Induction of Apoptosis by Tosyl-JM3 in HL-60 cells

Kun Jung Kim, Sung Min Ju, Chai Ho Lee, Won Sin Kim, Yong Gab Yun[2], Han Sol Jeong[3], Sung Hoon Kim[4]*, Sung Joo Park[5], Byung Hun Jeon[1]*

Division of Natural Science and Technology, College of Natural Sciences,
1: Department of Pathology, College of Oriental Medicine, Wonkwang University,
2: Department of Oriental medical prescription, Wonkwang University, 3: Department of Pathology, College of Oriental medicine, Woosuk University,
4: Department of Oncology, Graduate School of East-West Medical Science,
5: Department of Herbology, College of Oriental Medicine, Wonkwang University

The Tosyl-JM3 (TJM3) is a modified compound from one of 1,2,3,4-Tetra- hydroisoquinoline (THIQ) derivatives. The THIQs include potent cytotoxic agents that display a range of anti-tumor activities, antimicrobial activity, and other biological properties. In this study, we investigated the effect of TJM3 on the cytotoxicity, induction of apoptosis in human promyelocytic leukemia cells (HL-60 cells). TJM3 showed a significant cytotoxic activity in HL-60 cells (IC50 = approximately 60 ㎍/㎖) after a 24 hr incubation. Treatment of HL-60 cells with TJM3 exhibited several features of apoptosis, including formation of DNA ladders in agarose gel electrophoresis, morphological changes of HL-60 cells with DAPI stain. Here we observed that TJM3 caused a decrease of procaspase-3 protein. Further molecular analysis demonstrated that TJM3 led to cleavage of poly(ADP-ribose) polymerase (PARP) by western blot and increase of hypodiploid (Sub-G1) population in the flow cytometric analysis. In conclusion, these above results indicate that TJM3 dramatically suppresses HL-60 cell growth and induces apoptosis. These data may support a possibility for the use of TJM3 in the prevention and treatment of leukemia.

Key words : Apoptosis, TJM3, caspase-3, PARP

Introduction

The isoquinoline (IQ) and tetrahydroisoquinoline (THIQ) alkaloids, which are widely distributed in plant and animal kingdoms, have received much attention because of their valuable biological activities (i.e., calcium antagonistic, cardiovascular, β-adrenergic receptor antagonism, antibacterial, antiplasmodial, antitumor, and antibacterial activity)[1-5]. Markedly, THIQs are strong complex I inhibitor of mitochondrial respiration after their transformation into N-methyl-isoquinoline ions[6]. The Tosyl-JM3 (TJM3) is a modified compound from one of 1,2,3,4-Tetrahydroisoquinoline derivatives. However, the potential mechanisms underlying the anti-tumor function is not well known. In recent years, we discovered that TJM3 has not effective of inducing apoptosis and cytotoxicity in the various solid tumor cells (data not shown). After tested on solid tumor cells, we used human promyelocytic leukemia cells (HL-60) to investigate the molecular mechanism of apoptosis. In addition, HL-60 cells have been provided a valid model for studying the mechanisms and relationships involved in the induction of differentiation and apoptosis in response to anti-leukemic or general anti-tumoral agents[7].

Apoptosis, or programmed cell death, is an intensively controlled process that is related with activation of a series of dramatic molecular events. It is characterized by cell shrinkage, blebbing of the plasma membrane, and chromatin condensation that are compatible with DNA cleavage in ladders revealed by electrophoresis[8-10]. Caspases, cysteine proteases, play a critical role in apoptosis. Among them, caspase-3 has been shown to be an important component of leading the result of apoptosis11). Especially, specific substrates for caspase-3 such as poly (ADP-ribose) polymerase (PARP) are cleaved into 85 kDa fragment, and lead cellular mechanism into apoptosis[12,13].

In order to examine the cytotoxicity which involved apoptosis, we first examined the effects of TJM3 on cell viability, morphology and DNA ladder formation. We further investigated the molecular mechanism of TJM3 by flow cytometry for apoptotic DNA contents and detected caspase-3

* To whom correspondence should be addressed : Byung Hun Jeon, Department of Pathology, College of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea, Sung Hoon Kim, Department of Oncology, Graduate School of East-West Medical Science, Kyunghee University, 1 Secheon-ri, Kiheung-eup, Yongin 449-701, Korea
· E-mail : omdjbh@wonkwang.ac.kr, sungkim7@khu.ac.kr
· Tel : 063-850-6843, 031-201-2179
· Received : 2005/07/25 · Revised : 2005/08/26 · Accepted : 2005/09/23

activation, PARP cleavage by western blotting.

