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Biological evaluation of 9-[(6-chloropyridin-4-yl)methyl]-9*H*-carbazole-3-carbinol as an anticancer agent

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Abstract. Most conventional anticancer drugs exert either anti-proliferation or anti-angiogenesis activity. Recently, searching for potential multi-target agents has become an alternative strategy for cancer treatment. Several structurally different carbazole alkaloids from either natural or synthesized sources represent an important and heterogeneous class of anticancer agents. In the present study, we investigated the anticancer activity of a novel synthetic carbazole derivative, 9-[(6-chloropyridin-4-yl)methyl]-9*H*-carbazole-3-carbinol (HYL-6d), which is structurally different from other previously characterized carbazoles. HYL-6d-treated human breast cancer MCF-7 cells exhibited an increased population arrested at the sub-G_1 and S phases, as well as an increase of p53 and decrease of cyclin D1, A and CDK2. Also, HYL-6d treatment induced MCF-7 cell apoptosis and this was accompanied by a decreased expression of Bcl-2, increased levels of p53 and Bcl-X_S and the activation of caspase-9. Experimental results from human umbilical vascular endothelial cells (HUVECs) showed that HYL-6d also exerted its anti-angiogenic activity in HUVECs by inhibiting cell proliferation, migration, and tube formation induced by VEGF- or bFGF *in vitro*. In summary, the data indicate that HYL-6d exhibits both cytotoxic effects against human cancer cells and anti-angiogenic activities, which make it a potential therapeutic drug for cancer treatment.

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Key words: carbazole derivative, Bcl-X_S, apoptosis, anti-angiogenic activity

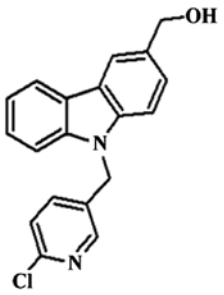
Introduction

Breast cancer is a common type of cancer affecting Taiwanese women and its incidence and mortality rates increase year by year (1). To eradicate cancer and its recurrence, a multifaceted therapeutic approach has been employed and includes targeting pathways that promote or sustain cancer cell growth and invasion (2,3). These therapeutic strategies are successful in preventing tumor progression with a minimal effect on normal cells (4). Several pathways are potential targets for cancer treatments, including pathways that induce cell apoptosis, prevent cell proliferation, modulate cellular re-dox states and detoxify carcinogens. These therapeutic targets are considered clinically useful (5).

New practical strategies have been used for cancer treatment, including the inhibition of cell cycle of tumor cells and induction of apoptosis in tumor cells. For example, through phosphorylation of target proteins to interfere with the interactions of cyclins with CDKs, it is possible to regulate the cell cycle and to stop the growth of progressive tumors (6). Therefore, it provides an opportunity for those chemicals capable of interfering with the abnormal cell proliferation to be used as chemotherapeutic agents. Similarly, intervention in the programmed cell death, known as apoptosis, is another possible strategy for controlling cell growth in cancer therapies (7). Two distinct apoptotic pathways have been identified: the intrinsic apoptotic pathway (also known as the p53-mitochondrial pathway) and the extrinsic pathway (activated by 'death receptors' and their corresponding ligands) (8). Unlike the extrinsic pathway, whose apoptotic pathway begins outside the cell through the activation of specific pro-apoptotic receptors, the intrinsic apoptotic pathway involves the regulation of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax and Bad) proteins of the Bcl-2 family to trigger the release of cytochrome *c* from the mitochondria, subsequently culminating in caspase-9-dependent apoptosis (9). The induction of apoptosis in tumor cells has become one of the most widely-used strategies of cancer therapy.

Targeting the angiogenesis of tumor cells is another common strategy for cancer treatment (10). Several pro-angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor, and platelet-derived endothelial growth factor, are known factors of tumor angiogenesis (11). These pro-angiogenic factors activate quiescent endothelial cells and promote their migration into the tumor (11). Therefore, preventing angiogenesis and consequent metastasis in tumors has become an attractive strategy in tumor treatment (12). In 2004, Miller reported that certain chemotherapeutic agents routinely used for breast cancer treatments have anti-angiogenic activity (12). Ma and Waxman also conceived the potential benefits of targeting angiogenesis in chemotherapy (11). Taken together, an agent combined with both anti-proliferative and anti-angiogenic activities would make an ideal drug for cancer therapy.

The carbazole alkaloids are widely distributed compounds in nature and have certain physiological roles in plants and microorganisms. Several structurally different carbazole alkaloids from either natural or synthesized sources have shown diverse pharmacological effects (13-16). Some of these carbazoles and their derivatives exhibit cytotoxic activities related to the inhibition of DNA-dependent enzymes such as topoisomerase I/II and telomerase (13-16). In addition, other reported cytotoxic effects of carbazoles include anti-proliferation (17,18), anti-angiogenesis (19), anti-HIV-activities (20,21) and anti-estrogenic activities (22). Previous studies have shown that LCY-2-CHO, an asymmetric substituted carbazole derivative, showed evident apoptosis effects in three leukemia cells, but had only slight effects on adherent cells including PC3 and MCF-7 (18). Another asymmetric substituted 9*H*-carbazole derivative, 9-[(6-chloropyridin-4-yl) methyl]-9*H*-carbazole-3-carbinol (HYL-6d) (Fig. 1), synthesized by the laboratory of Dr L.J. Huang, has demonstrated a greater cytotoxic effect on six adherent human cancer cell lines, particularly on MCF-7 cells (Fig. 2). The aim of the present study was to determine the underlying mechanisms of the *in vitro* cytotoxic effects of HYL-6d. This study analyzed different cell subsets of cell cycle in HYL-6d-treated human breast cancer MCF-7 cells, and determined whether HYL-6d induces apoptosis through the molecular parameters related to the Bcl-2 family, p53 and caspase pathways. Furthermore, we found that HYL-6d could suppress human umbilical vein endothelial cell (HUVEC) proliferation, migration, and tube formation *in vitro*.



