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Inhibition of Cell Cycle Progression and Induction of Apoptosis in HeLa Cells by HY558-1, a Novel CDK Inhibitor Isolated from *Penicillium minioluteum* F558

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Abstract In the course of screening for a novel inhibitor of CDC2, HY558-1 was isolated from a culture broth of *Penicillium minioluteum* F558. Moreover, it was found that HY558-1 had an effect on both the cell cycle regulation and apoptosis of human cervical adenocarcinoma HeLa cells. A flow cytometric analysis of HeLa cells revealed appreciable cell cycle arrest at the G1 and G2/M phases following treatment with HY558-1. Furthermore, DNA fragmentation due to apoptosis was observed in HeLa cells treated with HY558-1. To obtain further information on the cell cycle arrest and apoptotic induction induced by HY558-1, the expression of certain cell cycle and apoptosis-associated proteins was examined using a Western blot analysis. The results revealed that HY558-1 inhibited the phosphorylation of pRb and decreased the expression levels of CDK2, CDC2, and cyclin A in the cell cycle progression. It was also shown that the level of $p21^{WAF1/CIP1}$ was increased in HeLa cells treated with 0.52 mM of HY558-1. Accordingly, HY558-1 was found to inhibit the proliferation of HeLa cells through the induction of G1 phase arrest by inhibiting pRb phosphorylation via an upregulation of $p21^{WAF1/CIP1}$, and G2/M phase arrest by directly inhibiting CDC2 and cyclin A. Moreover, HeLa cells treated with 0.52 mM of HY558-1 exhibited apoptotic induction associated with the cleavage of Bid and release of cytochrome *c* from mitochondria into the cytosol. Subsequent investigation of the activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase (PARP) suggested that the mitochondrial pathway was primarily involved in the HY558-1-induced apoptosis in HeLa cells.

Key words: HY558-1, *Penicillium minioluteum* F558, cell cycle arrest, apoptosis

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The cell cycle progression in mammalian cells is governed by the orderly activation of cyclin-dependent kinase (CDK) regulated by induction or degradation of cyclin proteins [9, 12, 18, 20, 25]. Each cell cycle phase is characterized by the presence of distinct cyclin-CDK complexes [21]. The G1-S transition is dependent on the activation of G1 phase CDKs and phosphorylation of retinoblastoma protein (pRb) [13, 32]. Cyclin D associates with CDK4 and CDK6 and this association then leads to the activation of CDK4 and CDK6, which help in maintaining and progression through the early G1 phase of the cell cycle [18]. The cyclin E and CDK2 proteins both play a role in the transition from the G1 to S phase of the cell cycle. The G1 phase-specific cyclin and CDK complexes phosphorylate the pRb and repress its inhibitory activity by associating with the E2F transition factor, which mediates the expression of the S phase-specific gene [13, 32]. In addition, cyclin A is produced in the late G1 phase, then expressed during the S and G2 phases, while the expression of cyclin B is typically maximal during the G2 to M phase transition and controls the passage through the M phase by primarily associating with and activating CDK2 and CDC2, respectively [7].

Meanwhile, the activity of CDKs is inhibited by cyclin-dependent kinase inhibitors (CKIs), such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, whose expression regulates the G1/S phase CDKs [3, 8, 10, 23, 26, 27], thereby blocking cell proliferation and inducing cell growth arrest. As such, inhibitors of the cell cycle progression have the potential of being used as antiproliferation agents [12, 15, 28].

In the course of screening for a novel inhibitor of CDC2, we isolated HY558 and HY558-1 from a culture broth of *Penicillium minioluteum* F558. As reported previously, HY558 exhibited a selective antiproliferative effect on

various human cancer cell lines [14]. A flow cytometric analysis of HepG2 cells treated with HY558 revealed an appreciable arrest of cells at the G1 and G2/M phases of the cell cycle. Furthermore, DNA fragmentation due to apoptosis was observed in HeLa cells treated with HY558 [14]. As such, the current study reports on the isolation and characterization of HY558-1, which shows no structural correlation with HY558, as a cell cycle inhibitor and an apoptosis inducer.

