (DC81) was carried out by our previously reported method[15] via 6 steps starting from 4-(benzyloxy)-5-methoxy-2-nitrobenzoic acid (**1**, Scheme 1). The reaction of **2** with commercially available reagents *trans*-1,4-dibromobutene and *cis*-1,4-dichlorobutene in ethylmethylketone (MEK) and in the presence of potassium carbonate formed compound **3** and **4**, respectively. Compounds **3** and **4** reacted with sodium azide and catalytic amount of potassium iodide in methanol to give the corresponding PBD azide derivatives **5** and **6**.

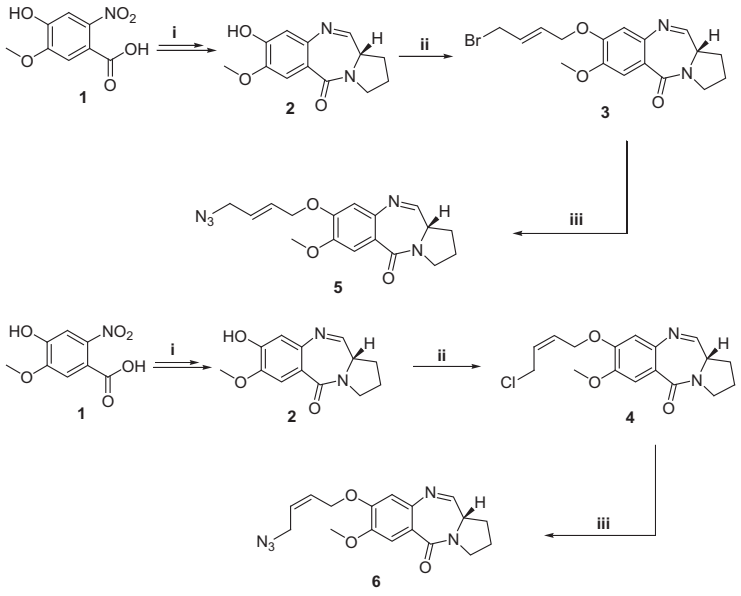
The terminal alkyne derivatives **13**–**15** were synthesized from the aryl iodides **7**–**9** as reported.[16] Finally, the azide compounds **5** and **6** were individually subjected to CuAAC protocol to obtain the corresponding *E*- and *Z*-PBD-triazole hybrid agents **16a**–**d** and **17a**–**d**, respectively (Scheme 2).

After preparing the new series of PBD-triazole derivatives, we evaluated the cytotoxicity of the PBD-triazoles analogues (**16a**–**d** and **17a**–**d**) in various human or mouse cells by MTT assay. As shown in Table 1, *cis*-form agents (**16a**–**d**) were potently cytotoxic in sensitive melanoma (A375, A2058, B16) and Ca9-22 oral cell lines compared to that of *trans*-form agents (**17a**–**d**).

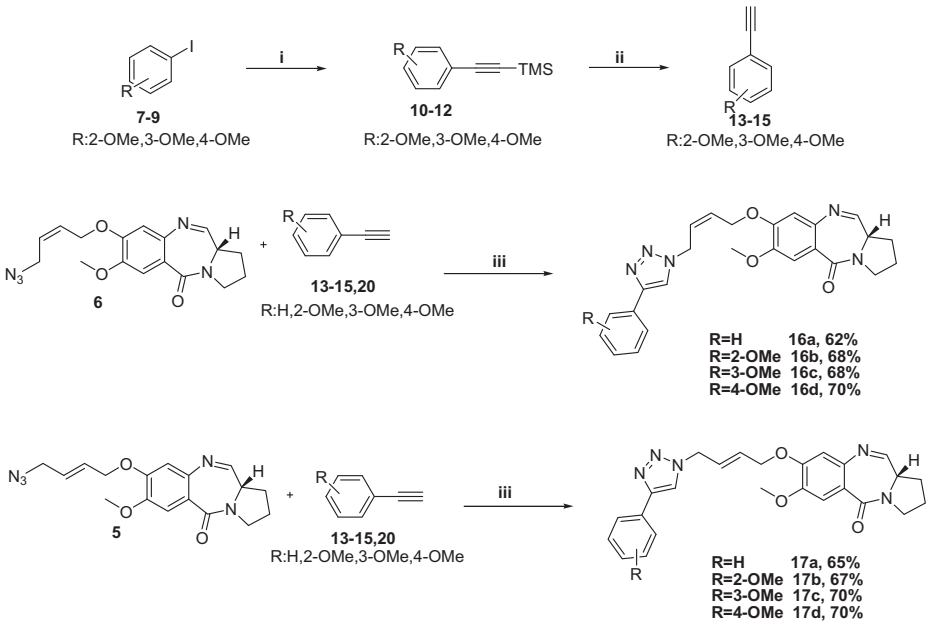
There was no significant cytotoxic effect in human dermal fibroblast cells. Because the agents **16a**–**d** exhibited a higher inhibitory activity on A375 cells compared to other cell lines at a concentration of 5 μM, the A375 cells were selected as a model for further studies. To investigate the most effective time in this study, A375 cells were treated with 0, 1, 3, and 5 μM of **16a**–**d** and **17a**–**d** for 0, 12, and 24 h. There was no significant cytotoxic effect against A375 cells for 12 h (see Supplementary data). *cis*-Form agents induced cytotoxic death of A375 cells in a dose- and time-dependent manner. Compound **16a** exhibited a higher inhibitory activity compared to that of other agents on A375 cells. Based on the cytoxicity data, IC_{50} were determined and shown in a subset table in Figure 4.

