

Mechanism of α -Lipoic Acid-Induced Apoptosis of Lung Cancer Cells

Involvement of Ca^{2+}

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α -Lipoic acid (LA) shows a protective effect on oxidative stress-induced apoptosis while it induces apoptosis in various cancer cells. Intracellular Ca^{2+} plays a central role in triggering apoptotic pathways. In the present study, we aim to investigate whether LA induces apoptosis in lung cancer cells and whether Ca^{2+} is involved in LA-induced apoptosis. We found that LA decreased cell viability and increased DNA fragmentation of the cells. LA activated the caspase-independent pathway, determined by upregulation of poly(ADP-ribose) polymerase (PARP) and increased the nuclear level of apoptosis-inducing factor and caspase-dependent apoptotic pathway, determined by increased levels of cytochrome *c* and PARP-1 cleavage product. LA-induced apoptotic alterations were inhibited in the cells treated with Ca^{2+} chelator BAPTA-AM. In conclusion, LA induces apoptosis through caspase-independent and caspase-dependent pathways, which is mediated by intracellular Ca^{2+} .

Key words: α -lipoic acid; apoptosis; lung cancer cells; Ca^{2+}

Introduction

α -Lipoic acid (LA), also known as thioctic acid, is a naturally occurring compound that is synthesized in small amounts by plants and animals, including humans.¹ Endogenously synthesized LA functions as cofactors for several important mitochondrial enzyme complexes.² In addition to the physiological functions of LA, there are increasing scientific and medical interests in potential therapeutic uses of LA. LA acts as an anti- or a proapoptotic agent in various cells. LA prevents oxidant-induced cell death and apoptosis, while LA induces apoptosis in cancer cells.³⁻⁷

LA has antioxidant activity by quenching a variety of intracellular reactive oxygen species (ROS).² On the other hand, LA stimulates mi-

tochondrial Ca^{2+} release and increases the intracellular Ca^{2+} level by oxidizing some protein vicinal thiols.^{8,9} Increased Ca^{2+} induces necrosis and/or triggers the controlled pathways of apoptotic cell death.¹⁰

There are two types of apoptosis.¹¹ One pathway is a death pathway that traditionally involves Bcl family proteins and caspases (caspase-dependent pathway). Increased Ca^{2+} stimulates mitochondrial stress through activating the Bcl family proteins and initiates apoptosis. Cytochrome *c* is released from the mitochondria of the cells undergoing apoptosis. The other pathway does not involve Bcl/Bax and caspase activation (caspase-independent pathway). In this type of cell death, apoptosis is often triggered by poly(ADP-ribose) polymerase (PARP). PARP is hyperactivated in the caspase-independent pathway.¹² DNA damage by various cell-death signaling, such as Ca^{2+} and ROS, induces hyperactivation and upregulation of PARP and translocation of apoptosis-inducing factor (AIF) into the

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nucleus and initiates apoptosis. Although LA induces apoptosis in several cancer cells, the mechanism of LA-induced apoptosis remains to be investigated.

We aim to investigate whether LA induces apoptosis in lung cancer cells and whether Ca^{2+} is involved in LA-induced apoptosis, by determining cell viability, DNA fragmentation, the levels of AIF, PARP, and cytochrome *c* in the cells, using Ca^{2+} chelator BAPTA-AM. In the present study, A549 cells, a commonly used human lung carcinoma cell line, were employed to elucidate the mechanism of LA-induced apoptosis.

Materials and Methods

A549 cells (human lung carcinoma, ATCC CCL185) were purchased from American Type Culture Collection (Rockville, MD). The cells were grown at 37°C at 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex, Walkersville, MD), and antibiotics (GIBCO). LA and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) were dissolved in ethanol and dimethylsulfoxide, respectively.

For concentration response of LA for cell viability and DNA fragmentation, the cells were treated with LA (0.5, 1 mmol/L) for 24 h. For time response of LA for the levels of AIF, PARP-1, PARP-1 cleavage product, and cytochrome *c*, the cells were treated with LA (1 mmol/L) for 0, 6, 12, and 24 h. Histone H1 was used for the nuclear control, while actin served as a loading control. To investigate the involvement of Ca^{2+} on LA-induced apoptosis, the cells were treated with or without BAPTA-AM (5, 10 $\mu\text{mol/L}$) for 2 h. After removing BAPTA-AM from the medium, the cells were treated with LA (1 mmol/L) for 24 h.

Cell viability was determined by the trypan blue exclusion test (0.2% trypan blue). Cells

(6×10^4) were seeded onto a 24-well culture plate and then treated with LA for 24 h. The number of viable cells was counted with a hemocytometer, using 0.2% trypan blue.

