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Biochemical and Biophysical Research Communications 329 (2005) 46–50
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Selective cytotoxicity of ascochlorin in ER-negative human breast cancer cell lines

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Received 17 January 2005
Available online 2 February 2005

Abstract

While agents targeting estrogen receptors are most effective in adjuvant therapy for human breast cancers expressing estrogen receptors after surgery, breast cancers lacking estrogen receptor are clinically serious, because they are highly malignant and exhibit resistance to the usual anti-cancer drugs, including estrogen receptor-antagonists and DNA breaking agents. Here, we found that MX-1, a human breast cancer cell line lacking estrogen receptors, exhibited higher AP-1 activity and expressed higher levels of c-Jun, c-Fos, and Fra-1 when compared with conventional estrogen receptor-positive human breast cancer cell lines. The prenylphenol antibiotic ascochlorin suppressed the AP-1 activity of MX-1 cells, and selectively killed MX-1 cells, partly due to induction of apoptosis. Our results suggest that AP-1 is an effective clinical target molecule for the treatment of estrogen receptor-negative human breast cancer.

Keywords: Ascochlorin; AP-1; c-Jun; c-Fos; Fra-1; Breast cancer; ER; Apoptosis; Cytochrome *c*; Caspase

Although systemic treatments for breast cancer including the combination of surgery, hormone therapy, chemotherapy, and irradiation are commonly believed to be effective in prolonging patient survival, despite the different genomic backgrounds of each breast cancer, the most important factors for prognosis are estrogen receptor (ER) expression and HER-2 gene expression [1,2]. It is now particularly clear that patients with ER-positive tumors benefit substantially from hormone therapy [3]. For patients with ER-negative tumors, on the other hand, no effective therapy regimens have been established and prognosis is generally poor. It is therefore important to find new agents that are selectively effective against ER-negative breast cancer cells.

Ascochlorin is a prenylphenol antibiotic, originally isolated as an anti-virus agent produced by an incomplete fungus, *Ascochyta visiae* [4,5]. Ascochlorin and its derivatives exhibit a large variety of physiological activities including hypolipidemic activity [6,7], suppression of hypertension [8], amelioration of type I and II diabetes [9,10], immunomodulation [11,12], and anti-tumor activity [12,13]. Ascochlorin and ascofuranone, one of its derivatives, inhibit oxidative phosphorylation by inhibiting ubiquinone-dependent electron transport in isolated mitochondria [14–16], and it is suggested that the anti-viral activity of ascochlorin and the macrophage activation by ascofuranone are caused by this inhibitory activity on mitochondria respiration

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.01.096

[14,15,17]. They also modulate activity of nuclear hormone receptors, and ascochlorin activates transcription of human ER [18,19], thus suggesting that mechanisms other than those involving the respiratory chain contribute to their physiological activities. We recently found that ascochlorin selectively suppressed the AP-1 activity of human renal carcinoma cells, and its downstream targets such as matrix metalloproteinase-9 promoter (Chang et al., unpublished results).

AP-1 is a family of homodimeric or heterodimeric transcription factors composed of basic region-leucine zipper proteins that belong to the Jun, Fos, and Jun dimerization partners and closely related transcription activating partners [20,21]. AP-1 has been implicated in transcriptional regulation of a wide range of genes participating in cell survival, proliferation, oncogenesis, and apoptosis [21,22].

In this study, we found that AP-1 activity and expression of components of AP-1 significantly increased in ER-negative breast cancer cell lines, and that inhibition of AP-1 activity by ascochlorin selectively reduced their survival. Our results suggest that AP-1 is an effective target for chemotherapy in ER-negative breast cancers.

Materials and methods

Cell culture. Human breast cancer cell lines, MX-1, MDA231, and ZR-75-1, were purchased from American Type Culture Collection (MD, USA) and KPL-1, KPL-4 were kindly provided by Dr. J. Kurebayashi (Kawasaki Medical College, Okayama, Japan) [23,24]. MCF-7 was purchased from Human Cell Science (Osaka, Japan). The human osteosarcoma cell line U2OS was obtained from Nicholas H. Heintz (University of Vermont, Burlington, VT, USA). All cells were cultured and maintained in DMEM supplemented with 5% fetal bovine serum.