MATERIALS AND METHODS

Chemicals

The phosphate-buffered saline (PBS) and RPMI 1640 medium were purchased from GIBCO, Ltd. (Grand Island, NY, U.S.A.), while the mouse monoclonal antibodies against caspase-3, PARP, cytochrome *c*, and β-actin, and polyclonal antibodies against caspase-8, -9, and Bid were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and the protein assay kit was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other materials were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell Line and Culture

The human cervical adenocarcinoma cell line HeLa was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured at 37°C in a humidified atmosphere of 5% CO_2-air using the RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin. The cell density in the culture did not exceed 1×10^6 cells/ml [15, 16].

Isolation and Purification of HY558-1

The ethanol extract of the culture broth (4 l) of strain *Penicillium minioluteum* F558 was concentrated *in vacuo*, extracted 3 times with an equal volume of ethyl acetate, then the organic phase was evaporated to dryness under reduced pressure. Thereafter, the solid residue was dissolved in 30% isopropyl alcohol (IPA) in water, and the solution applied to a Lichroprep RP-18 (Merck AG, Darmstadt, Germany) column for chromatography. The fraction was eluted with 50% IPA and further purified using semi-preparative reverse-phase HPLC (Vydac ODS, 25 mm×250 mm: Hesperia, U.S.A.) with 45% acetonitrile in water as a mobile phase. Finally, HY558-1 (10 mg) was isolated in a pure state.

Flow Cytometry Analysis

The HeLa cells ($1-2\times10^6$ cells /ml) were treated with 0.52 mM of HY558-1 for a time course (0–24 h). 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 in 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.). The HeLa cells ($1-2\times10^6$ cells/ml) were incubated in MEM with 10% FBS for 24 h after treatment with 0.52 mM of HY558-1. The HeLa cells were then washed in PBS and fixed in a 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 (PI) 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.

Western Blot Analysis

The HeLa cells were plated onto 60-mm dishes at a density of 2×10^5 cells/ml with or without HY558-1 (0.52 mM, 0–24 h) and then harvested. To prepare the whole-cell extract, the cells were washed with PBS and suspended in a protein lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 μg/ml PMSF, and protease inhibitors). The protein content was determined with a Bio-Rad protein assay reagent using bovine serum albumin as a standard. The protein extracts (30–50 μg) were analyzed based on 8–14% SDS-PAGE and transferred to a PVDF membrane (Millipore, Corp., Bedford, MA, U.S.A.). The membrane was blocked with 5% w/v nonfat dry milk, then incubation with the indicated antibodies was performed in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl/pH 7.6) containing 0.1% Tween-20, with gentle shaking at 4°C for 2–12 h. The secondary antibody was a peroxidase-conjugated goat antimouse, rabbit antibody. The signals were detected using an ECL Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

Preparation of Cytosolic Extractions for Cytochrome *c* Analysis

The HeLa cells treated with or without HY558-1 were washed with ice-cold PBS and resuspended in cold lysis buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol,

and protease inhibitor). After incubation on ice for 20 min, the cells were homogenized with 15 strokes in a douncer, and the homogenates centrifuged at 1,000 ×*g* for 10 min at 4°C. The supernatants were then transferred to a tube and centrifuged at 100,000 ×*g* for 20 min at 4°C for use in the cytochrome *c* analysis.

Structure Elucidation of HY558-1

All NMR measurements were performed on a Bruker Avance 400 spectrometer system (9.4 T, Karlsruhe, Germany) at a temperature of 298 K. The nuclear magnetic resonance (NMR) spectra of H-NMR, ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bonded connectivity (HMBC) were collected in methanol-d4 with TMS as an internal reference. The concentration of the samples was 1 mM. For ^{1}H-NMR analysis, 128 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 19,048 Hz, collecting 64 K data points. The 90° pulse was 9.8 μsec. Two-dimensional spectra were acquired with 2,048 data points for t2 and 256 for t1 increments.

