Curcumin mediated apoptosis in AK-5 tumor cells involves the production of reactive oxygen intermediates

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Abstract Curcumin, the active ingredient of the rhizome of *Curcuma longa* has anti-inflammatory, antioxidant and anti-proliferative activities. Although its precise mode of action remains elusive, studies have shown that chemopreventive action of curcumin might be due to its ability to induce apoptosis in cancer cells. Curcumin was shown to be responsible for the inhibition of AK-5 tumor (a rat histiocytoma) growth by inducing apoptosis in AK-5 tumor cells via caspase activation. This study was designed to investigate the mechanism leading to the induction of apoptosis in AK-5 tumor cells. Curcumin treatment resulted in the hyperproduction of reactive oxygen species (ROS), loss of mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome c release to the cytosol, with the concomitant exposure of phosphatidylserine (PS) residues on the cell surface. This study suggests redox signalling and caspase activation as the mechanisms responsible for the induction of curcumin mediated apoptosis in AK-5 tumor cells.

Key words: Reactive oxygen metabolite; AK-5 cell; Curcumin

1. Introduction

Turmeric (*Curcuma longa*), a common Indian dietary pigment and spice has been shown to possess a wide range of therapeutic utilities in the traditional Indian medicine [1]. Its role in wound healing, urinary tract disease, liver ailments, hepatitis, are well documented in addition to its use as a cosmetic [2,3]. The active component of turmeric, identified as curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)1,6-heptadiene-3,5-dione] exhibits a variety of pharmacological effects including anti-inflammatory, anticarcinogenic and anti-infectious activities [2,4,5]. As a known antioxidant, curcumin also exhibits antiproliferative capability and is thus identified as a potent tool in cancer therapy [6–8]. The tumoricidal activity of curcumin has been observed in a wide range of cell lines like NIH3T3, mouse sarcoma S180, human kidney cancer cell 293 [9], Chinese hamster ovary [2] and human basal cell carcinoma [6]. Studies have shown that dietary administration of curcumin significantly suppressed development of chemically induced tumors in mice [10]. Its chemopreventive activity is observed when it is administered during the promotion/progression stages of colon cancer [7].

A recent study from our laboratory demonstrated antitumor activity of curcumin when it was injected intraperitoneally in rats bearing the AK-5 tumor [11]. The antitumor activity of curcumin was attributed to its ability to induce apoptosis in AK-5 cells, via the activation of caspase-3. The present study implicates reactive oxygen species as the key apoptotic signalling molecules in AK-5 cells leading to the loss of mitochondrial membrane potential, PS externalization and release of apoptogenic proteins like cytochrome c.

2. Materials and methods

2.1. Materials

AK-5 tumor was maintained as ascites in an inbred colony of Wistar rats by injecting 5×10^6 tumor cells i.p. We also used a single cell clone of AK-5 called BC-8 in these studies to avoid ambiguity in results due to tumor heterogeneity [12]. Dulbecco's modified Eagle's medium (DMEM), FCS, curcumin, cytochrome c (type VI), superoxide dismutase (SOD), *N*-acetyl-L-cysteine (NAC), Annexin V-FITC apoptosis detection kit, nitroblue tetrazolium salt (NBT) and 5′-bromo-4-chloro-3′-indolyl phosphate (BCIP) were procured from Sigma Chemical Co., USA. Propidium iodide (PI) was purchased from Calbiochem, $DiOC_6(3)$ from Molecular Probes and mouse anti-cytochrome c monoclonal antibody (clone 7H8.2C12) was purchased from Pharmingen.

2.2. Curcumin treatment

BC-8 cells (1×10^6) were plated in DMEM-FCS and treated with 50 μM curcumin. The cells were collected at different time points, washed with PBS, fixed in 70% ethanol, stained with propidium iodide (PI) and analyzed by flow cytometry.

2.3. Comet assay

BC-8 cells (5×10^6 cells) were subjected to 50 μM curcumin treatment for 4 h. The cells were washed, resuspended in 100 μl of low melting agarose (2%), maintained at 37°C and layered on a frosted slide. The slides were placed on ice (5 min) followed by treatment with lysis buffer (150 mM NaCl, 10 mM Tris (pH 8), 10 mM EDTA, 0.5% *n*-lauroyl sarcosine) for 5 min and distilled water (5 min). The slides were subjected to electrophoresis in 0.03 M NaOH, 2 mM EDTA (5 min at 20 Volt). The cells were stained with PI and analyzed by a fluorescent microscope [13].