Plasmids and antibodies. A luciferase reporter plasmid for AP-1 (pTRE-luc), which contains the AP-1-binding region of the collagenase promoter, was a kind gift from Yvonne M.W. Janssen-Heininger (University of Vermont, Burlington, VT, USA). An expression plasmid for β-galactosidase (pCMV-β-gal) was provided by Nicholas H. Heintz. Antibodies for c-Jun were purchased from Pharmingen (Transduction Laboratories, Lexington, KY, USA), those for ERα, c-Fos, Fra-1, JunB, JunD, caspase-3, and cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), those for poly(ADP-ribose)polymerase were purchased from Oncogene Science (Boston, MA, USA), and those for β-actin were purchased from Abcam (Cambridgeshire, UK).

Western blotting. Exponentially growing cells (2×10^5) were trypsinized and suspended in 50 μl SDS sample buffer (120 mM Tris, 4% SDS, 20% glycerol, 0.1 mg/ml BPB, and 100 mM dithiothreitol, pH 6.8). For cytochrome *c*, the cytoplasmic fraction prepared as described previously [25] was dissolved in SDS sample buffer. After SDS–PAGE, Western blotting of all samples was performed as described previously [26] using first antibodies and the corresponding second antibodies for whole immunoglobulins from mouse or rabbit (Amersham Biosciences, Buckinghamshire, UK). Protein signals were visualized by fluorescence emission using a commercial kit (Roche Diagnosis, Mannheim, Germany).

Reporter assay. Cells were transfected with the plasmids using Fugene transfection reagents (Roche Diagnosis) according to the manufacturer's instructions. One microgram of luciferase reporter plasmids and 100 ng of pCMV-β-gal per 30-mm diameter dish were used for transfection. Luciferase activity (Promega, Madison, WI, USA) and β-galactosidase activity (Promega) were measured using commercial kits as described previously [18,19].

MTT-dye reduction assay. Cell viability of drug-exposed breast cancer cells was determined by dimethylthiazol diphenyltetrazolium (MTT) dye reduction assay, which is based on the measurement of mitochondrial respiratory function [27,28]. Briefly, breast cancer cells (5×10^4 cells/well) were plated in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark), allowed to adhere for 24 h. After incubation, cells were exposed to various concentrations of ascochlorin for 48 h and were then incubated with MTT dye (Roche Diagnosis, 100 μg/well) for 2 h. The resultant formazan deposits were solubilized with 20 μl of 10% SDS, and the absorbance at 590 nm was measured. All data were calculated and expressed as percent A590 of control cells (without treatment, set at 100%).

Nuclear staining. Cells (2×10^5 cells) cultured on a coverslip were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and stained with 3 μg/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) for 15 min. Nuclear morphology of the stained cells was observed using a fluorescence microscope.

Results and discussion

Expression of AP-1 proteins in human breast cancer cell lines was examined by Western blotting (Fig. 1). MX-1 is an ER-negative human breast cancer cell line, while MCF-7 and ZR75-1 are cell lines that express ERα. MX-1 expressed higher levels of c-Fos, Fra-1, and c-Jun when compared with MCF-7 and ZR75-1. However, amounts of JunB and JunD were not significantly different among the cell lines. Although a human osteosarcoma cell line (U2OS) also expressed c-Jun at comparable levels as MX-1, expression of c-Fos and Fra-1 was as low as that in MCF-7 and ZR75-1. This suggests that the high-level expression of Fos-family proteins is one of the features distinguishing ER-negative and ER-positive breast cancer cell lines.

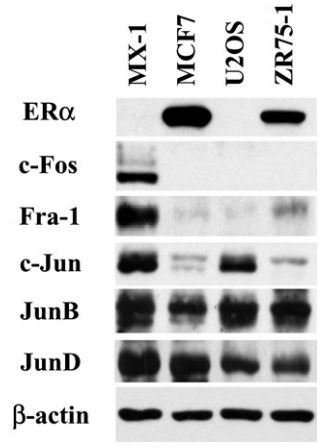

Fig. 1. Selective expression of AP-1 proteins in ER-negative or positive human breast cancer cell lines. Exponentially growing cells (5×10^5 cells) were lysed in 50 μl SDS sample buffer and subjected to Western blotting.

Cellular AP-1 activity was assessed by a reporter plasmid containing AP-1 responsive elements in the collagenase promoter region (Fig. 2). Consistent with the expression level of AP-1 proteins, AP-1 was highly active in MX-1 cells and the activity was enhanced by stimulation with phorbol myristate acetate (PMA), a known activator of AP-1. On the other hand, relative luciferase activity, i.e., luciferase activity normalized against β-galactosidase activity as an internal control, induced by the collagenase promoter in ZR75-1 lysate was less than 1/100 of that seen in MX-1, and stimulation by PMA did not increase the activity. These results suggest that AP-1 is highly and selectively active in ER-negative breast cancer cells. It should be noted that β-galactosidase induced by the CMV-promoter was also enhanced by PMA in MX-1 but not in ZR75-1. This enhancement might be mediated by activation of AP-1, because PMA had no effect on β-galactosidase activity in ZR75-1.