HYL-6d

9-[(6-Chloropyridin-4-yl)methyl]-9*H*-carbazole-3-carbinol

Figure 1. Chemical structure of HYL-6d.

Materials and methods

Reagents. HYL-6d (purity >95%), was synthesized and dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, USA). Further dilutions were made immediately prior to each experiment with a final DMSO concentration of <0.1% in culture media.

Cell lines and cell cultures. Five human cancer cell lines, including breast carcinoma (MCF-7 and MDA-MB-231), cervical carcinoma (CaSki and HeLa) and ovarian carcinoma (SKOV3) were obtained from the Bioresource Collection and Research Centre, Taiwan (BCRC; formerly the Culture Collection and Research Centre). Human ovarian carcinoma 2774 and HUVECs were provided by China Medical University, Taichung, Taiwan. MCF-7, MDA-MB-231 and HeLa cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA). CaSki cells were maintained in RPMI-1640 medium (Gibco-BRL). SKOV3 and 2774 cells were maintained in DMEM/F12 (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS; Kibbutz Beit Haemek, Israel), 5% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL). HUVECs were cultured in M199 medium (Sigma) containing 20% FBS and 15% endothelial cell growth supplements. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Medium was changed every 2 days, and cells were passaged after treatment with a 0.05% trypsin/0.02% EDTA solution. Experiments were conducted on HUVECs that had gone through two to five passages.

Cell viability assay. For cell viability assays, cells were seeded at a density of $5x10^4$ cells/well in 12-well culture plates. After 24 h, cells were treated with various concentrations of HYL-2d-6d (from 1 to 100 μM in 1 μl DMSO) for the indicated times. Cell viability was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay and the trypan blue exclusion assay.

Cell cycle analysis. MCF-7 cells were treated with or without 30 μM HYL-6d and harvested at different times. After washing with phosphate-buffered saline (PBS), the cells were fixed in 70% ethanol/PBS for 30 min on ice. Approximately $4x10^5$ cells were centrifuged and the cell pellets were re-suspended in PBS and further treated with RNase (DNase free, 100 μg/ml, final concentration in PBS) and propidium iodide (40 μg/ml, final concentration in PBS) at room temperature for 30 min. The cells were centrifuged and the cell pellets were re-suspended in PBS. The cell suspension was passed through a 19-gauge needle and kept on ice until analysis. The number of cells in different phases of the cell cycle was analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Distributions of different cell cycle phases were determined using CellFIT software (Becton-Dickinson).

Apoptosis. Cell apoptosis was determined using TUNEL/DAPI double stains and DNA fragmentation. HYL-6d-treated and untreated cells were harvested at 36 h, after washing twice

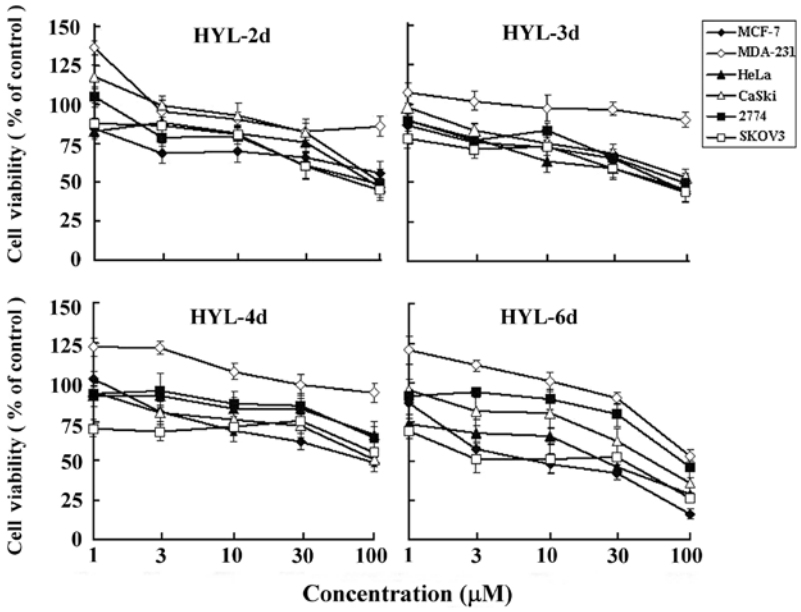


Figure 2. Effects of HYL-6d on the cell viability of 6 human cancer cell lines after 48 h of treatment using the MTT assay. Values represent % of control. Experiments were carried out in triplicate and each value is the mean ± SD of three independent experiments.

with PBS. These cells were first fixed with 2% formaldehyde for 30 min and then permeabilized by treating with 0.1% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS, the TUNEL assay was performed according to the manufacturer's instructions (Boehringer Mannheim, Germany). Briefly, cells were incubated in TUNEL reaction buffer in a 37˚C humidified chamber for 60 min in the dark, then rinsed twice with PBS and incubated with DAPI (1 μg/ml) at 37˚C for 30 min. Cells were visualized using a fluorescence microscope. TUNEL positive cells were considered apoptotic.