2.4. Cytochrome c reduction

Superoxide induced reduction of ferricytochrome c to ferrocytochrome c was monitored spectrophotometrically at 550 nm [14]. 5×10^4 cells were suspended in complete phenol red-free DMEM and plated in 96-well plates. The cells were treated with curcumin (50 μM) and the time dependent superoxide anion release was estimated in the presence of 80 μM cytochrome c with and without SOD (300 U/ml).

In a parallel experiment NAC (1 mM) inhibitable cytochrome c reduction was estimated after 1 h of curcumin treatment. The cellular proteins were estimated with Bradford reagent using BSA as the standard.

2.5. Release of cytochrome c from mitochondria

In order to monitor the release of mitochondrial cytochrome c to the cytosol during curcumin mediated apoptosis, BC-8 cells (1×10^7) treated with curcumin for 4 h, were washed in cold PBS, suspended in 0.5 ml ice-cold cytoplasmic extraction buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonylfluoride and 1 mM DTT) and incubated on ice

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for 30 min [15]. The cells were disrupted with 30 strokes in a dounce homogenizer and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was further centrifuged at 100 000 rpm and frozen in aliquots.

Cytoplasmic extracts were mixed with Laemmli's sample buffer containing β-mercaptoethanol and the proteins were separated on 12% SDS-polyacrylamide gel. The separated proteins were transferred onto nitrocellulose membrane, blocked with blocking buffer (3% BSA, 0.02% Tween-20 in Tris buffer pH 7.5) at 4°C. The blots were incubated with anticytochrome c antibody diluted 1:1000 in blocking buffer for 3 h at room temperature. The bound antibody was detected after washing and treatment with alkaline phosphatase linked anti-mouse IgG and developed using BCIP substrate with NBT.

2.6. Annexin V staining

BC-8 cells (2×10^6) were cultured in DMEM-FCS and treated with 50 μM curcumin for 3 h. Control and treated BC-8 cells were suspended in 80 μl of binding buffer (100 mM HEPES/NaOH, pH 7.5, containing 1.4 M NaCl and 25 mM $CaCl_2$). Annexin V-FITC conjugate (5 ul) was added to each tube and incubated at room temperature for 15 min. The cells were visualized by laser scanning confocal microscopy [16].

2.7. Status of mitochondrial transmembrane potential

BC-8 cells were treated with curcumin under similar conditions at 37°C. The $\Delta\Psi_m$ was estimated by staining cells with 50 nM $DiOC_6(3)$, a cationic lipophilic dye [17] for 15 min at 37°C. The dye accumulates in actively respiring mitochondria depending on the membrane potential. The cells were washed with the culture media and visualized by laser scanning confocal microscope at 488 nm.

3. Results

3.1. Curcumin mediated apoptosis

The effect of curcumin on tumor cell apoptosis was monitored at different time points by flow cytometry (Table 1). The initiation of apoptosis was observed as early as 1 h. There was a progressive increase in pre-G0/G1 peak with time, which comprised of the apoptotic cells. The cell death reached 84.5% by 4 h of curcumin treatment. Subsequently the cells showed cytoplasmic blebbing, nuclear fragmentation and disintegration.

3.2. Curcumin induced DNA fragmentation

In contrast to the routine methods used to assess DNA fragmentation like TUNEL assay or internucleosomal DNA laddering which are assayed in a total cell population, we have used the comet assay procedure to monitor DNA fragmentation at the single cell level [13]. The induction of apoptosis in AK-5 cells led to the fragmentation of the cellular DNA that migrated out of the cells during electrophoresis. The fragmented DNA formed a typical comet tail-like pattern after staining with propidium iodide (Fig. 1B). The control BC-8 cells, with intact DNA do not show this pattern (Fig. 1A).

Table 1
Curcumin induced apoptosis in AK-5 cells[a]

Time (hr)		Apoptotic cells (%)
Control		1.00
Curcumin treatment:	1	7.13
	2	28.14
	3	47.16
	4	86.52

[a]AK-5 tumor cells (1×10^6) treated with curcumin (50 μM) were collected at different time points, fixed, stained with PI and analyzed by flow cytometry. The apoptotic cells represent pre-G0/G1 peak.