We previously observed that a prenylphenol antibiotic, ascochlorin, selectively suppressed AP-1 activity, and expression of its downstream targets in a human renal carcinoma cell line (Chang et al., unpublished results). This observation prompted us to use ascochlorin for suppression of AP-1 activity in MX-1. Ascochlorin was added 30 min before PMA-stimulation of MX-1 cells transfected with the AP-1 reporter. Treatment with ascochlorin at 1 μM significantly suppressed the basal luciferase activity detected in the absence of PMA, and completely inhibited the PMA-mediated stimulation of luciferase activity in MX-1 (Fig. 2). Although β-galactosidase activity was significantly suppressed by ascochlorin because transcription activity of the CMV-promoter was also increased by AP-1 activation as described above, the inhibitory effect on luciferase induction was much stronger, as was evidenced by relative luciferase activity. These results suggest that ascochlorin selectively inhibits the active elevation of AP-1 in ER-negative human breast cancer cells.

The growth of breast cancer cells is assumed to be dependent on estrogen, and antagonists of estrogen effectively suppress the growth and expansion of breast cancer cells expressing ER. Because a signal from ER receptor is necessary for the growth and survival of breast cancer cells with ER, other signals are likely to compensate for these signals in ER-negative breast cancer cells. AP-1 is one of the candidates for such signals, because the transcription factor is involved in proliferation and oncogenesis in various mammalian cell systems. To confirm this possibility, we compared the

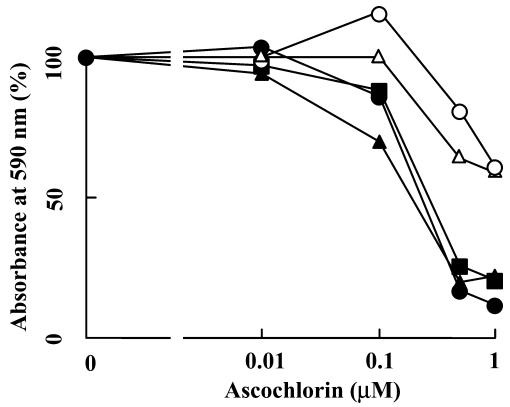

Fig. 3. Selective cytotoxic effect of ascochlorin on ER-negative human breast cancer cell lines. Cells (5×10^4/well) were cultured in the presence of ascochlorin for 48 h. MTT reagent was added 2 h prior to the termination of cultures. Formazan deposits were quantified by measuring the absorbance at 590 nm. Each point represents the average of triplicate independent cultures. Symbols: open circles, ZR75-1; open triangles, KPL-1; closed circles, MDA231; closed triangles, KPL-4; and close squares, MX-1.

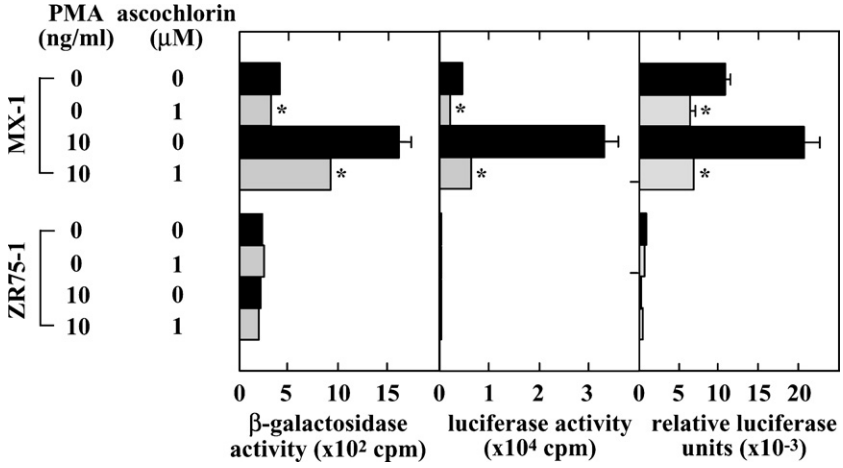

Fig. 2. Suppression of AP-1 activity elevated in ER-negative human breast cancer cells by ascochlorin. Cells (1×10^5/ml) cultured overnight were transfected with TRE-luc and pCMV-β-gal, and further incubated for 20 h. Ascochlorin was added 30 min before the addition of PMA and cells were cultured for additional 20 h before harvesting. Relative luciferase units are defined as luciferase cpm/β-galactosidase cpm. Values are averages of triplicate independent cultures. Bars are standard deviations. *$p < 0.05$ (double-sided t test), as compared with controls cultured without ascochlorin.