The triazoles moiety **21** was synthesized according to the reported procedure (Scheme 3).[17] In order to confirm whether conjugate agent is more effective as an antiproliferative agent than **2** (DC-81), **21** (triazoles moiety) or a combination of **2** and **21**, we used conjugate agent **16a** for this issue. Our data indicate that compound **16a** exhibited a higher inhibitory activity compared to that of other agents on A375 cells (Fig. 5).

Meikrantz and Schlegel reported that the control of cell death is linked to the cell cycle.[18] Cells with a defective cell cycle are more



Scheme 1. Syntheses of *E*/*Z*-PBD-azides. Reagents and conditions: (i) construction of PBD scaffold via 6 steps, Ref. 15; (ii) *trans*-1,4-dibromobutene or *cis*-1,4-dichlorobutene, K_2CO_3, ethylmethylketone (EMK), reflux, 4 h, 94–95%; (iii) NaN_3, KI, MeOH, rt, 12 h, 90–91%.



Scheme 2. Synthesis of PBD-triazole and presynthesis for diversity of phenylalkynes. Reagents and conditions: (i) $Pd(PPh_3)_4$, CuI, Et_3N, 90 °C, 6–12 h; (ii) K_2CO_3, THF, rt, 12 h; (iii) 13–15 and 20, CuI, DMF:THF = 1:4, rt, 2 h, 65–70%.

Table 1
Cytotoxic activities of compounds in different human cells and mouse cell line[a]

Survival (%control)	Compound (5 μM)								
	DC81	**16a**	**16b**	**16c**	**16d**	**17a**	**17b**	**17c**	**17d**
A375 (melanoma)	52.2 ± 0.5	22.4 ± 0.1	58.4 ± 0.2	48.4 ± 0.2	38.7 ± 0.2	69.6 ± 0.2	58.4 ± 0.2	58.3 ± 0.2	69.2 ± 0.3
RPMI7951 (melanoma)	61.6 ± 0.1	55.9 ± 0.1	75.1 ± 1.1	65.3 ± 2.1	66.4 ± 3.2	79.1 ± 1.4	83 ± 0.7	87.2 ± 0.2	80.1 ± 1.1
A2058 (melanoma)	54.8 ± 0.2	45.7 ± 0.7	63.1 ± 1.7	40.7 ± 1.2	42.0 ± 2.1	53.3 ± 2.1	57.9 ± 2.1	62.8 ± 3.3	49.2 ± 3.1
A549 (lung)	85.4 ± 0.3	65.6 ± 1.0	102.5 ± 0.6	100.2 ± 0.9	98.2 ± 0.9	70.0 ± 1.4	91.0 ± 0.5	86.0 ± 1.7	81.0 ± 1.2
293T (kidney)	55.2 ± 0.1	74.1 ± 0.7	51.1 ± 5.1	42.8 ± 2.5	46.7 ± 1.7	78.0 ± 1.3	55.0 ± 1.7	40.0 ± 1.9	40.0 ± 0.5
Ca9-22 (oral)	94.4 ± 0.1	25.6 ± 2.4	104.8 ± 2.4	50.0 ± 0.4	55.5 ± 0.7	61.2 ± 1.3	95.6 ± 0.9	97.5 ± 4.3	84.1 ± 2.0
B16 (mouse melanoma)	66.8 ± 0.2	33.9 ± 3.8	66.3 ± 1.6	74.2 ± 0.9	62.8 ± 1.8	33.9 ± 3.8	98.9 ± 1.4	107.2 ± 2.6	75.2 ± 4.6
Fibroblast	89.7 ± 0.3	87.7 ± 1.2	84.7 ± 8.1	90.4 ± 4.5	90.3 ± 4.8	93.0 ± 2.0	100.0 ± 6.7	107.0 ± 5.9	104.0 ± 3.3

