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Isolation and Biological Properties of Novel Cell Cycle Inhibitor, HY558, Isolated from *Penicillium minioluteum* F558

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Abstract In the course of screening for a novel cell cycle inhibitor, a potent Cdk 1 inhibitor, HY558, was found from the culture broth of *Penicillium minioluteum* F558 isolated from a soil sample. The molecular ion of HY558 was identified at m/z 329 (MH+) with a molecular formula of $C_{20}H_{44}ON_2$. HY558 exhibited selective antiproliferative effects on various human cancer cell lines. Its IC_{50} values were estimated to be 0.29 mM on HepG2, 0.30 mM on HeLa, 0.30 mM on HL60, 0.33 mM on HT-29, and 0.25 mM on AGS cells. Interestingly, HY558 demonstrated no antiproliferative effect with normal lymphocytes used as the control, and a low level of inhibition on the proliferation of A549 cancer cells. A flow cytometric analysis of HepG2 cells revealed an appreciable arrest of cells at the G1 and G2/M phases of the cell cycle following treatment with HY558. Furthermore, DNA fragmentation due to apoptosis was observed in HeLa cells treated with 0.46 mM of HY558.

Key words: HY558, cell cycle inhibitor, *Penicillium minioluteum* F558

In recent years, there have been major developments in the understanding of cell cycle regulations, especially cyclin-dependent kinases (Cdks), and their role as cell cycle checkpoints. The cell cycle is an evolutionarily conserved process functioning in all eukaryotic cells to control growth and division [7, 10, 13]. The cell cycle consists of four distinct stages: two gap-phases (G1 and G2), where RNA and protein syntheses occur, an S-phase, where DNA synthesis and replication occur, and an M-phase where the cell undergoes mitosis and divides into two daughter cells. Normal cellular proliferation is ordered and tightly controlled by a series of regulatory mechanisms that either permit or prevent cell cycle progression through each phase, thereby playing an important role in maintaining the balance between old and new cells within an organism. Proliferative disorders, such as cancer, are recognized as diseases of the cell cycle. The involvement of cell cycle regulations in malignant cancers where the cellular regulations have been lost has resulted in a search for specific inhibitors of the cell cycle [11, 12, 14]. Accordingly, it is likely that manipulating the activity of cell cycle regulatory proteins, such as Cdks, in diseased states will provide an important route for treating proliferative disorders, along with the opportunity to develop a novel class of future medicines. Several synthetic and natural compounds have already been identified as inhibitors of these enzymes [4]. Therefore, such compounds would seem to be ideal targets for controlling cell proliferation and have significant advantages over conventional therapies for cancer, which are not usually tissue- or cell-type specific; however, they are frequently highly toxic to non-tumourigenic cells.

In the course of a screening program for novel microbial metabolites that inhibit the progression of the cell cycle, HY558 was identified as a Cdk 1 inhibitor from the culture broth of *Penicillium minioluteum* F558. Therefore, the current study reports on the taxonomy of the producing strain, and the fermentation, isolation, and biological properties of HY558 as a selective cell cycle inhibitor and apoptosis inducer.

Materials and Methods

Preparation of Cdk 1 Fraction from HeLa Cells for Inhibitor Screening

HeLa cells, synchronized in the M-phase, were washed twice with PBS and centrifuged at 2,000 rpm for 10 min.

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The pellet was then resuspended in a 1.5 pellet volume of an extraction buffer (50 mM Tris-HCl/pH 7.4, 250 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1% Triton X-100, one tablet of protease inhibitor, and 0.5 mM PMSF). The lysate was obtained by centrifugation (15,000 rpm) at 4°C for 30 min, and the supernatant was used as the enzyme source for screening Cdk 1 inhibitors.

Screening for Cdk 1 Inhibitor

To isolate a novel cell cycle inhibitor from the culture broths of various soil microorganisms, their Cdk 1 inhibitory activities were assayed as follows: A kinase reaction mixture (25 μl) containing 2.5 μg of histone H_1 peptide (1 μg/μl) as the substrate, 10 μg of crude Cdk 1 fraction from HeLa cells, 2.5 μl of a 10× kinase buffer (250 mM Tris-HCl/pH 7.4, 2 mM $MgCl_2$, 20 mM DTT, 50 mM β-glycerophosphate, and 1 mM Na_3VO_4), the culture broth of soil microorganisms, 1 μCi of [γ-^{32}P] ATP(5 Ci/μmol), and 2.5 μl of 0.5 mM ATP was incubated for 15 min at 30°C. After the incubation, the reaction was stopped by the addition of 12 μl of 7.5 M guanidine hydrochloride. Subsequently, the reaction mixture was centrifuged, and 10 μl aliquots of the supernatant were spotted onto Whatman P-81 phosphocellulose paper. After 10 min, the paper was washed 3 times with 1% phosphoric acid for 15 min, dried, and counted for radioactivity using a liquid scintillation counter (Wallac, Perkin Elmer, Boston, U.S.A.).