For DNA fragmentation assay, MCF-7 cells were grown in the presence or absence of 30 μM HYL-6d for 36 h, and treated cells were harvested and lysed in extraction buffer (50 mM Tris pH 7.5, 10 mM EDTA, and 0.3% Triton X-100) for 30 min on ice. RNAs were removed by treating with RNase (100 μg/ml) for 30 min at 55˚C. Cell lysates were subsequently treated with proteinase K (400 μg/ml) for 1 h at 55˚C. The supernatants were then prepared by centrifugation, and the soluble proteins were extracted by phenol/chloroform. DNA was ethanol-precipitated and subjected to electrophoresis on a 2% agarose gel.

Western blot analysis. HYL-6d-treated cells were also used for western blot analysis. Following incubation, 5x10^4 cells were lysed in a 100 μl modified protein lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% 2-mercaptoethanol, 1% Triton X-100, 0.25% sodium-deoxycholate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 0.2 mM phenylmethylsulfonylfluoride). Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of sample lysates were separated by SDS-PAGE on an 8-12% polyacrylamide gel, and the gel was transferred to a PVDF membrane (NEN Life Science Products Inc., Boston, MA, USA). The proteins were detected using mouse and rabbit specific primary antibodies (BD Biosciences Pharmingen, San Diego, CA, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by RDye 800 anti-mouse Molecular Probes (Rockland Immunochemicals Inc., Gilbertsville, PA, USA), and Alexa Fluor® 700 anti-rabbit (Molecular Probes, Eugene, OR, USA) as fluorescent secondary antibodies. The resulting membrane was imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences, St. Lincoln, NE, USA) and analyzed with their software program as specified in the Odyssey software manual. An equal loading in the lanes was evaluated by probing with an anti-β-actin antibody (Santa Cruz Biotechnology).

HUVEC proliferation assay. DNA synthesis in proliferating cells was determined by measuring BrdU incorporation with the commercial Cell Proliferation ELISA system (Chemicon). Confluent HUVECs were trypsinized, suspended in M199 medium supplemented with 20% FBS, and seeded at 2x10^4 cells/well in 96-well plates. After 24 h, cells were washed twice with PBS and starved with 2% FBS-M199 medium for 24 h. These cells were then incubated with or without the indicated reagents and growth factors (such as VEGF and bFGF at 10 ng/ml). After 24 h, cells were analyzed using the BrdU Cell Proliferation kit and quantified with an ELISA reader at $OD_{450/540}$. Culture medium without any additions was used as a control for non-specific binding.

Migration assay. To measure the ability of HUVECs to migrate, cells were seeded into a Boyden chamber with 8 μm pore polycarbonate filters (Costar, Cambridge, MA, USA). Briefly, HUVECs were pretreated with various concentrations of HYL-6d. After 24 h, HUVECs were detached by trypsin and resuspended in a serum-free medium. VEGF or bFGF was diluted to 10 ng/ml in 100 μl M199/2% FBS and added to the lower well chamber as a chemoattractant, and HUVECs were seeded on the upper chamber at a density of 1x10^5 cells/well in 150 μl of serum-free medium. The chamber

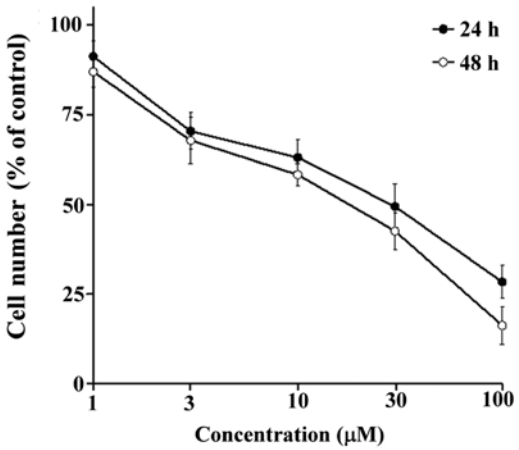


Figure 3. Concentration-dependent and time-dependent effects of HYL-6d-induced cell death in MCF-7 cells. Cells were incubated in the absence or presence of the indicated concentrations of HYL-6d for different incubation times. Then, the cell number was determined using the trypan blue dye exclusion method. Experiments were carried out in triplicate and each value is the mean ± SD of three independent experiments.

was incubated for 4 h at 37˚C. Following incubation, HUVECs in the upper surface of the membrane were carefully removed with a cotton swab and cells that migrated to the lower surface of the membrane were fixed with methanol and stained with 5% Giemsa solution. Migratory cells on the lower surface of the membrane filter were counted in six randomly chosen high-power fields (magnification, x400). Cell migration was calculated as the difference between the number of migrated cells in the HYL-6d-treated samples and the number of migrated cells in the control samples. Each experiment was carried out in triplicate.