[a] Cells were cultured with agents at a concentration of 5 μM for 24 h before growth and viability were assessed using the MTT assay.

vulnerable to some anticancer agents according to numerous preclinical studies.[19] Compounds manipulating the cell cycle may be able to promote growth arrest, inhibit differentiation and induce apoptosis. To verify whether cell damage might be attribute to the cell cycle program or might have become arrested at any cell cycle phase by PBD-triazole-induced apoptosis in A375 cells, the DNA content of cell nuclei was measured by flow cytometric analysis. As shown in Table 2, cells-treated with 5 μM PBD-triazole for 24 h significantly increased cell percentage at G1 phase and concomitantly, decreased cell populations at S and G2/M phase. In addition, *cis*-form agents (**16a**–**d**) induced more G1 cell cycle arrest in A375 cells than did *trans*-form agents (**17a**–**d**).

To investigate if the antiproliferative effect of **16a** was a consequence of DNA intercalation, we performed an in vitro DNA unwinding assay. DNA intercalators enhance the unwinding of negatively supercoiled DNA, which leads to a decreased mobility in agarose gel during electrophoresis.[20] As shown in Figure 6, it showed that PBD-triazole compound **16a** between 0 and 1000 μM exhibited a dosage response. These data indicate that **16a** may bind to DNA through intercalation.

Cyclin-dependent kinases (CDKs) play a crucial role in the inhibition of cancer cell proliferation by chemotherapeutic agents.[21] Overexpression of cyclin D1 has been reported in several types of human cancer.[22] p27 is considered the main regulator of G1 phase CDK activity, and p21 function as a sensor of cytostatic signals and is mainly regulated by the p53 tumor suppressor gene.[23] In our study, we observed that PBD-triazoles caused cell cycle G1 arrest. To further evidence the expression of possible G1 cell cycle-associated regulators, four genes (p53, p21, p27, and cyclin-D1) were detected by quantitative RT-PCR (qPCR) experiments. A375 cells were treated with agents at a concentration of 5 μM for 24 h. As shown in Figure 7, we observed that **16a**–**d**-induced G1 cell cycle arrest in A375 cells might be through induction of p53, p21, and p27 followed by a decrease in the expression of cyclin D1.

Apoptosis is an important phenomenon in cytotoxicity induced by anticancer drugs. The execution of apoptosis, or programmed cell death[24] is associated with characteristic morphological and biochemical changes mediated by a series of gene regulation and cell signaling pathways. Fluorescein isothiocyanate (FITC)-conjugated annexin V has been utilized to detect the externalization of phosphatidylserine that occurs at an early stage of apoptosis. Propidium iodide (PI) is used as a marker of necrosis due to cell membrane destruction.[25] To elucidate whether compound-induced cell death involved apoptosis or not, we performed a bi-parametric cytofluorimetric analysis using annexin V and PI double-staining as shown in Figure 8. Treatment of A375 cells with 5 μM agents for 24 h induced apoptosis effects in 2.6% (**Control**), 23.1% (**16a**), 11.1% (**16b**), 16.4% (**16c**), and 12.1% (**16d**) of annexin V-FITC cells,

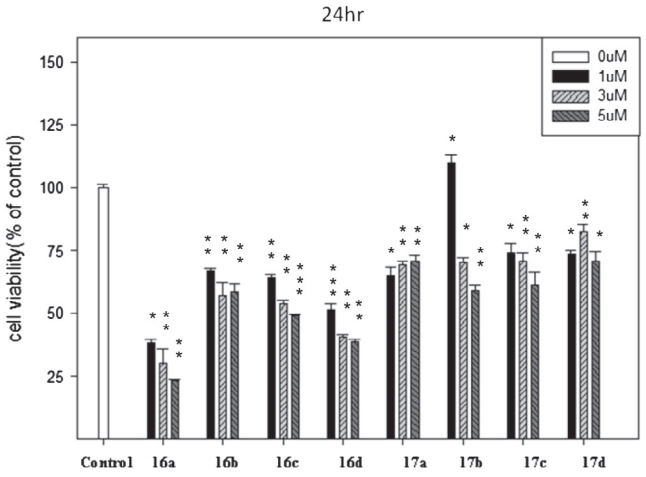
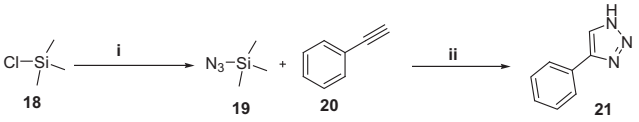