Taxonomic Studies of Strain F558

For identification of strain F558, the rDNA sequence was analyzed using the PHYDIT program of MicroID Co. (Daejon, Korea) according to ITS (Internal transcribed spacer) region sequencing. The percentage of similarity was determined by sequence alignment. The evolutional distance was calculated by the Jukes' and Cantor method [8], and a phylogenic tree was created using the neighbor-joining method [15]. The morphological properties of strain F558 were characterized by observation under a light microscope.

Fermentation of Strain F558

The culture medium consisted of 15 g/l soluble starch, 3 g/l yeast extract, 5 g/l $KHPO_4$, and 0.5 g/l $MgSO_4$. A single colony of strain F558 was inoculated into a 250-ml flask containing 50 ml of the medium, and incubated at 30°C for 4 days, shaking at 250 rpm. Five milliliters of the broth was transferred into a 500-ml baffled flask containing 100 ml of the culture medium and cultivated at 30°C for 3 days at 250 rpm. This seed culture was then transferred into a 7-l fermentor (BioG-12, BioTron Co., Puchon, Korea) containing 5-l of the culture medium and cultivated at 30°C for 120 h. After this main culture, the fermentation broth was mixed with the same volume of ethanol, and extracted at room temperature for 4 h with stirring. The pH of the culture broth was not controlled during the fermentation. However, the concentration of dissolved oxygen was maintained above 20% air saturation by adjusting the agitation speed within a range of 300 to 800 rpm. The aeration rate was 1.0 vvm during the fermentation.

Analytical Methods

The dry cell weight was estimated using a calibration curve derived from the relationship between the absorbance at 600 nm after grinding with a sonicator and the dry cell weight. The concentration of soluble starch was measured using the phenol-sulfuric acid method [6].

Isolation of HY558

The ethanol extract of the culture broth (3 liters) of strain F558 was concentrated *in vacuo*. The concentrate was extracted 3 times with an equal volume of ethyl acetate, then the organic phase was evaporated to dryness under reduced pressure. The solid residue was dissolved in 30% isopropyl alcohol (IPA) in water, then the solution was applied to a Lichroprep RP-18 (Merck AG, Darmstadt, Germany) column for chromatography. The fraction eluted with 50% IPA was further purified by a semi-preparative reverse-phase HPLC (Vydac ODS, 25 mm×250 mm; Hesperia, U.S.A.), using 50% acetonitrile in water as the mobile phase. Finally, HY558 (32 mg) was isolated in a pure state.

Cell Culture

The human cervical adenocarcinoma cell line HeLa, human hepatocellular carcinoma cell line HepG2, human myelocytic leukemia cell line HL-60, human stomach cancer cell line AGS, human lung cancer cell line A549, and human colon cancer cell line HT-29 were all obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in MEM (for HeLa) and RPMI 1640 (for HepG2, HL-60, AGS, A549, HT-29) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin at 37°C in a humidified atmosphere of 5% CO_2.

MTT Assay

The antiproliferative effect of HY558 was determined by an MTT assay [9]. An MTT reagent kit was purchased from Sigma Chemical (St. Louis, U.S.A.) to count the viable cells. The cells (1.4×10^5 cells per well) were seeded in a 96-well plate in 100 μl of the cell culture medium. After incubation for 24 h, 1 μl containing various concentrations of HY558 was added to the cancer cells to be tested, with normal lymphocytes as a control. After incubation at 37°C for 24 h, the culture medium was carefully removed without disturbing the cells, and replaced by 100 μl of a fresh cell medium. Subsequently, fifteen microliters of an MTT reagent was added to each well, and the plates

incubated again in a CO_2 incubator at 37°C for 3 h. After the incubation, the supernatant was removed from each well, then 100 ml of DMSO was added to dissolve the colored formazan crystals produced by MTT. Subsequently, the optical density was measured at 570 nm using an ELISA Reader (Molecular Devices Corp., Sunnyvale, U.S.A.).