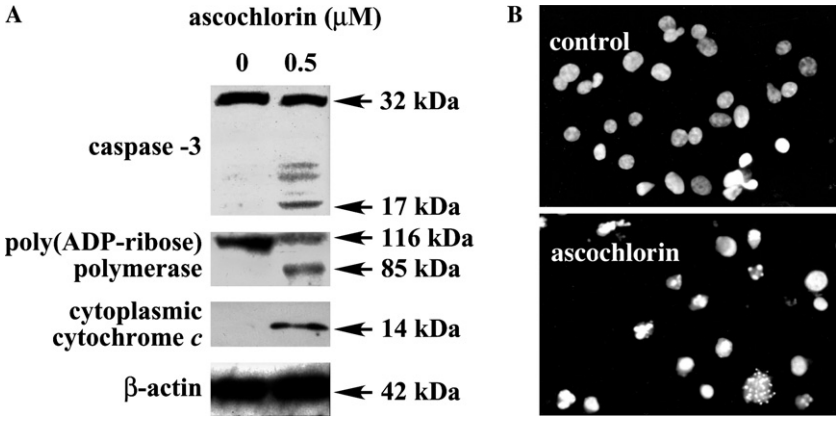

Fig. 4. Induction of apoptosis in ER-negative human breast cancer cells by ascochlorin. (A) MX-1 cells (1×10^6 cells) were treated with 500 nM ascochlorin for 4 h, and the resultant cell lysate, or cytoplasm fraction for cytochrome *c*, was subjected to Western blotting. (B) MX-1 cells cultured on a coverslip were treated with or without 500 nM ascochlorin for 48 h and were stained with Hoechst 33342. Nuclear morphology was observed under a fluorescent microscope.

growth of ER-negative and ER-positive cell lines isolated from human breast cancers. Cells were incubated with ascochlorin for 48 h and further cultured for 2 h in the presence of MTT reagent in order to assess the cytotoxic activity of ascochlorin (Fig. 3). We found an 80% reduction in formazan deposits by 48-h treatment with 1 μM ascochlorin in an ER-negative breast cancer cell line, MX-1, while ascochlorin only partially suppressed formazan deposits in ZR-75-1, a cell line expressing ER (less than 40% at 1 μM ascochlorin). We also tested the cytotoxic effects of ascochlorin in an additional ER-positive cell line, KPL-1, and two ER-negative cell lines, KPL-4 and MDA231, derived from human breast cancers, and observed similar cytotoxic activity in the ER-negative cell lines.

We observed segmentation of some MX-1 cells treated with ascochlorin in culture wells, a characteristic morphological feature of apoptosis. To confirm this, we determined the biochemical features of apoptosis by Western blotting of MX-1 cells treated with 500 nM ascochlorin for 24 h. This treatment degraded 116 kDa poly(ADP ribose)polymerase, a major substrate for caspase-3 [29], to 85 kDa (Fig. 4A). Consistent with the degradation of poly(ADP ribose)polymerase, processing of 32-kDa procaspase-3 was also detected, generating a 17-kDa active form. Furthermore, cytochrome *c* in mitochondria was released into the cytoplasm. Morphologically, we observed DNA-condensation and DNA-fragmentation in nuclei of ascochlorin-treated MX-1 cells stained with Hoechst 33342 (Fig. 4). These results suggest that ascochlorin selectively kills ER-negative breast cancer cells, at least in part, through the induction of apoptosis.

The consequences of activating AP-1 are dependent on cell type and other environmental factors. While it may promote apoptosis in some cell types, it is required for the survival of others. For instance, inhibition of c-Jun activity by dominant negative mutants or neutralizing antibodies can protect neuronal cells from apoptosis [30,31]. Ectopic expression of c-Jun or c-Fos can induce apoptosis in some types of neurons and fibroblasts [32,33]. JNK activation, on the other hand, protects cells from apoptosis induced by tumor necrosis factor-α or Fas-stimulation [34,35]. These observations indicate the homeostatic function of AP-1, which reacts to changes in growth and environmental conditions to adjust the gene expression profile and allows the cells to adapt to the new environment [22]. Ascochlorin may interrupt the homeostatic function of c-Jun required for ER-negative breast cancer cells to survive in an environment deficient of ER-mediated signals.