Materials and Methods

1. Drug.

Tosyl-JM3 (TJM3) was synthesized by Dr, Chai-Ho, Lee, Department of Chemistry, Wonkwang University (Iksan, Korea). TJM3 was dissolved in DMSO at a concentration 40 mg/ml and stored at -20℃ before use. The structure of TJM3 is illustrated in Fig. 1.

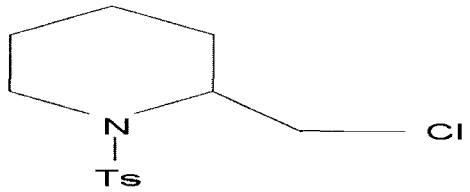

Fig. 1. Chemical structure of TJM3.

2. Cell culture

HL-60 cells were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea) and cultured in suspension at 37℃ in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere containing 5% CO_2 in air. The pH of the medium was adjusted to 7.2~7.4 with 2g/L of sodium bicarbonate. The cells were passaged twice a week.

3. Materials

Methanol and DAPI were purchased from CARLO ERBA REAGENTI and Roche, respectively. MTT and PI were from Sigma. RPMI 1640 and Fetal bovine serum were obtained from Gibco. The Wizard Genomic DNA Purification Kit from Promega was used for DNA ladder formation analysis. Rabbit polyclonal anti-human anti-PARP, and anti-caspase-3 were purchased from Cell Signaling Technology and Santa Cruz (Santa Cruz, CA) each other.

4. MTT assay

In the primary study, the cytotoxic effect of TJM3 was determined by MTT assay. MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml. MTT is introduced into cells and reduced in a mitochondria-dependent reaction to yield a formazan product. The ability of cells to reduce MTT is considered to be an indication of mitochondrial integrity and activity[14]. To assess the cytotoxicity of TJM3, cells were treated with TJM3 at concentration of 0, 20, 40, 60, 80 and 100 ㎍/㎖. After cells were seeded at $1x10^5$ cells/well in 500 ㎕ of medium in 24-well plates, those were incubated for 24 hr and 125 ㎕ of MTT were added to each well, and plates were gently shaken and incubated at 37℃ for 4 hr. The resulting blue formazan crystals were dissolved with the same volume (625 ㎕) of the solubilization solution containing (10% SDS /0.01 N HCl) added to each well. The results were read on a 96-well plate by enzyme-linked immunosorbent assay (ELISA) reader (575 nm).

5. DAPI staining

The change of cell morphology was analyzed to investigate the cytotoxic and apoptotic effect of TJM3 on HL-60 cells. Cells were treated with TJM3 in a concentrations-dependent manner for 24 hr, and collected by centrifugation. The pellet was resuspended and stained with the methanol solution containing 1 ㎍/㎖ DAPI for 15 min at 37℃. Cells were viewed by a fluorescence microscope at the magnification of x400, after centrifugation of the stained cell using the cytospin.

6. DNA fragmentation assay

Genomic DNA was obtained using the Wizard Genomic DNA Purification Kit of Promega and DNA fragmentation was assayed by agarose gel electrophoresis. The cells were treated with TJM3 at various concentrations. After exposure to TJM3, the cells were immediately incubated at 37℃ for 24 hr, and collected by centrifugation. After cells were washed once in ice-cold phosphate buffered saline (PBS), DNA was isolated according to the procedure provided by Wizard Genomic DNA Purification Kit of Promega. The DNA sample was loaded on a 1% agarose gel containing ethidium bromide (0.5 ㎍/㎖). Electrophoresis was carried out in TAE buffer. Gels were examined and photographed under UV light.