Results

Structure Determination of HY558-1

In order to determine the structure of HY558-1, several basic 1D and 2D NMR experiments were carried out on a Bruker Avance 400 NMR spectrometer. Eighteen ^{13}C signals were observed in the ^{13}C spectrum. The type of carbons was determined based on the DEPT experiments. The compound contains 4 methyl carbons, 2 methylene carbons, 7 methine carbons, and 5 quaternary carbons. According to the interpretation of HMQC, the ^{1}H signals connected to the corresponding ^{13}C signals were determined. Ten ^{13}C signals observed between 119 ppm and 156 ppm showed that this compound includes a naphthalene moiety. Two downfield signals, such as 153.3 ppm and 155.7 ppm, were caused by hydroxylated carbons. The quartet signal at 63.3 ppm gave the existence of a methoxyl group. The doublet and the triplet signals at 66.6 ppm and 70.6 ppm, respectively, were assigned as hydroxylated carbons. Two quartet carbons at 22.4 and 24.0 ppm were connected through the doublet carbon at 26.2 ppm. The triplet carbon at 49.1 ppm was connected to the ^{1}H signal at 3.92 ppm directly. Based on the interpretation of the COSY spectrum, six carbons at 22.4, 24.0, 26.2, 49.1, 66.6, and 70.6 ppm connected to the six protons at 0.98, 0.96, 1.77, 1.45/1.65, 5.09, and 5.09 ppm, respectively, mentioned above should be composed of 4-methylpentan-2-ol. It was decided that this

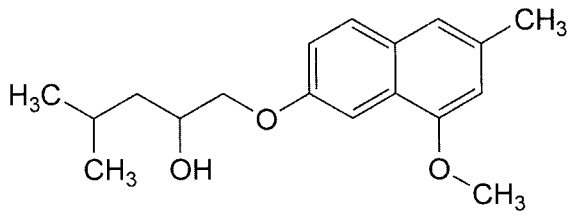

Fig. 1. Structure of HY558-1.

partial structure was connected to the carbon at 153.3 ppm through oxygen. The quartet carbon at 21.0 ppm was connected to the carbon at 136.2 ppm, based on the HMBC experiment. According to the same experiment, the methoxyl group observed at 63.3 ppm should be connected to the carbon at 155.7 ppm. The final structure of the compound should be 1-[(8-methoxy-6-methyl-2-naphthyl)oxy]-4-methylpentan-2-ol whose structure is shown in Fig. 1. In order to verify this result, we need to further pursue the analysis of the mass spectrum as well as the synthesis of that compound.

Inhibition of Cell Cycle Regulation by HY558-1

To investigate the effect of HY558-1 on the cell cycle progression of human cervical adenocarcinoma cells, the DNA content of the cell nuclei was measured using a flow cytometric analysis. HeLa cells were cultured with HY558-1 at the indicated time (0, 6, 12, and 24 h), washed, stained with propidium iodide, and then the cell cycle was analyzed. As shown in Fig. 2, the flow cytometric analysis revealed an appreciable arrest of cells in the G1 and G2/M

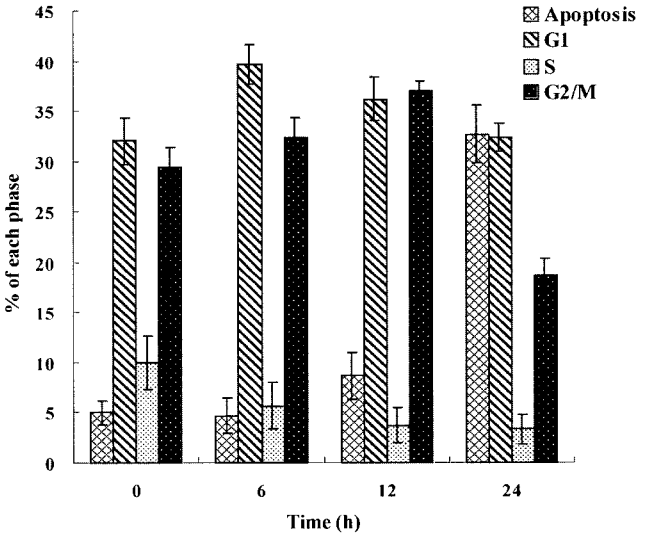

Fig. 2. Effect of HY558-1 on the DNA content of HeLa cells. HeLa cells were treated with 0.52 mM of HY558-1 for a time course. The cells were then stained with propidium iodide, and the nuclei analyzed for their DNA content using flow cytometry with Cell Quest software. A total of 10,000 nuclei was analyzed from each sample. Data are means±SD of three separate experiments.