Matrigel capillary tube formation assay. A 24-well culture plate was coated with 250 μl of Matrigel (Becton-Dickinson, Bedford, MA, USA), and incubated for 1 h to allow solidification. HUVECs (2x10^5 cells/ml) were treated with or without HYL-6d (3-30 μM) and VEGF or bFGF (10 ng/ml). After 24 h, trypsin-harvested HUVECs were suspended in 500 μl M199/2% FBS. The suspension was placed onto the surface of the Matrigel and incubated for 24 h. The cell morphology was evaluated using a phase-contrast microscope, and cells were photographed. The lengths and areas of tube-structured cells were then quantified using a MetaFluor Imaging System (Meta Imaging Series; Molecular Devices Corp., Silicon Valley, CA, USA).

Statistical analysis. All data presented in this study (as the means ± SD of nine replicates) are from three separate experiments. Figures are representative of at least three independent experiments with similar patterns. The Student's t-test was used for statistical analysis between the HYL-6d-treated group and the control group.

Results

Cytotoxicity of HYL-6d in various human cancer cell lines. To assess the cytotoxicity of carbazole derivatives, six human cancer cell lines from various organs were used, including breast carcinoma (MCF-7 and MDA-MB-231), cervical carcinoma (CaSki and HeLa) and ovarian carcinoma (SKOV3 and 2774). These cell lines were treated with HYL-2d, 3d, 4d and 6d at various concentrations (1-100 μM) for 48 h. As shown in Figs. 2 and 3, HYL-6d exhibited different degrees of dose- and time-dependent cytotoxic activities. HYL-6d appeared to be the most potent to inhibit MCF-7 breast cancer cells with an IC_{50} of 20-30 μM at 48 h.

Table I. Cell cycle analysis of HYL-6d-treated MCF-7 cells.

		Treatment (% of the cell no.)	
Time (h)	Cell cycle	Control	HYL-6d
0	G_1	57.5±2.0	
	S	34.0±1.5	
	G_2/M	8.5±1.2	
	Apoptosis	0.5±0.6	
12	G_1	59.0±2.0	58.5±0.9
	S	28.4±2.7	39.4±1.2[a]
	G_2/M	12.6±1.3	2.1±1.1[b]
	Apoptosis	0.4±0.4	0.2±0.2
24	G_1	61.8±4.0	58.9±2.1
	S	30.4±3.9	38.9±2.4
	G_2/M	7.8±1.9	2.3±1.5[a]
	Apoptosis	0.2±0.2	1.2±1.2
36	G_1	57.9±3.8	57.5±2.2
	S	33.5±2.4	39.5±3.0
	G_2/M	8.6±1.8	3.0±2.0[a]
	Apoptosis	0.3±0.4	1.7±1.6
48	G_1	59.7±3.1	51.8±3.6
	S	31.8±2.8	47.5±4.1[b]
	G_2/M	8.5±1.0	0.6±0.7
	Apoptosis	0.7±0.9	25.1±5.3[c]

Cells were treated with HYL-6d for up to 48 h, stained with propidium iodide and analyzed by FACScan, as described. The proportion of cells in the G_0/G_1, S and G_2/M phase of the cell cycle were calculated. Data are from one representative experiment (mean ± SD, n=3). Similar results were obtained in three other independent experiments. [a]P<0.05, [b]P<0.01, [c]P<0.001 compared with the untreated control group.

Effects of HYL-6d on cell cycle progression and regulatory proteins. To determine whether HYL-6d-induced cytotoxicity was associated with a disturbance of cell cycle regulation, the HYL-6d-treated MCF-7 cells were analyzed by flow cytometry. As illustrated in Table I, treatment of MCF-7 cells with HYL-6d resulted in a significant increase in the percentage of cells in the S phase, accompanied by a decrease in the percentage of cells in the G_2/M phase. HYL-6d-induced arrest in the S phase peaked at 48 h, with ~47.5% of the cells in the S phase arrested, compared to 31.8% in controls.

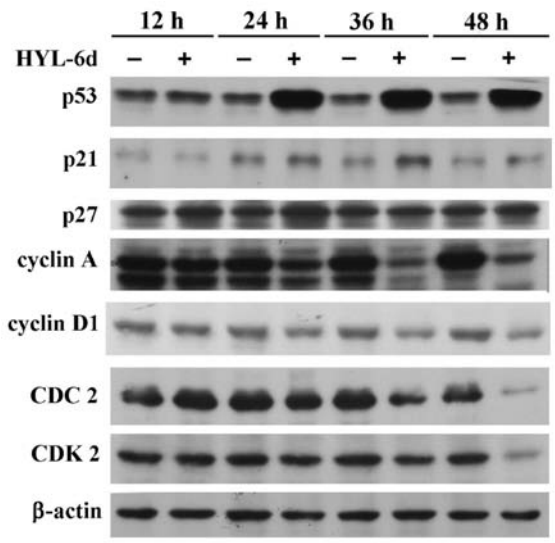


Figure 4. Expression of cell cycle regulatory proteins in MCF-7 cells. Cells were collected from HYL-6d treatments (30 μM) at indicated time points and used for western blot analysis as described. Equal amounts of total protein from each preparation were separated by SDS-PAGE on a 10% gel for subsequent western blot analysis. Specific antibodies against the indicated proteins were used in the western blot analysis. Blots were re-probed for β-actin, which was used as a control for equal protein loading.