7. Flow cytometric Analysis of Apoptosis by PI staining

To determine apoptosis, cells were stained with propidium iodide (PI). HL-60 cells ($1x10^6$) were incubated in a 6 well plate with different time of TJM3 (0, 6, 12, 18, 24, and 30 h). Cells were collected, washed twice in ice-cold PBS and then fixed with 70 % ethanol at 4℃ for 15 min. Cellular DNA was stained with PI staining solution (25 ㎍/ml PI, 100 ㎍/ml RNase, 0.05% NP-40 in 1 ml of PBS). Stained cells were incubated for 15 min at 37℃ in the dark. Analytic flow cytometric measurements were performed using a FACS vantage flow cytometer (Becton Dicknson), and the fluorescence was detected through a 564-606 nm band-pass filter. Ten thousand cells were analyzed in each sample using CELLQuest software (Becton Dickinson).

8. Western blot analysis

After treatment with TJM3 in the time-dependent manner , HL-60 cells were collected and lysed in ice-chilled 120 ㎕

lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5% Igepal and 150 mM NaCl) with 1% fresh protease inhibitor cocktail for 30 min. Cell lysates were prepared by centrifugation at 14,000 rpm for 20 min at 4℃, and protein concentration was determined by Bradford assay kit (Bio-rad). Equal amounts of protein were boiled in equal volume of SDS-PAGE sample loading buffer for 5 min at 100℃, and electrophoresed on SDS-polyacrylamide gel with Tris-glycine running buffer for 1 hr. After separation, proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell) for 3 hr at 40V. The membrane was blocked with 5% skim milk in Tirs-buffered saline (TBS) for 1 hr at room temperature. The blocked membrane was subsequently probed with rabbit polyclonal anti-human anti-PARP, and anti-human anti-caspase-3 at 4℃ for overnight. These primary antibodies were used at a dilution of 1:1000 in TBS containing 5% skim milk. After washing the membrane in TBS for 15 min 2 times, blots were detected using anti-rabbit (Zymed) peroxidase-conjugated secondary immunoglobulin G antibodies diluted 1:2000 in TBS containing 5% skim milk at 4℃ for 1 hr and rinsed in TBS again. The transferred protein were visualized with SuperSignal West Pico Chemilumi- nescent (PIERCE).

Results

1. Cytotoxic and apoptotic effect on HL-60 cells

The cells were incubated with different concentrations of TJM3 for 24 hr. MTT assay was carried out in TJM3 free media as the control. As shown in Fig. 2A, viabilities of cells incubated with TJM3 were significantly decreased as compared with the control value, respectively. The result of MTT assay clearly showed that the cytotoxic effect of TJM3 on HL-60 cells was increased in a concentration-dependent manner (Table 1). To investigate morphological changes, cells were observed by a fluorescence microscope after DAPI staining. Fig. 2B. shows that the morphological change of the cells were induced by TJM3. The rounding, cytoplasmic blebbing, and irregularities in shape were observed in the cells treated with TJM3 at various concentrations for 24 hr. It was thought that the CDDT induced morphological changes of cells indicated an apparent apoptotic cell death pattern. In order to determine the pattern of cell death, HL-60 (2x105/ml) cells were incubated with indicated concentrations of TJM3 for 24 hr. The endonucleolytic DNA cleavages were analyzed by agarose gel electrophoresis. DNA fragmentation, reflecting the endonuclease activity which was characteristic of apoptosis, was shown from 60 ㎍/㎖ TJM3 treatment and respond to a dose-dependent manner in Fig. 2C.

2. Apoptotic cells were increased in HL-60 cells

We examined DNA contents of HL-60 cells treated with TJM3. Apoptotic cells were confirmed by flow cytometric analysis of the DNA-stained cells. Flow cytometric analysis of treated HL-60 cells was informed the increase of hypodiploid apoptotic cells in a time-dependent manner (0, 6, 12, 18, 24, and 30 h) (Fig. 3. Table 2.).

Table 1. Cytotoxic effect of TJM3 on HL-60 cells for 24 hr, as measured by MTT assay.

TJM3 concentration(㎍/ml)	cell viability %
Un-treated (Control)	100±1.2
20	80±1.1
40	69.8±1.9
60	50.3±3.3
80	27.3±2.7
100	13.2±4.5

Values are represented of four or more independent experiments.