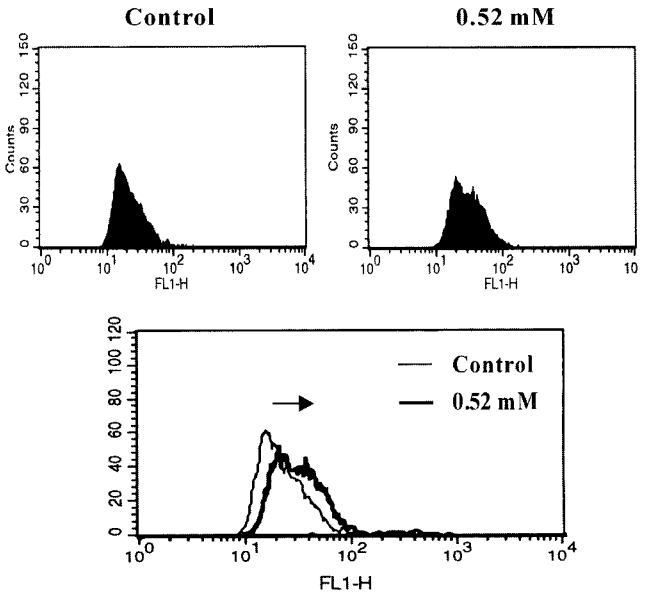

Fig. 3. Apoptotic induction in HeLa cells by HY558-1.
The cells were incubated for 24 h in 0.52 mM of HY558-1 and then fixed, permeabilized, and stained with a fluorescent TUNEL reaction. The cells were then analyzed using flow cytometry.

phases of the cell cycle after treatment with 0.52 mM of HY558-1. HeLa cell population increased gradually from 32% at 0 h, to 40% at 6 h in the G1 phase, and from 29% at 0 h, to 37% at 12 h in the G2/M phase, after exposure to 0.52 mM of HY558-1. Furthermore, eventual progression to apoptosis was first observed after 24 h (AP: 33%). The percentage of S phase cells was not profoundly affected. Accordingly, treatment with HY558-1 induced a G1 and G2/M phase arrest of the cell cycle progression of HeLa cells.

Induction of Apoptosis by HY558-1

To elucidate the apoptotic induction in cancer cells by HY558-1, the DNA fragmentation of HeLa cells due to apoptosis was measured directly by a terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay. As shown in Fig. 3, when HeLa cells were incubated with 0.52 mM of HY558-1 for 24 h, an apoptotic DNA fragmentation (shift of the curve to the right in contrast to the control region) was observed in the HeLa cells.

Effect of HY558-1 on pRb

The change in pRb phosphorylation in the cell cycle progression was studied in HeLa cells treated with HY558-1 using a Western blot analysis. As shown in Fig. 4, a significant amount of hypophosphorylated pRb (with a faster migration on the gel) accumulated after treatment with 0.52 mM of HY558-1 for 24 h, whereas the amount of hyperphosphorylated pRb (with a slower migration on the gel) was reduced. Thus, treatment of the cells with

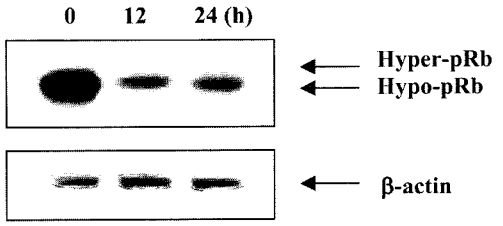

Fig. 4. Effect of HY558-1 on pRb phosphorylation.
Total cell lysates from HeLa cells treated with 0.52 mM of HY558-1 for 24 h were electrophoretically separated on an 8% polyacrylamide gel and immunoblotted with an antibody against pRb and β-actin, which served as an internal control.