Based on the fact that HYL-6d induced S phase arrest in MCF-7 cells, we assessed the effect of HYL-6d in cell cycle regulatory modules that play important roles in the S phase. The cyclin A-CDK2 complex is the primary regulator of the S phase progression and its expression serves as an index for the S phase arrest (23). Cyclin D1 is one of the most overexpressed oncogenes in breast cancer (6). As shown in Fig. 4, after 12 h of HYL-6d treatment in MCF-7 cells, an increase of p53 protein up to 48 h was observed. By contrast, cyclin D1, A and CDK2 levels were markedly decreased in HYL-6d-treated MCF-7 cells. These results indicate that the increase of p53, and the decrease of cyclin D1, A and CDK2, might be involved in the S phase arrest caused by HYL-6d.

Effects of HYL-6d on cell apoptosis. HYL-6d-treated cells also accumulated in the sub-G_1 phase. Accumulation in the sub-G_1 was time-dependent and indicative of apoptosis, consistent with the induction of cell death. To determine if HYL-6d-induced cell death was mediated by apoptosis, the nuclear morphology of dying cells was examined using the fluorescent DNA-binding agent DAPI. As shown in Fig. 5A, HYL-6d-treated MCF-7 cells displayed typical apoptotic morphological features, including condensed and fragmented nuclei. Homogeneous nuclear chromatin was evident in control cells (treated with 0.1% DMSO). To confirm the morphological findings, *in situ* TUNEL assay and DNA fragmentation analysis were performed. As shown in Fig. 5A, TUNEL positive cells (apoptotic cells) and internucleosomal DNA fragments (Fig. 5B) were observed after HYL-6d treatment. However, neither apoptotic cells nor DNA fragmentation were observed in control cell lines within the 36-h culture period.

Effects of HYL-6d on Bcl-2 family proteins and caspase-9. Accumulating evidence indicates that Bcl-2-related proteins play an important role in regulating apoptosis (24). To determine whether or not the expression of these cell death-associated molecules are crucial for HYL-6d-mediated apoptosis, MCF-7 cells were cultured in the presence or absence of HYL-6d for 12, 24, 36 and 48 h. In these assays, significant increases of Bcl-X_S and Bax protein levels following a 48-h treatment of MCF-7 cells with HYL-6d were found (Fig. 6). However, a decrease of Bcl-2 protein was also observed after 48 h of HYL-6d treatment. Bcl-X_L protein levels remained the same in these experiments (Fig. 6). These results suggest that HYL-6d-induced apoptosis might be mediated through the downregulation of Bcl-2 anti-apoptotic proteins and upregulation of Bcl-X_S apoptotic proteins in MCF-7 cells.

Activation of caspases during apoptosis is correlated with the cleavage and activation/inactivation of a range of critical cellular substrates, including activation of the DNA repair enzyme Poly (ADP-ribos) polymerase (PARP). In this study, the potential effects of HYL-6d on the cleavage of procaspase-9 and PARP in MCF-7 cells were also investigated. As shown in Fig. 7, procaspase-9 and PARP showed a time-dependent cleavage.

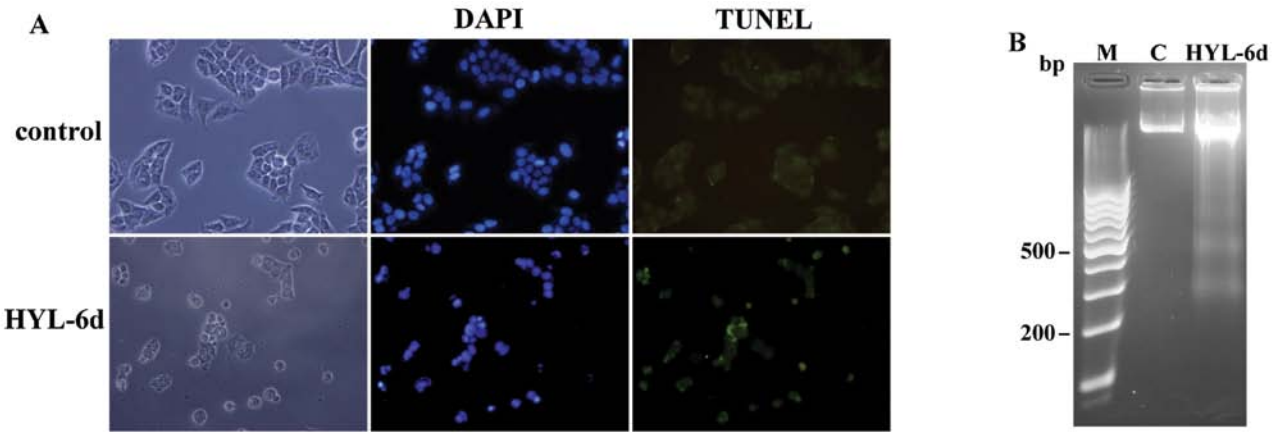


Figure 5. Induction of apoptosis by HYL-6d. (A) TUNEL/DAPI double stains. Cells were incubated with 1 μg/ml DAPI, or the TUNEL reaction mixture, and stained cells were examined by fluorescence microscopy. (B) DNA fragmentation. Cells were treated with HYL-6d for 36 h. Cellular DNA was extracted and analyzed by agarose gel electrophoresis as described.