Table. In vitro cytotoxicity of tested compounds on human melanoma A375 cells.[a]

Compound	IC_{50} (μM)
16a	2.2
16b	4.2
16c	3.6
16d	2.7
17a	4.5
17b	5.2
17c	5.3
17d	6.4

[a] IC_{50} (50% inhibitory concentration) represents the mean from dose-response curves of three experiments.

Figure 4. Effect of PBD-triazoles on cell viability. A375 cells were seeded in a 96-well plate at 10,000 cells per well and cultivated overnight until cell attachment. PBD-triazoles at the indicated concentration was added into the culture media in triplicate and incubated for 24 h before MTT reagent was added. The absorbance is directly proportional to the number of living cells. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 versus control group.



Scheme 3. Synthesis of 5-phenyltriazole. Reagents and conditions: (i) NaN_3, PEG400, 60 °C, 2 h, 82%; (ii) CuI, DMF:MeOH = 1:1, 100 °C, 11 h, 80%, Ref. 17.

respectively. Our results showed that **16a–d** agents induced more apoptotic cells than that of the untreated control.

The anti-apoptotic proteins (Bcl-2, Bcl-xL) and the death-promoting proteins (Bax, Bad) of the Bcl-2 family are considered as the requisite gateway to the mitochondrial apoptotic pathway.[26] Caspase-3 has been shown to be one of the most important cell executioners for apoptosis.[27] Poly (ADP-ribose) polymerase (PARP) has been identified as a substrate for caspase-3. The expression of apoptosis-related proteins was determined by Western blotting assay. Our results showed that **16a–d** triggering apoptotic cell death was associated with increased levels of Bax and Bad and decreased levels of Bcl-2 and Bcl-xL. In addition, cell treatment with **16a** induced a concentration-dependent increase in Bax and Bad, cleaved both caspase-3 and PARP whereas the levels of Bcl-2 and Bcl-xL were reduced (Fig. 9A and B).

In conclusion, the desired PBD-triazole hybrid analogues **16a–d** and **17a–d** have been synthesized in good yields. Initially, we used an MTT assay to evaluate the cytotoxicity of tested compounds in seven human and one mouse cell lines. Our results indicated that

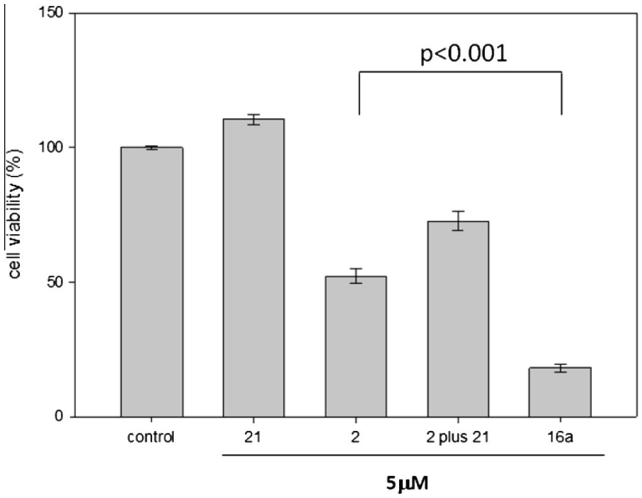


Figure 5. Cell viability of 5 μM compounds tested against A375 cells for 24 h (same experimental conditions as in Table 1).

Table 2
Effect of PBD-triazoles on cell cycle distribution of A375 melanoma cells[a]

Cell cycle	%sub-G1	%G1	%S	%G2/M
Control	7.7 ± 0.1	65.3 ± 0.1	11.8 ± 0.1	15.2 ± 0.6
16a	8.9 ± 0.3	83.0 ± 3.2	5.9 ± 1.8	2.2 ± 0.9
16b	11.7 ± 0.2	81.9 ± 8.3	5.1 ± 0.0	1.3 ± 0.4
16c	7.7 ± 0.1	82.3 ± 3.8	6.7 ± 1.6	3.3 ± 1.0
16d	7.1 ± 0.3	82.3 ± 0.6	7.2 ± 1.5	3.4 ± 1.4
17a	7.0 ± 0.2	77.8 ± 9.0	9.7 ± 4.1	5.5 ± 4.1
17b	17.3 ± 1.8	69.5 ± 12.2	8.1 ± 4.0	5.1 ± 2.1
17c	17.6 ± 2.5	71.4 ± 15.6	7.5 ± 4.6	3.5 ± 3.6
17d	13.9 ± 2.1	73.6 ± 13.8	7.4 ± 1.8	5.1 ± 4.1