HY558-1 was found to suppress the hyperphosphorylated form of pRb with a commensurate increase in the hypophosphorylated form. β-Actin served as an internal control.

Effect of HY558-1 on CDKs, Cyclins, and CKI

The expression levels of CDKs and cyclins by HY558-1 during cell cycle progression were investigated next. As shown in Fig. 5, the level of CDK2 during the G1 phase was gradually reduced in a time-dependent manner after treatment with 0.52 mM of HY558-1.

To determine the G2/M phase arrest in HeLa cells by HY558-1, the expression levels of CDC2, cyclin B1, and

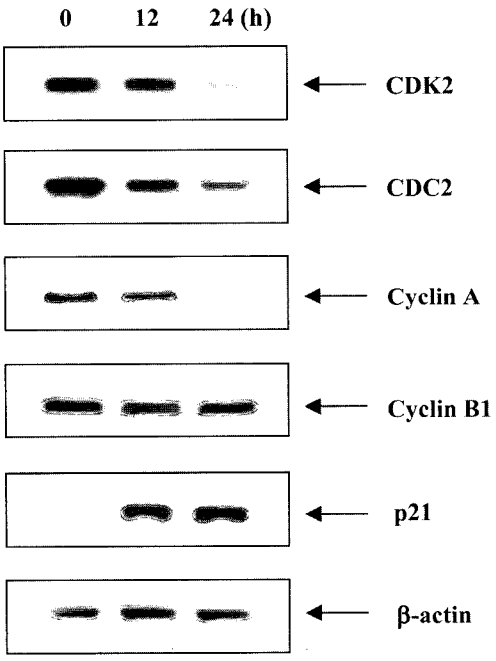

Fig. 5. Effect of HY558-1 on protein expression of CDKs, cyclins, and CKI.
Total cell lysates from HeLa cells treated with 0.52 mM of HY558-1 for 24 h were analyzed using 10–14% PAGE for CDKs, cyclins, and CKI, and subsequently immunoblotted with an antibody against CDKs, cyclins, CKI, and β-actin, which served as an internal control.

cyclin A were all assessed. The expression levels of CDC2 and cyclin A were gradually reduced in a time-dependent manner after treatment with 0.52 mM of HY558-1, however, the amount of cyclin B1 was not reduced.

In addition, the expression level of the CKI regulated G1/S phase CDKs was also examined. As shown in Fig. 5, Western blot analysis revealed a significant increase in the expression level of $p21^{WAF1/CIP1}$ 12 h after treatment with 0.52 mM of HY558-1.

Effect of HY558-1 on Caspases and PARP

Caspases are believed to play a central role in the apoptotic signaling pathway. Both caspase-9 and caspase-8 are defined as initiator caspases, which in turn activate caspase-3, the executor of apoptosis [28, 29]. Therefore, the expressions of caspase-3, -8, and -9 were evaluated in HeLa cells following treatment with 0.52 mM of HY558-1. A Western blot revealed a time-dependent cleavage of procaspase-8 and -3 into an active form and the decrease of procaspase-9 (Fig. 6). It is known that PARP, poly (ADP-ribose) polymerase, one of the substrates of caspase-3, is characteristically processed during apoptosis from its native 116 kDa form into a truncated 85 kDa product [2]. As shown in Fig. 6, treatment with HY558-1 resulted in the cleavage of PARP. Therefore, when taken together, the results clearly showed that the