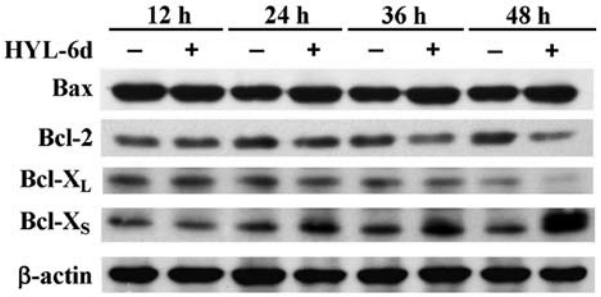


Figure 6. Regulation of Bcl-2 family proteins by HYL-6d. Cells were treated with 30 μM HYL-6d for the indicated times and prepared as in Fig. 3. Equal amounts of total proteins from each preparation were resolved by SDS -PAGE. Specific antibodies against Bcl-2 family members were used in the western blot analysis. The blots were re-probed for β-actin to test equal protein loading.

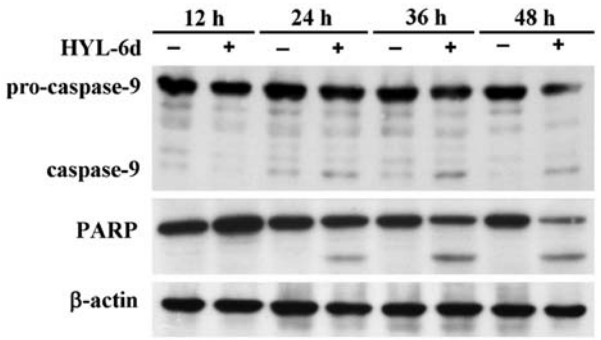


Figure 7. HYL-6d induced the cleavage of caspase-9 and PARP. MCF-7 cells were treated with HYL-6d (30 μM) for the indicated time points. After treatment, total proteins were extracted and separated by SDS-PAGE, and were then blotted using caspase-9 and PARP specific antibodies. The blots were re-probed for β-actin, which was used to test for equal protein loading.

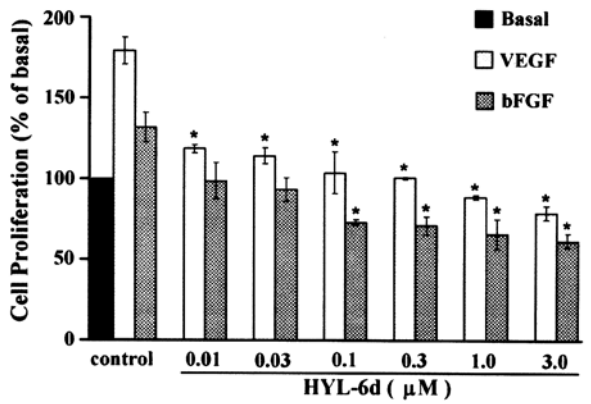
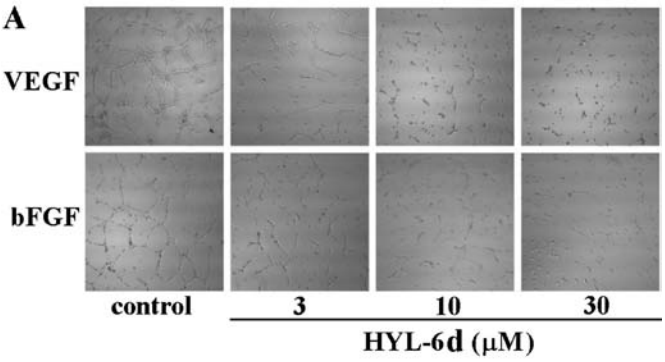


Figure 8. Effect of HYL-6d on VEGF- and bFGF-induced cell proliferation. HUVECs were incubated with the indicated concentrations of HYL-6d (0.01-3.0 μM) and with growth factor (10 ng/ml VEFG or bFGF) for 24 h. Cell proliferation was examined measuring BrdU incorporation with the commercial Cell Proliferation ELISA System (Chemicon). Each value represents the mean ± SD (n=3). *P<0.05 compared to the untreated control group.

Effect of HYL-6d on VEGF- and bFGF-induced cell proliferation of HUVECs. The ability of HYL-6d to influence the mitogen activity of VEGF and bFGF was also determined using the BrdU cell proliferation assay. In the presence of 10 ng/ml VEGF and bFGF, different concentrations of HYL-6d (0.3, 3 and 30 μM) showed inhibition of cell growth in a dose-dependent manner (Fig. 8). The data suggest that VEGF- and bFGF-induced cell proliferation is inhibited by HYL-6d.



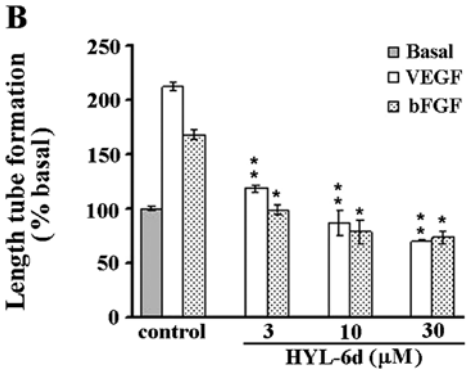


Figure 9. Inhibitory effect of HYL-6d on VEGF- and bFGF-induced cell migration. (A) HUVECs were allowed to migrate for 24 h with the indicated concentration of HYL-6d and 10 ng/ml growth factor (VEFG or bFGF) in the lower chamber to stimulate chemotaxia. (B) The migrated HUVECs were quantified by manual counting. Data are the means of three independent experiments performed in triplicate. *P<0.05, **P<0.01 compared with the untreated control.