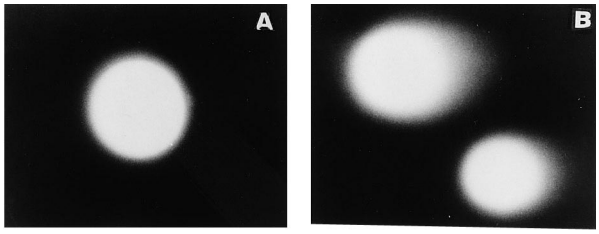

Fig. 1. Induction of DNA fragmentation after treatment of AK-5 cells with curcumin and formation of comet tail after electrophoresis. A: control; B: curcumin treated cells.

3.3. Release of superoxide anion

Treatment of BC-8 cells with curcumin resulted in a flush of superoxide release in the extracellular milieu within 20 min as noted by the reduction of ferricytochrome c to ferrocytochrome c by superoxide anion. The O_2^- release was initiated within 15 min and reached the peak at 1 h time point followed by progressive decrease. The specificity of the reduction was controlled by its inhibition by SOD (Fig. 2A). In addition, the superoxide production was inhibited by antioxidants like *N*-acetyl-L-cysteine (Fig. 2B), thus confirming that superoxide anion is regulating the downstream events of apoptosis. The BC-8 cells did not show superoxide release under normal conditions.

3.4. Change in membrane asymmetry

The hydrophobic property of curcumin enabled the molecule to pass through the plasma membrane and accumulate on the nuclear envelope and ER, depicting bright autofluorescence (Fig. 3A). Curcumin treatment resulted in the exposure of phosphatidylserine (PS) residues, which normally reside on the inner membrane, to the outer membrane leaflet on the treated cells. We analyzed the PS exposure by confocal mi-

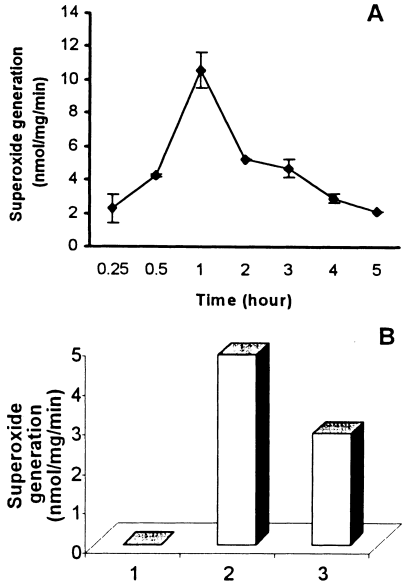

Fig. 2. Generation of extracellular superoxide anion by BC-8 cells after treatment with 50 μM curcumin. A: SOD inhibitable cytochrome c reduction at different time points; mean ± S.D. B: NAC inhibitable superoxide generation as detected after 1 h of curcumin treatment. 1, control cells; 2, curcumin treated positive control; 3, curcumin and NAC treated cells.