Flow Cytometry Analysis

HepG2 cells ($1-2\times10^6$/ml) were incubated in an RPMI medium with 10% FBS for 24 h after treatment with 0.46 mM of HY558. The cells were then washed twice with ice-cold PBS, harvested, fixed with ice-cold PBS in 70% ethanol, and stored at 4°C. For a flow cytometric analysis, the cells were incubated with 0.1 mg/ml RNase A at 37°C for 30 min, stained with 50 μg/ml propidium iodide for 30 min on ice, and then measured using a FASTAR flow cytometer (Becton Dickinson, San Diego, U.S.A.) with Cell Quest software.

TUNEL Assay

The detection of apoptotic cells was processed according to the manufacturer's protocol using an Apoptosis Detection System, Fluorescein (Promega Corp., Madison, U.S.A). HeLa cells ($1-2\times10^6$/ml) were incubated in MEM with 10% FBS for 24 h after treatment with 0.46 mM of HY558. The HeLa cells were then washed in PBS and fixed in 1% formaldehyde solution for 20 min on ice. Subsequently, the cells were washed again in PBS and resuspended in an equilibration buffer for 5 min at room temperature, then in a TdT reaction buffer (50 μl) at 37°C for 60 min. After the termination of the TdT reaction, the cells were incubated in 1 ml of PBS containing 25 μg/ml propidium iodide and 250 μg/ml RNase A at room temperature for 30 min in the dark. The fluorescein-12-dUTP-labeled DNA was quantitated using a FASTAR flow cytometer with Cell Quest software.

Structure Elucidation

All the NMR measurements were performed on a Bruker Avance 400 spectrometer (Karlsruhe, Germany) system (9.4 T) at a temperature of 298 K. The NMR spectra of ^{1}H-NMR, ^{13}C-NMR, DEPT [5], COSY [2], HMQC [3], and HMBC [1] were collected in MeOH-d_4 with TMS as the internal reference. The concentration of samples was 50 mM. For ^{1}H-NMR analysis, 16 transients were acquired with a 1 sec relaxation delay using 32 K data points. The 90° pulse was 9.7 μsec with a spectral width of 4,000 Hz. The ^{13}C-NMR and DEPT spectra were obtained for a spectral width of 8,000 Hz, collecting 64 K data points, where the 90° pulse was 9.8 μsec. The two-dimensional spectra were acquired with 2,048 data points for t2 and 256 points for t1 increments. The ESI/MS was carried out on a VG Micromass Autospec (Micromass, Manchester, U.K.).

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TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCG
GGCCCTCGTGGCCCAACCTCCCACCCTTGTCTCTATACACC
TGTTGCTTTGGCGGGCCCACCGGGGCCACCTGGTCGCCGG
GGGACATCTGTCCCCGGGCCTGCGCCCGCCGAAGCGCTCT
GTGAACCCTGATGAAGATGGGCTGTCTGAGTACTATGAAA
ATTGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCA
TTTCTGCCCTCAAGCACGGCTTGTGTGTTGGGTGCGGTCC
CCCCGGGGACCTGCCCGAAAGGCAGCGGCGACGTCCGTC
TGGTCCTCGAGCGTATGGGGCTTTGTCACTCGCTCGGGAA
GGACTGGCGGGGGTTGGTCACCACCAAAATTTTACCACGG
TGACCTCGGA
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Fig. 1. ITS rDNA sequence of strain F558.

Results and Discussion

Taxonomy

The conidia of strain F558 were smooth and ellipsoidal with a size of 2.5–3.5×2.0–3.5 μm, while the conidiophores were biverticillate with a size of 60–130 μm. The ITS sequencing of the rDNA was performed using the PHYDIT program (Fig. 1), and the size found to be 532 bp, showing 99.43% similarity with *Penicillum minioluteum* (Table 1). A phylogenic tree was constructed using the neighbor-joining method (Fig. 2). The scar bar of the tree indicated 0.1 substitutions per site. Accordingly, strain F558 was found to belong to *P. minioluteum* with a high bootstrap value based on an analysis of the phylogenic tree. Strain F558 was also identified to be closely related to *P. funiculosum* and *P. purpurogeum* in the phylogenic tree. However, the growth rates of *P. minioluteum* and strain F558 were lower than those of *P. funiculosum* and *P. purpurogeum*, and the conidiophores of strain F558 exhibited a longer strip with more regular arrangement than the others. Based on its morphological characteristics and ITS sequencing, strain F558 was classified as *P. minioluteum* F558.