Effect of HYL-6d on VEGF- and bFGF-induced cell migration and tube formation of HUVECs. As cell migration is necessary for endothelial cells in angiogenesis and for cancer cells in tumor growth and metastasis (10,25,26), the potential regulatory role of HYL-6d on cell migration was also measured in this study. Treatment of HYL-6d (ranging from 3 to 30 μM) significantly inhibited VEGF- and bFGF-induced HUVEC cell migration in a dose-dependent manner (Fig. 9). Angiogenesis is a complex process involving several types of cells and tube formation of endothelial cells is the key step (27). To determine if HYL-6d-inhibited angiogenesis was mediated by its effect on endothelial cell tube formation, assays were performed using HUVECs ($2x10^5$ cells) in 500 μl M199/2% FBS treated with different concentrations of HYL-6d and plated onto Matrigel layers. Following 24 h of incubation, the ability of endothelial cells to form tube-like structures was assessed by an inverted photomicroscope (Fig. 10A and B). Our results illustrated that HYL-6d blocks the formation of capillary tubes in a concentration-dependent manner without affecting HUVEC viability (Fig. 10C).

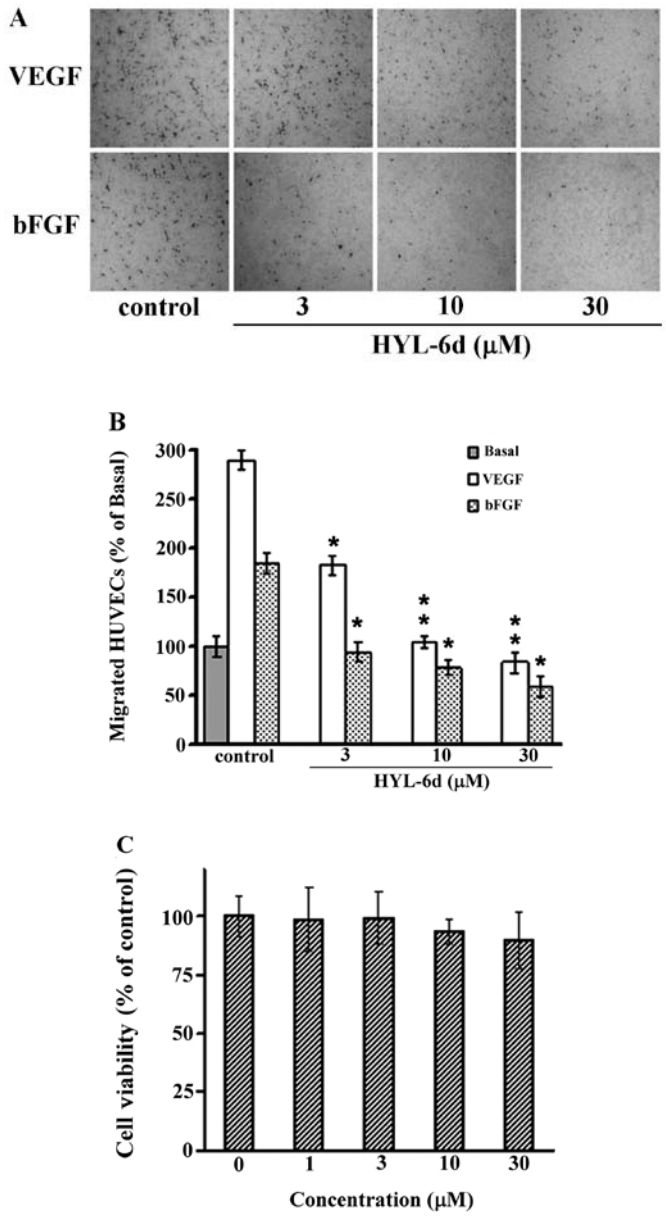


Figure 10. Inhibitory effect of HYL-6d on VEGF- and bFGF-induced cell tube formation. (A) Endothelial cell cord formation assays in which HUVECs in M199 medium are grown on Matrigel for 24 h in the presence or absence of the indicated HYL-6d concentrations. (B) The lengths and areas of structured cells were quantified using a MetaFluor Imaging System. (C) HUVEC viability was assayed using the MTT assay method described in Materials and methods. Data are the means of three independent experiments performed in triplicate. $^{*}P< 0.05$, $^{**}P<0.01$ compared with the untreated control group.

Discussion

Previous studies have shown that carbazole alkaloids isolated from the alcohol extract of the *Rutaceae* root bark display cytotoxic activity (28). The antitumor activity of carbazole, and its ability to selectively inhibit tumor growth, has been previously reported in human lung cancer cells, colon cancer cells and monocytic leukemia cells (15-17). In the present study, HYL-6d, a novel synthetic carbazole derivative, was evaluated for its cytotoxic activities in six human cancer cell lines. Our data revealed that HYL-6d is the most effective antitumor agent in the human breast cancer cell line MCF-7. *In vitro* studies have also demonstrated that HYL-6d causes cell growth inhibition, cell cycle arrest and induction of apoptosis in MCF-7 cells (Table I, Figs. 1 and 5). Moreover, HYL-6d also displayed anti-angiogenic activity. Therefore, we concluded that HYL-6d might be a potent carbazole derivative for breast cancer therapy.