In conclusion, our results suggest that suppression of AP-1 is an effective strategy for chemotherapy in ER-negative breast cancers, and ascochlorin is a promising candidate for such chemotherapy.

References

[1] D.C. Allred, J.M. Harvey, M. Berardo, G.M. Clark, Prognostic and predictive factors in breast cancer by immunohistochemical analysis, Mod. Pathol. 11 (1998) 155–168.
[2] V. Stearns, B. Singh, T. Tsangaris, J.G. Crawford, A. Novielli, M.J. Ellis, C. Isaacs, M. Pennanen, C. Tibery, A. Farhad, R. Slack, D.F. Hayes, A prospective randomized pilot study to evaluate predictors of response in serial core biopsies to single agent neoadjuvant doxorubicin or paclitaxel for patients with locally advanced breast cancer, Clin. Cancer Res. 9 (2003) 124–133.
[3] Early Breast Cancer Trialists' Collaborative Group, Tamoxifen for early breast cancer: An overview of the randomized trials, Lancet 351 (1998) 1451–1467.
[4] G. Tamura, S. Suzuki, A. Takatsuki, K. Ando, K. Arima, Ascochlorin, a new antibiotic, found by the paper-disc agar-diffusion method. I. Isolation, biological and chemical properties of ascochlorin. (Studies on antiviral and antitumor antibiotics. I), J. Antibiot. 21 (1968) 539–544.