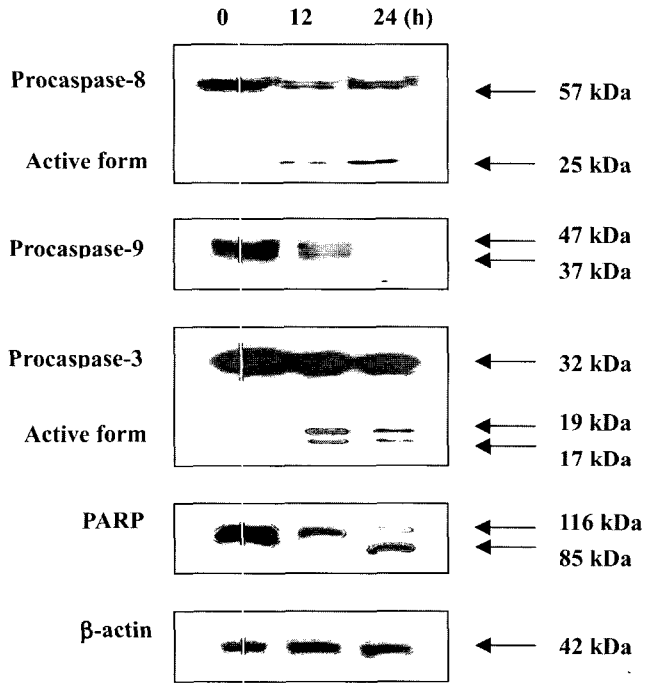

Fig. 6. Effect of HY558-1 on activation of caspases and cleavage of PARP.
HeLa cells were exposed to 0.52 mM of HY558-1 for 24 h. Thereafter, the cells were lysed and equal amounts of the cellular proteins separated by 10-14% SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with antibodies against caspases, PARP, and β-actin and the proteins visualized using an ECL detection system.

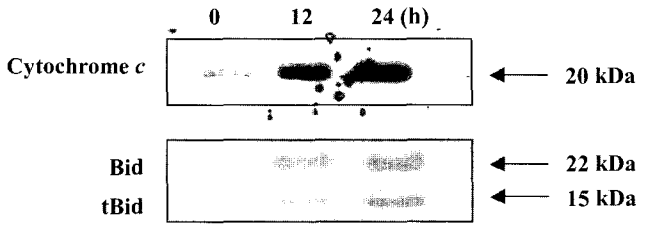

Fig. 7. Effect of HY558-1 on cleavage of Bid and release of cytochrome *c*.
HeLa cells treated with 0.52 mM of HY558-1 for 24 h were washed with PBS, lysed, separated by 14% SDS-PAGE, and immunoblotted with the corresponding antibodies. The proteins were then visualized using an ECL detection system.

HY558-1-induced apoptosis involved the activation of caspase-3.

Effect of HY558-1 on Bid and Cytochrome *c*

Death promoting members of the Bcl-2 family, such as Bax and Bid, play key roles in many drug-induced cytochrome *c* releases. For example, Bid receives death signals in the cytosol from upstream events and is then processed into a p15 subunit fragment (tBid), which induces the release of cytochrome *c*, thereby activating the mitochondrial apoptotic pathway [6, 19]. Thus, to further understand the molecular pathway of the HY558-1-induced apoptotic cell death, the effect of HY558-1 on the fragmentation of Bid and free-release of cytochrome *c* into the cytosol was investigated. As shown in Fig. 7, Bid was time-dependently cleaved, then cytochrome *c* was released from mitochondria into the cytosol in the HY558-1-treated HeLa cells.

DISCUSSION

In the course of screening for novel microbial metabolites that inhibit the progression of the cell cycle, we isolated HY558 [14] and HY558-1 from a culture broth of *Penicillium minioluteum* F558. In the present study, it was found that HY558-1 has an effect on both cell cycle regulation and apoptotic induction in human cervical adenocarcinoma HeLa cells. The flow cytometric analysis revealed that HY558-1 arrested the cell cycle progression at the G1 and G2/M phases (Fig. 2). Furthermore, DNA fragmentation due to apoptosis was observed in cells treated with HY558-1 (Fig. 3). Therefore, the changes in multiple regulatory molecules in the cell cycle were investigated to elucidate the mechanism of this action. Since eukaryotic cellular proliferation is regulated by the expression and sequential activation of cell cycle dependent cyclins, CDKs, and CKIs [9, 12, 18, 20, 25], the expression of certain cell cycle-associated proteins was examined by Western blot analysis. The results revealed that HY558-1 induced cell cycle arrest in the G1 phase through the inhibition of pRb

phosphorylation and in the G2/M phase through the direct inhibition of CDC2 and cyclin A. It was also found that the level of $p21^{WAF1/CIP1}$, an important CKI, was increased in the HeLa cells treated with HY558-1.