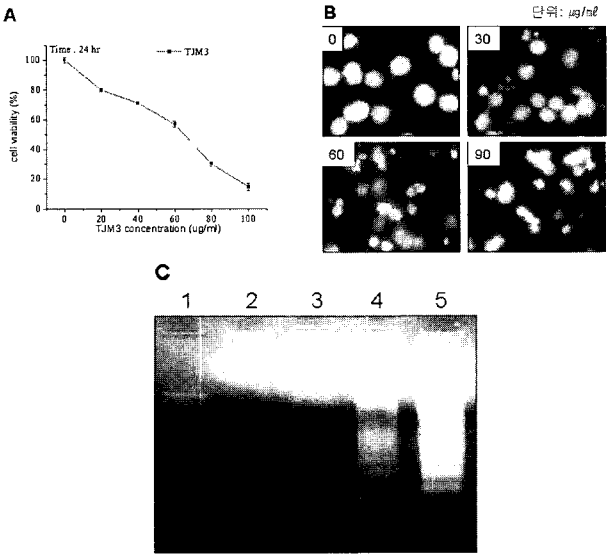

Fig. 2. Apoptosis-inducing effect of TJM3. (A) Cytotoxic effect of TJM3 on HL-60. Cells were incubated with TJM3 at various concentrations prior to the determination of cellular viability through MTT assay. Results are presented as mean ± standard error. Asterisk (**) represents p<0.01 compared to the control. (B) Characterization of TJM3 - induced cell death in HL-60. Cells were cultured without TJM3 (control) and with various concentrations for 24 hr in each case. Cells were stained with DAPI. (C) DNA ladder formation following exposure of HL-60 cells to TJM3 for 24 hr. Genomic DNA was extracted and analyzed by electrophoresis on 1% agarose gels electrophoresis; molecular-weight markers (lane1), control cells (lane2), cell treated with 30, 60, 90 ㎍/㎖ (lane3-5).

Table 2. Increase of apoptotic cells by TJM3 treatment.

TJM3 concentration(㎍/ml)	Apoptotic cells (%)
20	5.39±1.1
40	5.7±0.2
60	18.3±3.8
80	41.4±15.9
100	51.2±13.7

3. Treatment with TJM3 causes Degradation of PARP, an

endogenous substrate of caspase-3.

Activation of caspase-3 degrades numerous proteins, one of which is PARP (116 kDa). Cleaved PARP (85 kDa) fragment is a typical evidence of apoptotic process[15]. Accordingly, we first carried out an experiment in the effects of TJM3 on cleavage of caspase-3 by Western blot analysis in HL-60 cells. TJM3-treated cells revealed to decrease of the 35-kDa pro-enzyme caspase-3 by time-dependent manner(Fig. 4). In a second step, we assessed the degradation of the DNA-repair enzyme PARP, the known substrate of caspase-3. As shown in Fig. 4, incubation with different period of time with TJM3 resulted in marked degradation of PARP, a typically 85-kDa band, which was almost complete degraded at 80 and 100 $\mu g/m\ell$ concentrations of TJM3. These data shows that caspase-3 cleaves PARP in time-dependent manner at same concentrations.

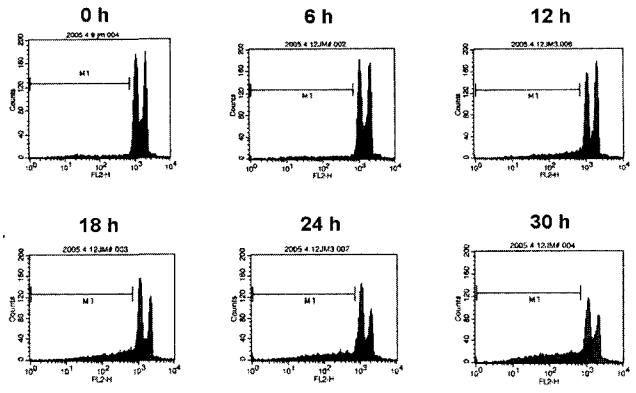

Fig. 3. Determination of hypodiploid cells (apoptotic cells) by flow cytometry. HL-60 cells were cultured with TJM3 for 0, 6, 12, 18, 24, and 30 hr. After incubation, cells were stained with PI and analyzed by flow cytometry. Data represent the result from one of three similar experiments. (M1 displays quantity of hypodiploid cells.)