[a] Human A375 melanoma cells were treated with compounds, in triplicate at 5 μM concentration for 24 h. Cells were harvested for cell cycle analysis by using flow cytometer. Values are expressed as mean ± SD.

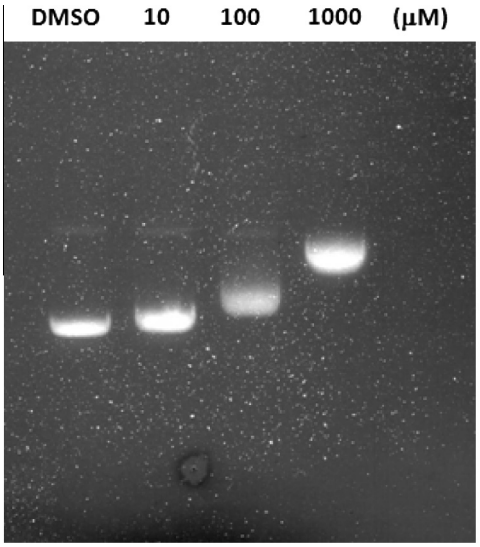


Figure 6. Compound **16a** unwind plasmid DNA. Increasing concentration of compound **16a** were incubated with negatively supercoiled pBR322 (0.4 μg) for 12 h in TE buffer and then run for 2.5 h on a 1% gel.

cis-form agents **16a–d** were more effective as an anti-proliferative agent than *trans*-form agents **17a–d**. We hypothesized that the anti-proliferative effects of **16a–d** may be associated with cellular

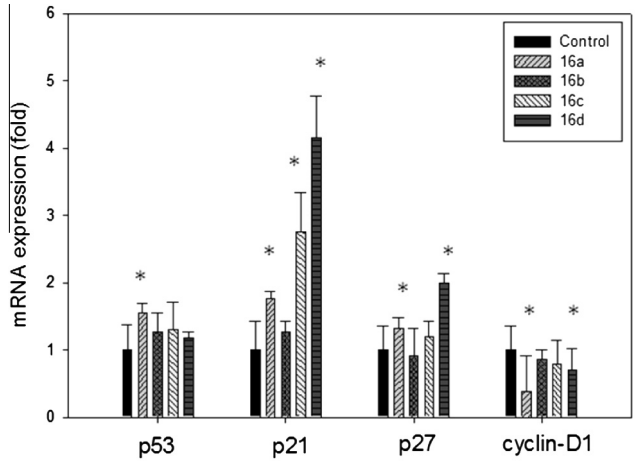


Figure 7. Relative expression of cell cycle G1-related genes. A375 cells were treated with 5 μM **16a–d** for 24 h. Gene expression was determined by qPCR and normalized with β-actin expression in each sample. *P <0.05 versus control group.