DNA fragmentation was determined by mononucleosome- and oligonucleosome-bound DNA in the cell lysates, using a sandwich ELISA (cell death detection ELISA plus kit; Roche GmbH, Mannheim, Germany). The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor.

For Western blot analysis, whole-cell extracts were prepared for the detection of PARP-1, PARP-1 cleavage product, cytochrome *c*, and actin. Nuclear extracts were prepared for the detection of AIF and histone H1 by the methods previously described.¹³ Briefly, the harvested cells were extracted with lysis buffer (10 mmol/L Tris-HCl, pH7.4, 10% NP-40, and protease inhibitor cocktail) and centrifuged. The supernatants were used for whole cell extracts. To prepare nuclear extracts, the cells were extracted in buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1.5 mmol/L MgCl_2 , 0.2% NP-40, 1 mmol/L dithiothreitol (DTT), and 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer (20 mmol/L HEPES, 420 mmol/L NaCl, 0.1 mmol/L EDTA, 1.5 mmol/L MgCl_2 , 25% glycerol, 1 mmol/L DTT, and 0.5 mmol/L PMSF).

The extracts (50 μg protein/lane) were subjected to 8–10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ) by electroblotting. The membranes were blocked using 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.2% Tween 20) for 2 h at room temperature. The proteins were detected with antibodies for PARP-1, actin, AIF (Santa Cruz Biotechnology, Santa Cruz, CA), cytochrome *c* (BD Biosciences Pharmingen, San Diego, CA), and histone H1 (Millipore Corporation, Bedford, MA)

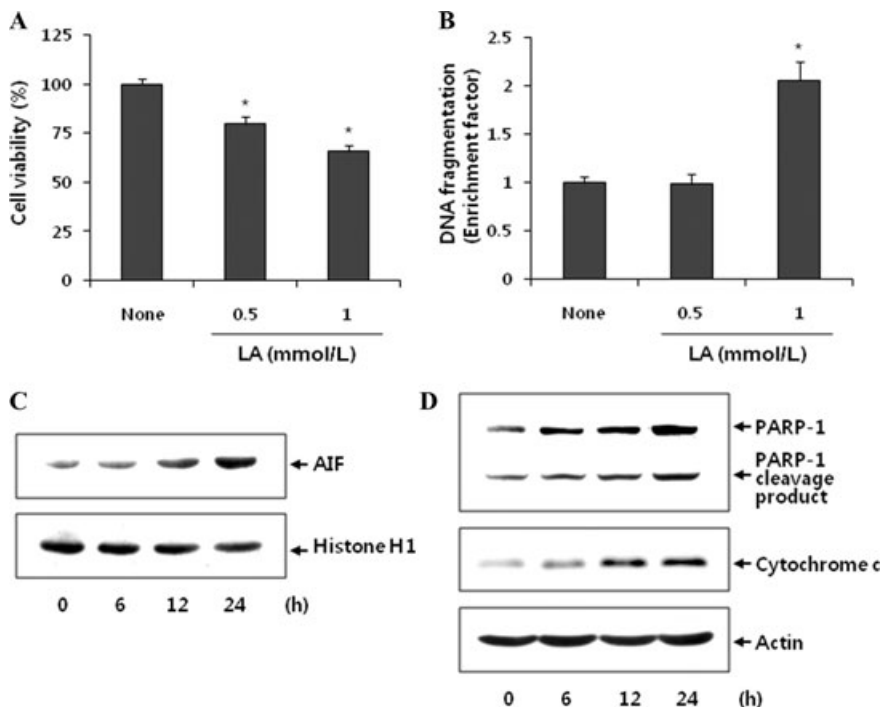


Figure 1. Cell viability, DNA fragmentation, nuclear apoptosis-inducing factor (AIF) level, and levels of poly(ADP-ribose) polymerase (PARP)-1, PARP-1 cleavage product, and cytochrome c in α -lipoic acid (LA)-treated A549 cells. **(A)** The cells were treated with LA for 24 h. Viable cell numbers were determined by the trypan blue exclusion assay. Cell viability was expressed as percent cells. **(B)** The cells were treated with LA for 24 h. DNA fragmentation was determined as the content of nucleosome-bound DNA by ELISA. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. Each bar represents mean \pm SE of three separate experiments. *, $P < 0.05$ versus none (cells treated without LA). **(C)** AIF in the nucleus was determined by Western blotting. Histone H1 served as a nuclear marker. The cells were treated with LA (1 mmol/L) for indicated time points. **(D)** The levels of PARP-1, PARP-1 cleavage product, and cytochrome c in whole-cell extracts were determined in the cells treated with LA (1 mmol/L) for indicated time points. Actin was used for a loading control.

diluted in TBS-T containing 5% dry milk, and incubated at 4°C for 16 h. After washing with TBS-T, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and visualized by the electrogenerated chemiluminescent (ECL) detection system (Santa Cruz Biotechnology), according to the manufacturer's instructions.