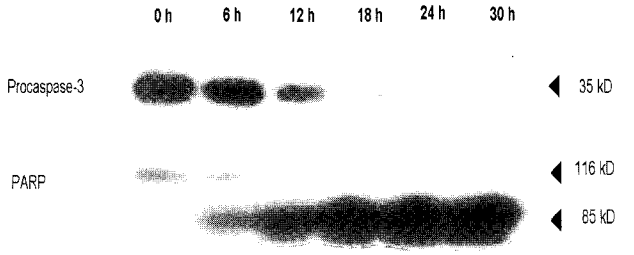

Fig. 4. Procaspase-3 and PARP cleavage in HL-60 cells in time-dependent manner. Cytosolic extracts (30 $\mu g/ml$ of protein for caspase-3 and PARP respectively) from HL-60 cells treated with TJM3 for indicated different time were resolved by SDS-PAGE. These experiments were repeated three times with similar results, and typical data are presented here.

Discussion

Our current report demonstrated that CDDT, a kind of THIQ derivatives, strongly induced apoptosis in the HL-60 cells[16]. TJM3 is structurally related to CDDT as described above. In this study, we observed that TJM3 inhibited the growth of HL-60 cells and induced apoptosis in a dose- and time-dependent manner. The aim of this study was to find the apoptotic inducing effect in HL-60 cells. Apoptosis displays some features including cell shrinkage, formation of cytoplasmic vacuoles, nuclear and plasma membrane blebbing, chromatin condensation, and formation of apoptotic bodies[17]. After HL-60 cells were treated with TJM3 for 24 hr, MTT assay showed that TJM3 exert a cytotoxic effect in a concentration-dependent manner. The fluorescencemicroscopic observations by DAPI stain demonstrated the apoptotic characters. We have also observed DNA fragment ladder formation, a characteristic gel electrophoretic band pattern associated with apoptosis. DNA ladder, the biochemical hallmark of apoptosis, informs degradation of DNA by endogenous DNase, which cleaves the internucleosomal DNA into fragmented products of 180-200 base pairs[18]. Based on the DNA cleavage, we investigated the sub-G1 population, usually considered apoptotic cells[19], using flow cytometry analysis. It was shown that TJM3-treated cells were notably increased apoptotic DNA matters in a time-dependent manner (0, 6, 12, 18, 24, and 30 h).

Apoptosis is regulated by a intensive genetic mechanism and a distinct morphology is detected. During the apoptotic process, caspases are recruited for activation of the apoptotic cascade. Especially, caspase-3 is a major component in the apoptotic pathway for leukemia cell apoptosis[20-22]. In our study, TJM3 showed that the caspase-3 activation was increased at the indicated period of time. It is certain that TJM3 induce activation of caspase-3 and causes apoptosis in HL-60 cells. In the apoptotic pathway, PARP, an plenty nuclear enzyme, is cleaved from 116 kDa into 85 kDa[23]. After time-dependent exposure, we found that TJM3-treated cells show the PARP cleavage (85 kDa) which is considered to be a hall mark of apoptosis[24].

This is the first report to demonstrate that TJM3 inhibit the cell growth and induces apoptosis in HL-60 cells by a dose- and time- dependent manner. Although more insight is needed to determine the cell signaling and biological importance of TJM3-induced apoptosis, our results provide a possibility of chemotherapeutic potency in human blood cancer cells.

Acknowledgements

This paper was supported by Wonkwang University in 2005.