[5] Y. Nawata, K. Ando, G. Tamura, K. Arima, Y. Iitake, The molecular structure of ascochlorin, J. Antibiot. 22 (1969) 511–512.
[6] T. Hosokawa, M. Sawada, K. Ando, G. Tamura, Enhanced excretion of fecal neutralsterols and the hypercholesterolemic properties of 4-O-methylascochlorin, Agric. Biol. Chem. 44 (1980) 2461–2468.
[7] T. Hosokawa, M. Sawada, K. Ando, G. Tamura, Alteration of cholesterol metabolism by 4-O-methylascochlorin in rats, Lipids 16 (1981) 433–438.
[8] T. Hosokawa, T. Okutomi, M. Sawada, K. Ando, G. Tamura, Unusual concentration of urine and prevention of polydipsia by fungal prenylphenols in DOCA hypertensive rats, Eur. J. Pharmacol. 69 (1981) 429–438.
[9] T. Hosokawa, K. Ando, G. Tamura, An ascochlorin derivative, AS-6 potentiates insulin action in streptozotocin diabetic mice and rats, Agric. Biol. Chem. 46 (1982) 775–781.
[10] T. Hosokawa, K. Ando, G. Tamura, An ascochlorin derivative, AS-6, reduces insulin resistance in the genetically obese diabetic mice, Diabetes 34 (1982) 267–274.
[11] J. Magae, S. Suzuki, K. Nagai, M. Yamasaki, K. Ando, G. Tamura, *In vitro* effects of an antitumor antibiotic, ascofuranone, on the murine immune system, Cancer Res. 46 (1986) 1073–1078.
[12] J. Magae, J. Hayasaki, Y. Matsuda, M. Hotta, T. Hosokawa, S. Suzuki, K. Nagai, K. Ando, G. Tamura, Antitumor and antimetastatic activity of an antibiotic, ascofuranone and activation of phagocytes, J. Antibiot. 40 (1988) 959–965.
[13] J. Magae, T. Hosokawa, K. Ando, K. Nagai, G. Tamura, Antitumor protective property of an isoprenoid antibiotic, ascofuranone, J. Antibiot. 35 (1982) 1547–1552.
[14] A. Takatsuki, G. Tamura, K. Arima, Antiviral and antitumor antibiotics. XIV. Effects of ascochlorin and other respiration inhibitors on multiplication of Newcastle disease virus in cultured cells, Appl. Microbiol. 17 (1969) 825–829.
[15] J. Magae, K. Munemura, C. Ichikawa, K. Osada, T. Hanada, R.F. Tsuji, M. Yamashita, A. Hino, T. Horiuchi, M. Uramoto, M. Yamasaki, T. Endo, K. Nagai, Effects of microbial products on glucose consumption and morphology of macrophages, Biosci. Biotechnol. Biosci. 57 (1993) 1628–1631.
[16] N. Minagawa, K. Meguro, S. Sakajo, A. Yoshimoto, Effects of ascofuranone on the mitochondria isolated from *Hansenula anomala*, Biosci. Biotechnol. Biosci. 58 (1994) 1334–1335.
[17] T. Ashikaga, M. Honma, K. Munemura, T. Kataoka, T. Endo, M. Yamasaki, J. Magae, K. Nagai, Selective induction of interleukin-1 production and tumor killing activity of macrophages through apoptosis by the inhibition of oxidative respiration, Biosci. Biotchnol. Biochem. 62 (1998) 1115–1121.
[18] M. Togashi, H. Masuda, T. Kawada, M. Tanaka, K. Saida, K. Ando, G. Tamura, J. Magae, PPARγ activation and adipocyte differentiation induced by AS-6, a prenyl-phenol antidiabetic antibiotic, J. Antibiot. 55 (2002) 417–422.
[19] M. Togashi, S. Ozawa, S. Abe, T. Mishimura, M. Tsuruga, K. Ando, G. Tamura, S. Kuwahara, M. Ubukata, J. Magae, Ascochlorin derivatives as ligands for nuclear hormone receptors, J. Med. Chem. 46 (2003) 4113–4123.
[20] P. Angel, M. Karin, The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation, Biochim. Biophys. Acta 1072 (1991) 129–157.
[21] P.K. Vogt, Jun, the oncoprotein, Oncogene 20 (2001) 2365–2377.
[22] E. Shaulian, M. Karin, AP-1 in cell proliferation and survival, Oncogene 20 (2001) 2390–2400.
[23] J. Kurebayashi, M. Kurosumi, H. Sonoo, A new human breast cancer cell line, KPL-1 secretes tumour-associated antigens and grows rapidly in female athymic nude mice, Br. J. Cancer 71 (1995) 845–853.
[24] J. Kurebayashi, S. Yamamoto, T. Otsuki, H. Sonoo, Medroxyprogesterone acetate inhibits interleukin 6 secretion from KPL-4 human breast cancer cells both in vitro and in vivo: A possible mechanism of the anticachectic effect, Br. J. Cancer 79 (1999) 631–636.
[25] M. Tsuruga, H. Nakajima, S. Ozawa, M. Togashi, Y.-C. Chang, K. Ando, J. Magae, Characterization of 4-O-methyl-ascochlorin-induced apoptosis in comparison with typical apoptotic inducers in human leukemia cell lines, Apoptosis 9 (2004) 429–435.
[26] Y.S. Lee, H. Nakajima, M. Tsuruga, J. Magae, Elimination of cell cycle regulators during caspase-3-dependent apoptosis by an immunosuppressant, FTY720, Biosci. Biotechnol. Biochem. 67 (2003) 467–474.
[27] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[28] D. Gerlier, N. Thomasset, Use of MTT colorimetric assay to measure cell activation, J. Immunol. Methods 94 (1986) 57–63.
[29] M. Tewari, L.T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D.R. Beidler, G.G. Poirier, G.S. Salvesen, V.M. Dixit, Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase, Cell 81 (1995) 801–809.
[30] J. Ham, C. Babij, J. Whitfield, C.M. Pfarr, D. Lallemand, M. Yaniv, L.L. Rubin, A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death, Neuron 14 (1995) 927–939.
[31] Z. Xia, M. Dickens, J. Raingeaud, R.J. Davis, M.E. Greenberg, Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis, Science 270 (1995) 1326–1331.
[32] G.A. Preston, T.T. Lyon, Y. Yin, J.E. Lang, G. Solomon, L. Annab, D.G. Srinivasan, D.A. Alcorta, J.C. Barrett, Induction of apoptosis by c-Fos protein, Mol. Cell. Biol. 16 (1996) 211–218.
[33] E. Bossy-Wetzel, L. Bakiri, M. Yaniv, Induction of apoptosis by the transcription factor c-Jun, EMBO J. 16 (1997) 1695–1709.
[34] H. Nishina, K.D. Fischer, L. Radvanyi, A. Shahinian, R. Hakem, E.A. Rubie, A. Bernstein, T.W. Mak, J.R. Woodgett, J.M. Penninger, Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3, Nature 385 (1997) 350–353.
[35] G. Natoli, A. Costanzo, A. Ianni, D.J. Templeton, J.R. Woodgett, C. Balsano, M. Levrero, Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway, Science 275 (1997) 200–203.