Table 1. Similarity to reference strains.

Strain	Accession No.	Similarity (%)
Penicillium minioluteum	L14505	99.43
P. funiculosum	L14503	98.77
P. aculeatum	NRRL2129	98.04
Talaromyces purpureus	L14527	97.55
P. marneffei	L37406	97.17
P. pinophilum	AF176660	97.16
P. duclauxii	L14534	96.86
T. stipitatus	L14514	95.14
T. helicus	NRRL2106	92.91
P. dendriticum	L14502	92.49
P. purpurogenum	L14506	91.68
P. chermesinum	NRRL735	85.63

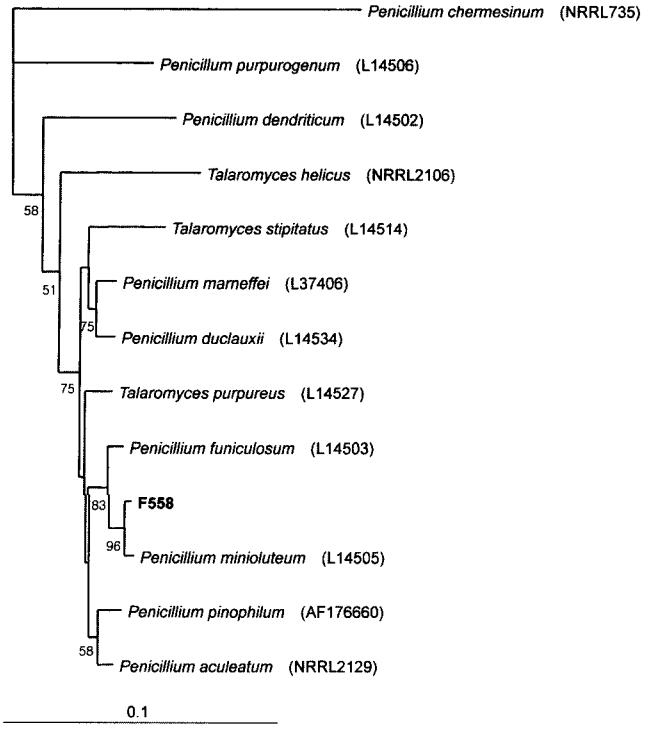

Fig. 2. Phylogenetic tree of strain F558.

Fermentation

The results from the typical batch fermentation of *P. minioluteum* F558 with an initial soluble starch concentration of 15 g/l are illustrated in Fig. 3. During the fermentation, the soluble starch was completely consumed within 40 h, yet a small part of the residual soluble starch still remained unutilized after 120 h of cultivation. A final cell concentration of 6.3 g/l was obtained after 120 h, at which point the residual soluble starch was 1.5 g/l.

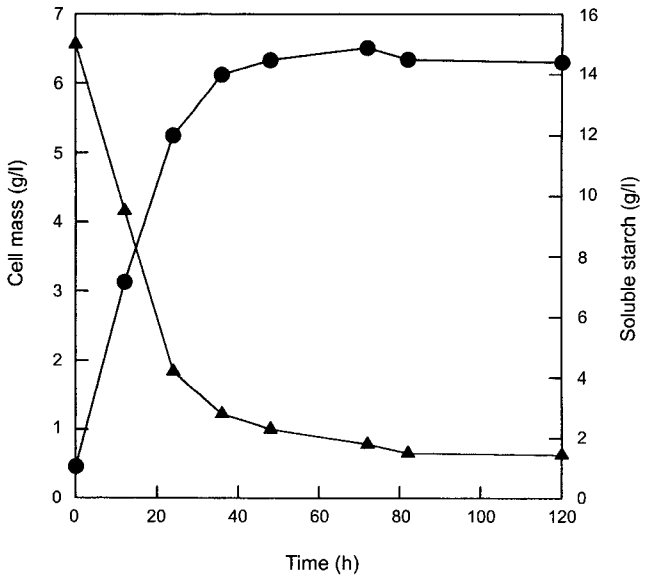

Fig. 3. Time course of growth of *P. minioluteum* F558 and consumption of soluble starch.
(●) Cell mass; (▲) Soluble starch.