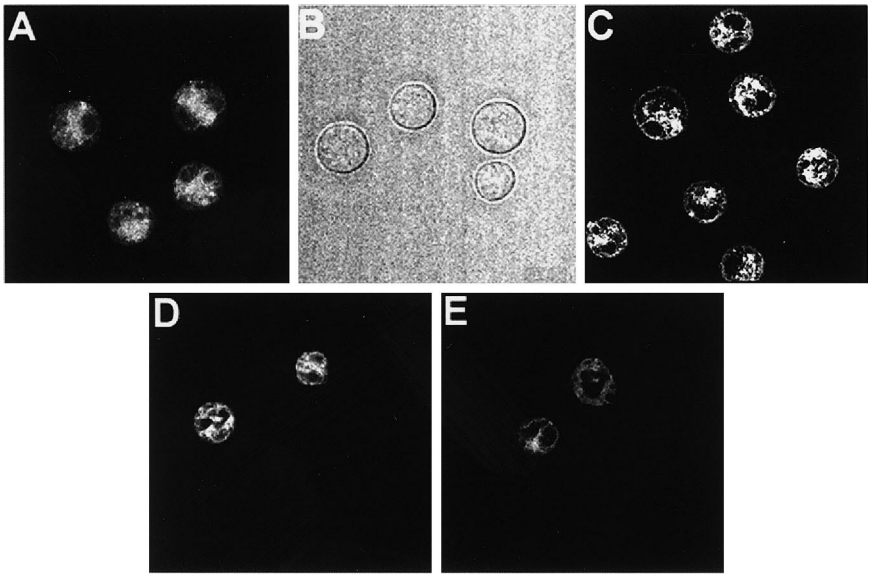

Fig. 3. Effect of curcumin on AK-5 membrane asymmetry and changes in mitochondrial transmembrane potential. A: accumulation of curcumin in AK-5 cells; B: phase contrast picture of AK-5 cells treated with curcumin; C: cells treated with 50 μM curcumin and stained with Annexin V-FITC showing the cell membrane as a ring, indicating exposure of PS on the outer plasma membrane; D: control cells treated with membrane potential sensitive dye $DiOC_6(3)$; and E: cells treated with curcumin for 4 h and stained with $DiOC_6(3)$.

croscopy using Annexin V-FITC conjugate. Annexin binds to the negatively charged phosphatidylserine exposed on the cellular membrane and the conjugated FITC showed a ring-like stain along the cellular boundary (Fig. 3C). The control BC-8 cells did not show any such staining pattern (Fig. 3A). PS externalization is an early marker for apoptotic induction, that may allow cells to be recognized by macrophages [18,19].

3.5. Loss of mitochondrial membrane potential

In order to study the mechanism of ROI mediated apoptosis by curcumin in BC-8 cells, we also studied the effect on the cellular mitochondrial transmembrane potential. The uptake of mitochondrial membrane potential sensitive dye, $DiOC_6(3)$ was visualized by confocal microscopy (Fig. 3D). The mitochondrial membrane potential was lost after 2–4 h of treatment of cells with curcumin (Fig. 3E) prior to the visualization of the apoptotic features in these cells, whereas normal cells retained the membrane potential and were brightly fluorescent.

3.6. Analysis of mitochondrial cytochrome c

The release of mitochondrial cytochrome c to the cytosol, and the initiation of a cascade of proteolytic events in the cytosol subsequent to cytochrome c release has been documented earlier [20]. The superoxide anion release and reduction in mitochondrial membrane potential in BC-8 cells was followed by release of cytochrome c to the cytosol after 3 h of treatment with curcumin. The accumulation of cytochrome c in the cytosol of curcumin treated cells was confirmed by Western blot analysis.

4. Discussion

In the present investigation we have studied the mechanism of curcumin mediated apoptotic death in AK-5 tumor cells. This polyphenolic, hydrophobic compound, can diffuse easily into the cytosol, triggering specific apoptotic events. We have implicated the involvement of ROS in curcumin mediated apoptosis in AK-5 cells earlier [11]. ROS includes free radicals such as superoxide (O_2^-), hydroxyl radicals ($^{\bullet}OH$) and non-radical derivatives of oxygen like H_2O_2. They cause damage in most of the biomolecules including DNA, protein, lipid membrane etc. [21]. Superoxide may also react with nitric oxide to yield peroxynitrite, a more powerful and damaging oxidant than either of the two [22]. Thus ROS could play an important role in the antitumor events leading to apoptotic tumor cell death [23]. Curcumin induced alterations in membrane dynamics have been observed recently [24].

Due to its unique physicochemical property curcumin passes easily through the plasma membrane (Fig. 3A) and evokes membrane perturbation by affecting the membrane lipid bilayer. Closer study by Jaruga et al. [24] on curcumin treated erythrocytes showed possibility of membrane lipid rearrangement leading to transient exposure of phosphatidylserine. Similar events of PS exposure in AK-5 cell membrane were observed after curcumin treatment (Fig. 3C). We have also quantitated simultaneous hyperproduction of superoxide anion in the extracellular milieu during the first hour of curcumin treatment (Fig. 2). The correlation between superoxide hypergeneration and PS exposure was also observed by Hirsch et al. during apoptotic events [25]. Curcumin may cause struc-