Reference

1. Silveira, C.C., Bernardi, C.R., Braga A,L., Kaufman, T.S. Thioorthoesters in the activated PictetSpengler cyclization. Synthesis of 1-thiosubstituted tetrahydroisoquinolines and carbon---carbon bond formation via sulfonyl iminium ions generated from N,S-sulfonyl acetals. Tetrahedron Lett. 44, 6137-6140, 2003.
2. Padwa, A., Beall, L.S., Heidelbauugh, T.M., Liu, B., Sheehan, S.M. A One-Pot Bicycloannulation Method for the Synthesis of Tetrahydroisoquinoline Systems. J. Org. Chem 65, 2684-2695, 2000.
3. Glunewald, G.L., Dahanukar, V.H., Teoh, B., Criscione, K.R. 3,7-Disubstituted-1,2,3,4-tetrahydroisoquinolines Display Remarkable Potency and Selectivity as Inhibitors of Phenylethanolamine N-Methyltransferase versus the ■ 2-Adrenoceptor. J. Med. Chem 42, 1982-1990, 1999.
4. Shinohara, T., Toda, J., Sano, T. An Improved Synthesis of 1,2,3,4- Tetrahydroisoquinolines via Intramolecular Cyclization of N-Acyl-N-(aryl) methyl-2-(phenylsulfinyl) ethylamine by Pummerer Reaction. Chem. Pharm. Bull 45, 813-819, 1997.
5. Ornstein, P.L., Arnold, M.B., Allen, N.K.T., Borromeo, P.S., Lugar, C.W., Leander, J.D., Lodge, D., Schoepp, D.D. Structure-Activity Studies of 6-(Tetrazolylalkyl)-Substituted Decahydroisoquinoline-3-carboxylic Acid AMPA Receptor Antagonists. 1. Effects of Stereochemistry, Chain Length, and Chain Substitution. J. Med. Chem 39, 2219- 2231, 1996.
6. Morikawa, N., Naoi, M., Maruyama, W., Ohta, S., Kotake, Y., Kawai, H., Niwa, T., Dostert, P., Mizuno, Y. Effect of various tetrahydroisoquinoline derivatives on mitochondrial respiration and the electron transfer complex. J. Neural Transm 105, 667-668, 1998.
7. Paillard, F., Finot, F., Mouche, I.A. Prenez and Vericat, J.A. Use of Primary Cultures of Rat Hepatocytes to Predict Toxicity in the Early Development of New Chemical Entities. Toxicol. In Vitro 13, 693-700, 1999.
8. Gupta, S. Molecular steps of death receptor and mitochondrial pathways of apoptosis. Life Sci 69, 2957-2964, 2001.
9. Chen, M., Wang, J. Initiator caspase in apoptosis signaling pathways. Apoptosis 7, 313-319, 2002.
10. Robertson, J.D., Orrenius, S. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. Crit. Rev. Toxicol 30, 609-627, 2000.
11. Salvesen, G.S., Dixit, V.M. Caspases: intracellular signaling by proteolysis. Cell 91, 443-446, 1997.
12. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X. Cytochrome c and dATP dependent formation of Apaf-1/ caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479-489, 1997.
13. Ogata, S., Takeuchi, M., Okumura, K., Taguchi, H. Apoptosis induced by niacin-related compounds in HL-60 cells. Biosci Biotechnol Biochem 62, 2351-2356, 1998.
14. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63, 1983.
15. Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S., Dixit, V.M. Yama/CPP32 beta, a mammalian homology of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81, 801-809, 1995.
16. Kim, K.J., Ju, S.M., Kim, M.W., Lee, C.H., Kim, W.S., Yun, Y.G., Jeon, B.H. In vitro cytotoxicity and apoptotic effect of Chloromethyl-2-dihydro- xyphosphinyl-6,7-dimethoxy-1,2,3, 4-tetrahydroisoquinoline on HL-60 cells. Korean J. Oriental Physiology & Pathology 19, 772-778, 2005.
17. Buttke, T.M., Sandstrom, P.A. Oxidative stress as a mediator of apoptosis. Immunology Today 15, 7-10, 1994.
18. Loo, D.T., Rillema, J.R. Measurement of cell death. Methods In Cell Biology 57, 251-264, 1998.
19. Telford, W.G., King, L.E., Fraker, P.J. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. Cytometry, 13, 137-142, 1992.
20. Tibbetts, M.D., Zheng, L., Lenardo, M.J. The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol 4, 404-409, 2003.
21. Datta, R., Banach, D., Kojima, H., Talanian, R.V., Alnemri, E.S., Wong, W.W., Kufe, D.W. Activation of the CPP32 protease in apoptosis induced by 1-beta-D-arabinofuranosylcytosine and other DNA-damaging agents. Blood 88, 1936-1943, 1996.
22. Ibrado, A.M., Huang, Y., Fang, G., Liu, L., Bhalla, K. Overexpression of Bcl-2 or Bcl-xL inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. Cancer Research 56, 4743-4748, 1996.
23. Kaufmann, S., Desnoyers, S., Ottaviano, Y., Davidson, N., Poirier, G., Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy induced apoptosis. Cancer Res 53, 3976-3985, 1993.
24. Patel, T., Gores, GJ., Kaufmann, S. H., The role of proteases during apoptosis. FASEB J 10, 587-597, 1996.