Biological Activities of HY558

Inhibition of cancer cell proliferation. The antiproliferative effects of HY558 were assessed in six human cancer cell lines and in normal lymphocytes as the control. HepG2, HL-60, AGS, A549, HT-29, HeLa, and normal lymphocyte cells were all cultured in varying concentrations of HY558 for 24 h, and analyzed using an MTT assay. All assays were performed in triplicate. As shown in Fig. 4, the growth of all cancer cells tested, except for A549 and the normal lymphocyte cell, was inhibited in a concentration-dependent manner by HY558. The estimated IC_{50} values determined for HY558 in these cancer cell lines ranged from 0.25 to 0.33 mM. Interestingly, HY558 demonstrated no antiproliferative effect on the normal lymphocyte cells which were used as the control, and a low level of inhibition was observed on the A549 cells, due to the deletion of the p16 gene. Accordingly, these results would seem to indicate that the inhibition of cancer cell proliferation by HY558 was associated with the level of p16.

Inhibition of cell cycle regulation. To investigate the effect of HY558 on the cell cycle progression of cancer cells, the DNA content of the cell nuclei was measured using a flow cytometric analysis. The cell cycle analysis

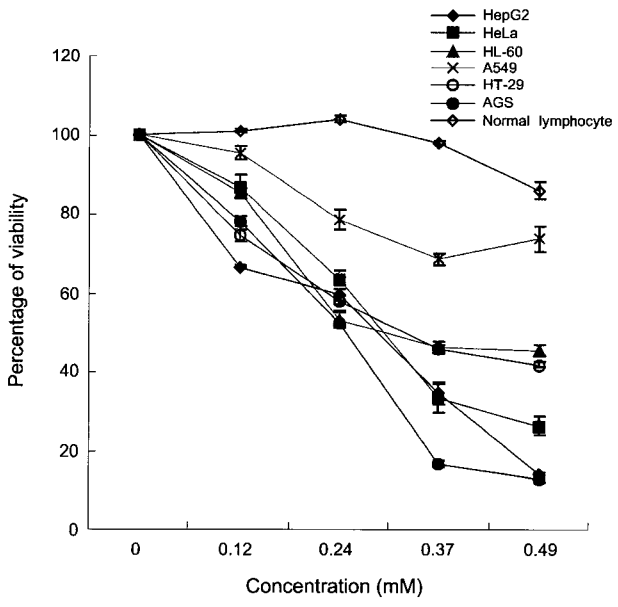

Fig. 4. Antiproliferative effect of cell growth of HY558 in HepG2, HeLa, HL-60, A549, HT-29, AGS, and normal lymphocyte cells as determined by MTT assay.
IC_{50}: 0.29 mM±0.001 in HepG2 cells, 0.30 mM±0.024 in HeLa cells, 0.30 mM±0.030 in HL-60 cells, 0.33 mM±0.009 in HT-29 cells, and 0.25 mM± 0.017 in AGS cells. Data points represent means of three experiments.

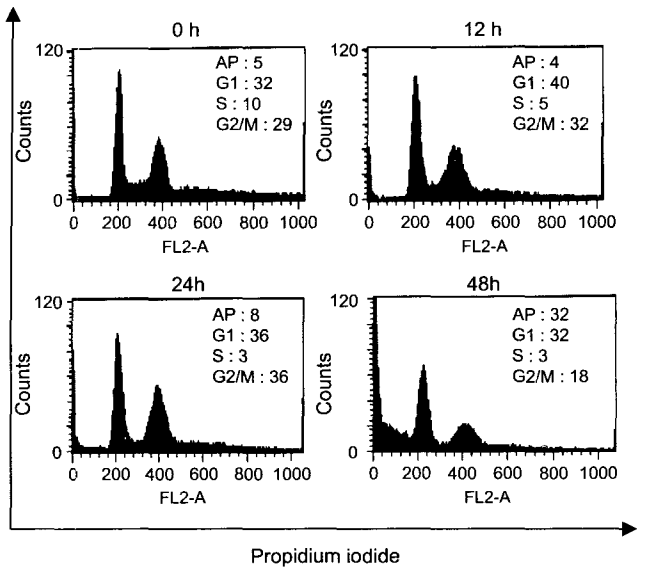

Fig. 5. Histogram of flow cytometric analysis showing the effect of HY558 on cell cycle in HepG2 cells.
HepG2 cells were treated with 0.46 mM of HY558 for a time course. The cells were then stained with propidium iodide, and the nuclei analyzed for their DNA content using flow cytometry with Cell Quest software. A total of 10,000 nuclei were analyzed from each sample (Ap, apoptotic cells; G1, cells in the G1 phase; S, cells in the S phase; G2/M, cells in the G2/M phase).

was performed on HepG2 cells, after exposure to HY558 at 0.46 mM for 0, 12, 24, and 48 h (Fig. 5). The flow cytometric analysis revealed an appreciable arrest of cells in the G1 and G2/M phases of the cell cycle after treatment with HY558. The HepG2 cell population increased gradually from 32% at 0 h to 40% at 12 h in the G1 phase, and from 29% at 0 h to 36% at 24 h in the G2/M phase, after exposure to 0.46 mM of HY558. Furthermore, eventual progression to apoptosis was first observed after 48 h (AP: 32%). The percentage of S phase cells was not profoundly affected. Accordingly, treatment with HY558 induced G1 and G2/M phases arrest during cell cycle progression of HepG2 cells.