Previous research has established that $p21^{WAF1/CIP1}$ plays a major role in regulating early and late cell-cycle-phase CDKs; for example, its overexpression and association with CDKs is implicated in the induction of a blockade at a specific stage of the cell cycle [3, 8, 23, 26].

As such, pRb phosphorylation plays a central role in controlling cellular proliferation by regulating the G1/S transition of the cell cycle. Upon commitment of a cell to continuous proliferation, pRb is phosphorylated by the activity of G1 CDKs, such as CDK4/6 and CDK2, thereby liberating the factors which govern the S phase entrance [13, 32]. Accordingly, the current results suggest that the increase in the $p21^{WAF1/CIP1}$ protein level was accompanied by a decrease in the CDK2 expression level and pRb hypophosphorylation, eventually leading to cell cycle arrest of the G1 phase in the HeLa cells treated with HY558-1 (Figs. 4 and 5). However, the detailed mechanism still needs to be studied further.

Apoptosis is a highly organized physiological mechanism for eliminating damaged cells and basically occurs via two distinct major activation pathways [4, 5]. One pathway involves changes in the mitochondrial transmembrane potential, leading to the release of cytochrome *c*, which then activates caspase-9 based on the proteolytic cleavage of procaspase-9. Meanwhile, the other pathway starts with death receptor ligation or Fas/FasL interaction, followed by recruitment of the Fas-associated death domain protein (FADD) and activation of caspase-8 [1]. Both caspase-9 and caspase-8 are defined as initiator caspases, which in turn activate caspase-3, the executor of apoptosis [28, 29]. Recently, a cross-communication was found between the two pathways, as caspase-8 can activate caspase-9 via Bid, a member of the bcl-2 family [33]. In the current study, the expression levels of caspase-8, -9, and -3 were all examined in the HeLa cells following treatment with HY558-1, and a Western blot revealed a time-dependent cleavage of procaspase-8, -9, and -3 into the active forms (Fig. 6). It was also found that HY558-1 cleaved Bid in a time-dependent manner and that this was followed by the release of cytochrome *c* from mitochondria into the cytosol to induce apoptosis in the HeLa cells (Fig. 7). Furthermore, HY558-1 was also shown to cause apoptosis via the proteolytic cleavage of PARP in a time-dependent manner (Fig. 6). Accordingly, the apoptotic pathway triggered by HY558-1 was as follows: First, HY558-1 activated caspase-8, which then activated caspase-9 via processing the cytosolic factor Bid, a proapoptotic member of the Bcl-2 family, to play a role in caspase-8 mediated mitochondrial damage and cell death [30]. Bid cleavage can induce the release of cytochrome *c* into the cytosol [17], whereas truncated Bid can be translocated from the cytoplasm into mitochondria, resulting in the activation of caspase-9 and caspase-3 [19, 22, 31]. Therefore, the current results suggest that HY558-1 induced the activation of caspase-8, followed by the temporary cleavage of Bid, release of cytochrome *c*, activation of caspase-9 and -3, cleavage of PARP, and final entry into the execution phase of apoptosis.

In conclusion, HY558-1 was found to inhibit the growth of human cervical adenocarcinoma HeLa cells through the induction of G1 cell cycle arrest by inhibiting pRb phosphorylation via an increase in the $p21^{WAF1/CIP1}$ level, and G2/M phase arrest by directly inhibiting CDC2 and cyclin A. In addition, it was shown that HY558-1 induced apoptosis through the activation of caspase-9 via Bid cleavage by the activation of caspase-8. Thus, the current results suggest that HY558-1 may be a promising chemopreventive or therapeutic agent against cervical adenocarcinoma.

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