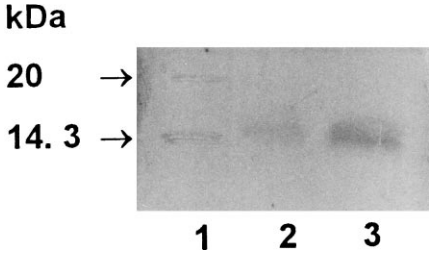

Fig. 4. Cytochrome c release from the AK-5 cell mitochondria after treatment with curcumin. Lane 1, molecular weight markers; lane 2, AK-5 (control); lane 3, curcumin treated AK-5 cells.

tural and functional changes in cellular membrane integrity leading to the flipping of PS to the outer cell surface which participates in the free radical release. Superoxide anions are reported to penetrate the cell membrane through anion channels [26]. Such extracellular ROS may also diffuse non-specifically into the cells causing direct damage to various biomolecules [27].

NADPH oxidase and xanthine oxidase, endoplasmic reticulum, peroxisome and mitochondrial electron transport chain (ETC) are some potential sources of superoxide generation in cells [22]. Superoxide generating membrane bound NADPH oxidase requires the presence of PS as an effector molecule [28]. Inhibition of NADPH oxidase by diphenyliodinium has been reported to protect HL-60 cells from apoptosis, when treated with H_2O_2, UV or actinomycin D [29]. Tsang et al. have reported $^{\bullet}OH$ mediated induction of internucleosomal DNA fragmentation [30]. To determine the initiation of apoptotic events, curcumin treated AK-5 cells were monitored by flow cytometry. The release of superoxide anion within the first hour of curcumin treatment initiated cell death (Table 1). The cells underwent complete DNA fragmentation within 6–8 h of treatment (data not shown).

The apoptotic events in AK-5 cells were controlled by the antioxidant NAC, proving its redox sensitivity. The treatment of cells with curcumin led to the activation of caspase-3-like proteases which trigger the downstream cascade of apoptotic events [14]. Similar ROS mediated apoptotic events have been documented earlier [25]. We also observed the release of cytochrome c in the cytosol (Fig. 4). Overexpression of Bcl-2, an integral mitochondrial membrane protein blocked cytochrome c release [15,31], thereby inhibiting release of apoptogenic proteases possibly by binding to cytochrome c [32].

We have also observed loss of the mitochondrial transmembrane potential, prior to the visualization of the apoptotic events in AK-5 cells, treated with curcumin (Fig. 3E). Such loss of mitochondrial membrane potential has been correlated to the induction of apoptosis via caspases. Mitochondrial depolarization is associated with the permeability transition (PT). Induction of PT leads to liberation of apoptogenic proteins like AIF (apoptosis inducing factor) and cytochrome c, which in turn activate caspase-3-like proteases and show apoptosis associated changes in cellular redox potential [33].

Nitric oxide, another well known short lived free radical has been recently identified to be a potent inhibitor of apoptosis [34]. This radical is produced by NOS isoforms and is capable of inhibiting caspase-3 activity. Nitric oxide mediated suppression of apoptosis is also associated with inhibition of Bcl-2 cleavage and cytochrome c release [35]. Curcumin treated AK-5 cells did not release NO and there was no expression of iNOS seen in these cells (data not shown). Therefore it seems that the AK-5 cells have preferentially chosen ROS mediated apoptotic pathway on treatment with curcumin.