Alterations in the expression and activity of cell cycle regulators have been associated with the occurrence of breast cancer (29). In 2003, a synthetic pyrrolo[3,4-c]carbazole was reported to induce cell cycle arrest in G_1 through cyclin D1/CDK4 inhibition in human lung cancer cell lines (17). Accordingly, HYL-6d is also able to induce cell cycle arrest. Exposure of MCF-7 cells to 30 μM HYL-6d led to cell cycle arrest in the S phase, and this arrest was accompanied by a marked decrease in the number of cells in the G_2/M phase (Table I). Several cell cycle regulators including cyclins, CDK, CDKI and the tumor suppressor protein p53 were examined to determine their roles in HYL-6d-induced cell cycle arrest. Using immunoblot analysis, we showed a marked increase of p53 and p21 in MCF-7 cells treated with HYL-6d (Fig. 4). Furthermore, expression of cyclin D1, A and CDK2 decreased in a time-dependent manner (Fig. 4). Collectively, these data indicated that HYL-6d-induced S-phase arrest in MCF-7 cells was caused by the inhibition of cyclins and CDK2, along with the induction of p21 and p53.

Apoptosis plays an important role in cell development and tissue homeostasis. The defects in cell death during cell development might be associated with certain problems such as autoimmune diseases and cancer, while excessive cell death can lead to degenerative diseases in the nervous system (29). Induction of apoptosis is one of the best strategies to treat cancer. Numerous current cytotoxic drugs that mediate their effects through induction of apoptosis in cancer cells have been reported (30). In this study, we revealed that HYL-6d is another anticancer drug that elicits apoptotic cell death as characterized by morphological changes, chromatin condensation and internucleosomal DNA fragmentation (Fig. 5). Bcl-2 family proteins, including the anti-apoptotic members Bcl-2 and Bcl-X_L and the pro-apoptotic members Bax and Bcl-X_S, are key regulators of apoptosis that act by either inhibiting or promoting apoptosis (24). Among these Bcl-2 family proteins, Bcl-X is an apoptotic effector (31), arising from an alternatively spliced form of the *Bcl-X* transcript that generates either a long Bcl-X_L or short Bcl-X_S form of the protein (32). Bcl-X_L is associated with decreased apoptosis in cancer cells, increased risk of metastasis and resistance to chemotherapeutic drugs (33,34). By contrast, the overexpression of Bcl-X_S can induce apoptosis and sensitize cells to chemotherapeutic agents (35,36). In this study, our data showed that the expression levels of the Bcl-2 and Bcl-X_L proteins decreased, while Bcl-X_S expression increased, after 48 h of HYL-6d treatment (Fig. 6). These expression changes were accompanied by the caspase activation (Fig. 7). Bcl-2, Bcl-X_L, Bcl-X_S and Bax are located on the outer mitochondrial membrane, leading to the release of pro-apoptotic factors from the mitochondrial inner-membrane space into the cytosol (37,38). These pro-apoptotic proteins lead to the subsequent activation of caspase-9 and the executioner caspases, an event associated with the induction of programmed cell death. These observations are consistent with our findings that elevation of Bcl-X_S protein levels in

HYL-6d-treated MCF-7 cells might alter the permeability of the mitochondrial membrane, facilitating the passage of cytochrome *c*, triggering cleavage, and activation of downstream caspases and the onset of apoptosis.

The ability of tumor cells to produce various cytokines, chemokines, angiogenic and growth factors is crucial for tumor cell proliferation and the formation of stroma and blood vessel networks that provide oxygen and nutrients to support progressive tumor growth (39). The level of angiogenic activity in breast cancer is a determinant of disease progression and survival (40), suggesting that inhibition of blood vessel formation could be a therapeutic target in this solid tumor (41). Invasive breast cancer generally expresses certain angiogenic factors, including VEGF and bFGF (42). VEGF and bFGF stimulate migration and proliferation of endothelial cells and formation of blood vessels (43). Therefore, a drug which targets angiogenesis has therapeutic value for cancer treatment. In fact, there are several chemotherapeutic agents used routinely in breast cancer treatment that are known to have anti-angiogenic activity (44). In this study, we demonstrated that HYL-6d significantly inhibited angiogenic processes including proliferation and migration in VEGF- or bFGF-stimulated HUVECs under pathological angiogenic conditions (Figs. 8 and 10A). Stimulation of angiogenic activator VEGF or bFGF can promote differentiation of HUVECS to form capillary-like tubes (45), and HYL-6d can effectively suppress this angiogenesis tube formation in human endothelial cells (Figs. 9 and 10). These data indicate that HYL-6d is a promising drug for the treatment of cancer by inhibiting angiogenesis.

In conclusion, our data revealed that HYL-6d, a novel carbazole derivative, possesses potent activity against various human cancer cell lines. HYL-6d treatment results in an arrest in the S phase and triggers apoptosis in MCF-7 cells through a p53-dependent pathway. Furthermore, HYL-6d exerts its anti-angiogenic activity by inhibiting HUVEC proliferation, migration, and tube formation induced by VEGF or bFGF *in vitro*. Thus, we have demonstrated that HYL-6d is a potent chemotherapy agent with anti-angiogenic activity and promotes apoptosis in MCF-7 cells.

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