Induction of apoptosis. To investigate the apoptotic induction in cancer cells by HY558, the DNA fragmentation of HeLa cells due to apoptosis was measured directly by a terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay (Fig. 6). When HeLa cells were incubated with 0.46 mM of HY558 for 24 h, an apoptotic DNA fragmentation (shift of the red curve to the right in contrast to the yellow control region) was observed in the HeLa cells.

In summary, the current study showed that HY558 can inhibit the growth of cancer cells *in vitro*. It was also shown by a flow cytometric cell cycle analysis that exposure to HY558 deregulated the cell cycle, thereby enhancing the proportion of HepG2 cells in the G1 and G2/M phases. Furthermore, it was demonstrated that HY558 could induce apoptotic DNA fragmentation in HeLa cells. These and other possible mechanisms for the action of HY558 are currently being investigated. The identification of molecular pathways that are altered by HY558 should lead to the development of agents with greater potency and specificity.

Structure Determination of HY558

The molecular ion of the compound was identified at m/z 329 (MH+). Based on the ^{1}H-NMR and ^{13}C-NMR spectra, the compound was found to contain a long hydrocarbon chain. The DEPT experiment revealed two methyl groups, two methine groups, and more than fourteen methylene groups. Two methane groups observed at 43.5 and 52.6 ppm were connected to nitrogen and oxygen, respectively. Since the molecular weight of the compound was an even number, there should be an even numbers of nitrogen atoms. From the ^{13}C-NMR spectrum, the compound must contain more than eighteen carbons, while based on the thirteen rule, the molecular formula of the compound was identified as $C_{20}H_{44}ON_2$ without a double bond. Interpretation of the MS spectrum provided us with information on the existence of a hydroxyl group. Even though the compound consisted of a long hydrocarbon chain and exhibited a positive Nihydrin test, since it was soluble in methanol, it was confirmed to contain a hydroxyl group and primary amine group, which consisted of a hydrazino group. This result satisfies the existence of two methine groups. Accordingly,

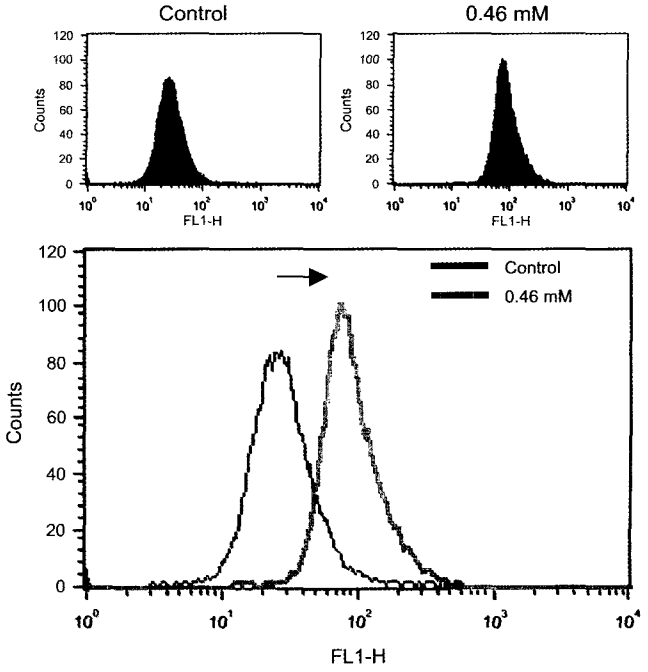

Fig. 6. Apoptotic induction in HeLa cells by HY558.
The cells were incubated for 24 h in 0.46 mM of HY558 and then fixed, permeabilized, and stained with a fluorescent TUNEL reaction. The cells were then analyzed using flow cytometry.

the compound was identified as a hydroxyhydrazinoicosane. However, to determine the branched positions for further experiments, more amounts of the compound are required.

Acknowledgments

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