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References

[1] Nadkarni, K.M. (1976) in: Indian Materia Media (Nadkarni, K.M., Ed.), Popular Prakashan, Bombay, pp. 414–417.
[2] Kuttan, R., Bhanumathy, P., Nirmala, K. and George, M.C. (1985) Cancer Lett. 29, 197–202.
[3] Pachuari, S.P. and Mukherjee, S.K. (1956) J. Res. Indian Med. 6, 27–31.
[4] Sharma, O.P. (1976) Biochem. Pharmacol. 25, 1811–1812.
[5] Kuo, M.-L., Huang, T.-S. and Lin, J.-K. (1996) Biochem. Biophys. Acta 1317, 95–100.
[6] Jee, S.H., Shen, S.C., Tseng, C.R., Chiu, H.C. and Kuo, M.L. (1998) J. Invest. Dermatol. 111, 656–661.
[7] Kawamori, T., Lubet, R., Steele, V.E., Kelloff, G.J., Kaskey, R.B., Rao, C.V. and Reddy, B.S. (1999) Cancer Res. 59, 597–601.
[8] Mehta, K., Pantazis, P., McQueen, T. and Agarwal, B.B. (1997) Anticancer Drugs 5, 470–481.
[9] Jiang, M.C., Yang-Yen, H.F., Yen, J.J. and Lin, J.K. (1996) Nutr. Cancer 26, 111–120.
[10] Huang, M.-T., Lou, Y.-R., Ma, W., Newmark, H., Rewhl, K. and Conney, A.H. (1994) Cancer Res. 54, 5841–5847.
[11] Khar, A., Ali, A.M., Pardhasaradhi, B.V.V., Begum, Z. and Anjum, R. (1999) FEBS Lett. 445, 165–168.
[12] Khar, A. and Ali, A.M. (1990) In vitro Cell. Dev. Biol. 26, 1024–1025.
[13] Gopalakrishna, P. and Khar, A. (1995) J. Biochem. Biophys. Methods 30, 69–73.
[14] Pick, E. (1986) Methods Enzymol. 132, 407–421.
[15] Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.-I., Jones, D.P. and Wang, X. (1997) Science 275, 1129–1132.
[16] Jaruga, E., Salvioli, S., Dobrucki, J., Chrul, S., Bandorowiez-Pikula, J., Sikora, E., Franceschi, C., Cossarizza, A. and Bartosz, G. (1998) FEBS Lett. 433, 287–293.
[17] Cai, J. and Jones, D.P. (1998) J. Biol. Chem. 273, 11401–11404.
[18] Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. (1995) J. Immunol. Methods 184, 39–51.
[19] Martin, S.J., Reutelingsperger, C.P.M., McGahan, A.J., Rader, J.A., Van Schie, C.A.A., Laface, D.M. and Green, D.R. (1995) J. Exp. Med. 182, 1545–1556.
[20] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell 86, 147–157.
[21] Halliwell, B. and Gutteridge, J.M.C. (1989) Free Rad. Bio. Med., 2nd Edn., Clarendon Press, Oxford.
[22] Wang, P. and Zweier, J.L. (1996) J. Biol. chem. 46, 29223–29230.
[23] Gorman, A., McGowan, A. and Cotter, T.G. (1997) FEBS Lett. 404, 27–33.
[24] Jaruga, E., Sokal, A., Chrul, S. and Bartosz, G. (1998) Exp. Cell Res. 245, 303–312.
[25] Hirsch, T., Marchetti, P., Susin, S.A., Dallaporta, B., Zamzami, N., Marzo, I., Genskens, M. and Kroemer, G. (1997) Oncogene 15, 1573–1581.
[26] Ikebuchi, Y., Masumoto, N., Tasaka, K., Koike, K., Kasahara, K., Miyake, A. and Tanizawa, O. (1991) J. Biol. Chem. 266, 13233–13237.
[27] Higuchi, M., Honda, T., Proske, R.J. and Yeh, E.T.H. (1998) Oncogene 17, 2753–2760.
[28] Tamura, M., Tamura, T., Tyagi, S.R. and Lambeth, J.D. (1988) J. Biol. Chem. 263, 17621–17626.
[29] Li, Y. and Trush, M.A. (1998) Biochem. Biophys. Res. Commun. 253, 295–299.
[30] Tsang, S.Y., Tam, S.C., Bremner, I. and Burkitt, M.J. (1996) Biochem. J. 317, 13–16.
[31] Kim, C.N., Wang, X., Huang, Y., Ibrado, A.M., Liv, L., Fang, G. and Bhalla, K. (1997) Cancer Res. 57, 3115–3120.
[32] Kharbanda, S., Pandey, P., Schefied, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z.-M., Saxena, S., Weichselbaum, R., Nalin, C. and Kufe, D. (1997) Proc. Natl. Acad. Sci. USA 94, 6932–6942.
[33] Castedo, M., Hirsch, T., Susin, S.A., Zamzami, N., Marchetti, P., Macho, A. and Kroemer, G. (1996) J. Immunol. 157, 512–521.
[34] Kim, Y.-M., Talanian, R.V. and Billar, T.R. (1997) J. Biol. Chem. 272, 31138–31148.
[35] Kim, Y.-M., Kim, T.-H., Seol, D.-W., Talanian, R.V. and Billiar, T.R. (1998) J. Biol. Chem. 273, 31437–31441.