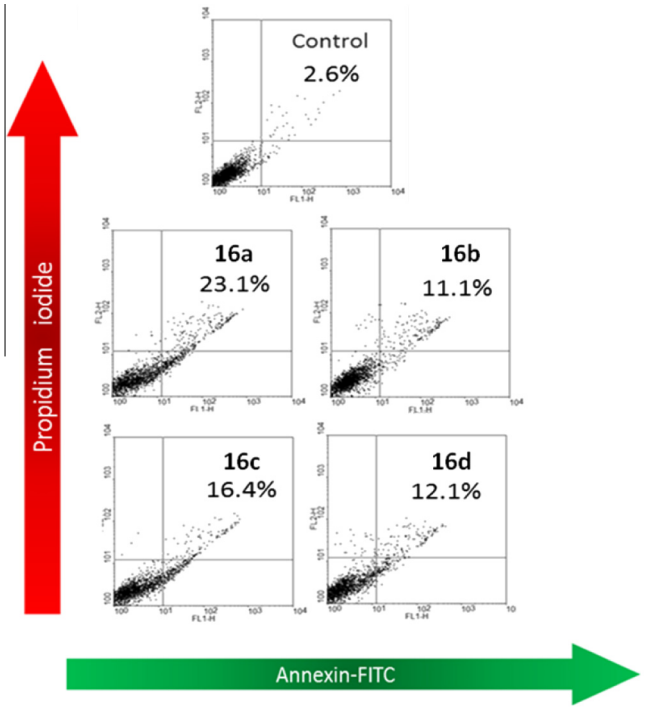


Figure 8. PBD-triazoles induced externalization of PS. Dot plots for A375 cells treated with **16a–d** at a concentration of 5 μM for 24 h and then stained with PI and an annexin V-FITC conjugate specifically detected the exposure of PS residues at the cell surface. Approximately 10,000 cells from each group were analyzed by flow cytometry. Data shown are of a representative experiment repeated three times with similar results.

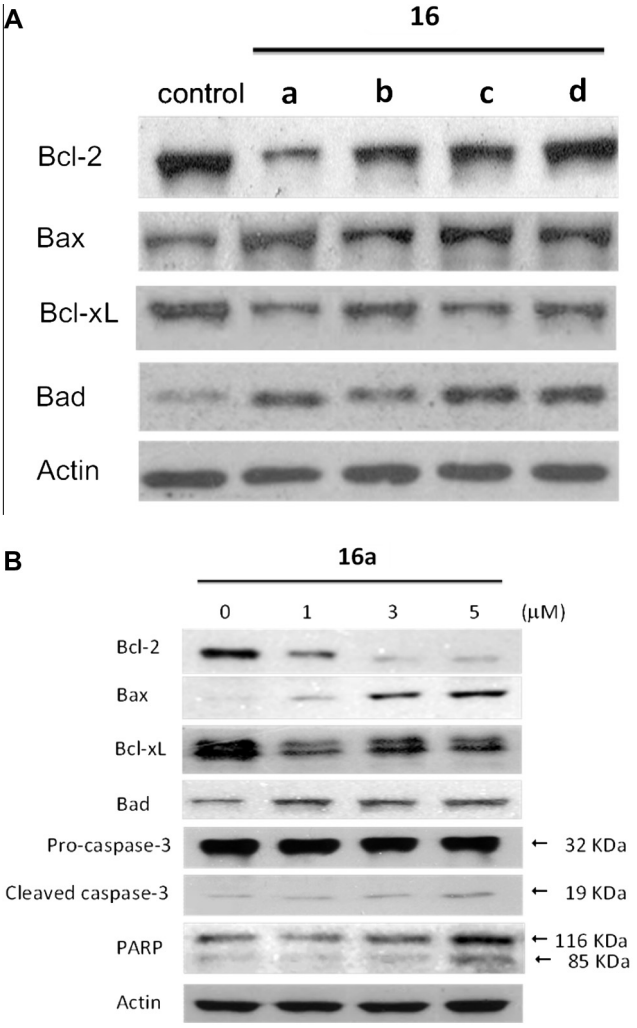


Figure 9. Western blot analysis showed the effect of compounds on the expression of apoptosis regulatory proteins. After exposure to 5 μM **16a–d** for 24 h (A) or incubated with various concentrations of **16a** (B), cell lysates were collected and Western blotted with specific antibodies as indicated. For the internal control, the same amounts of protein extract were also probed with antibody against actin. Similar results were observed in three separate experiments.

apoptosis. Because the cell cycle deregulation plays a critical role in malignant transformation and in the inefficacy of chemotherapy,[23b] the DNA content of cell nuclei was measured by flow cytometric analysis. Our results showed that PBD-triazole derivatives induce cell cycle G1 arrest on human melanoma A375 cells. Moreover, DNA unwinding assay indicated that PBD-triazole anologue **16a** may bind to DNA through intercalation. The *cis*-form hybrid agents **16a–d** induce p53 activation contributing to the over-expression of p21 and p27, both being strong cyclin-dependent kinase inhibitors, thereby inducing cell arrest G1 and blocking cell entry into the S phase. Furthermore, compound **16a–d** also induced a marked increase of annexin V binding, decreased protein expression of both Bcl-2 and Bcl-xL, increased levels of Bax and Bad and caspase/PARP degradation to identify apoptotic cells. Based on our results, compound **16a** was found to be the most effective of these PBD-triazole derivatives, which acts as a potential antimelanoma agent.

Acknowledgement

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Supplementary data

Supplementary data (preparation and characterization of compounds **16a–d** and **17a–d**; copies of the ^{1}H NMR and ^{13}C NMR for the new compounds. The purities of various resultant compounds determined by HPLC) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.10.004.

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