The statistical differences were determined using one-way ANOVA by the Newman-Keuls test. All values were expressed as means \pm SE of three separate experiments. $P < 0.05$ was considered statistically significant.

Results

To investigate the effect of LA on apoptosis, viable cell numbers and DNA fragmentation were determined in the cells treated with LA (0.5 or 1 mmol/L) for 24 h (Fig. 1A, B). LA induced a decrease in cell viability dose dependently. LA-induced DNA fragmentation was evident in the cells treated with 1 mmol/L of LA. As shown in Figure 1C, the nuclear level of AIF was increased by LA (1 mmol/L) time dependently, while histone H1, a nuclear control, was not changed by LA. LA increased the level

of PARP-1 and PARP-1 cleavage product in a time-dependent manner (Fig. 1D). These phenomena were in parallel with the increase in the cytochrome *c* level in the cells treated with LA (1 mmol/L).

For the study on possible involvement of Ca^{2+} on LA-induced apoptosis, the cells were pretreated with BAPTA-AM (5, 10 $\mu\text{mol/L}$) for 2 h and treated with LA (1 mmol/L) for a further 24 h (Fig. 2). DNA fragmentation and protein levels of AIF, PARP-1, PARP-1 cleavage product, and cytochrome *c* were determined in the cells treated with or without BAPTA-AM and cultured with or without LA. LA-induced DNA fragmentation of the cells was significantly inhibited by 10 $\mu\text{mol/L}$ of BAPTA-AM (Fig. 2A). BAPTA-AM inhibited not only PARP-1 induction but also PARP-1 cleavage caused by LA (1 mmol/L) (Fig. 2C). AIF levels in nuclear extracts and cytochrome *c* levels in whole-cell extracts were increased by LA and were inhibited by BAPTA-AM (5, 10 $\mu\text{mol/L}$) (Fig. 2B, C).

Discussion

In the present study, we showed that LA induced DNA fragmentation and cell death by inducing PARP-1 expression and translocation of AIF into the nucleus. PARP-1 mediates the release of AIF.¹² AIF directly binds DNA and induces large-scale DNA fragmentation.^{14,15} Therefore, LA induces apoptosis via the caspase-independent pathway. On the other hand, LA increased levels of both cytochrome *c* and PARP-1 cleavage product, which demonstrates that the caspase-dependent pathway is also involved in LA-induced apoptosis.

In the caspase-independent apoptotic pathway, Bcl/Bax and caspase are not required.¹¹ Mitochondria release proapoptotic proteins by a distinct mechanism that does not require caspase activation. A key molecule in this type of cell death is PARP-1, which mediates the release of AIF.¹² PARP-1 is an abundant nu-

clear protein that is involved in the DNA base excision-repair system. In response to DNA damage, PARP-1 activity and expression rapidly increased, resulting in NAD^+ and ATP depletion and cell death. Therefore, PARP-1 activation might be a key factor that regulates the fate of the cells to either die or survive following DNA damage.^{16,17}

Present results showed that LA increased PARP-1 expression, which was inhibited by a Ca^{2+} chelator (BAPTA-AM). The results clearly demonstrate the involvement of Ca^{2+} on LA-induced PARP-1 expression of lung cancer cells. LA was previously shown to stimulate mitochondrial Ca^{2+} release, presumably by oxidizing some protein vicinal thiols.⁹ Inositol 1,4,5-triphosphate (IP_3)-dependent Ca^{2+} mobilization induced PARP-1 activation in brain cortical neurons in the absence of DNA breaks.¹⁸ Other studies showed that Ca^{2+} mediated ROS-induced DNA damage, PARP-1 activation, and cell death.^{19,20} In our previous study, LA induced ROS production and apoptosis in lung cancer cells (data not shown). Therefore, further studies should be performed to investigate the relation between Ca^{2+} and ROS in the LA-induced apoptotic mechanism of lung cancer cells.

AIF is a key mediator of cell death downstream of PARP-1 activation because PARP-1 stimulates translocation of AIF into the nucleus.^{16,17,21} In response to apoptotic stimuli, AIF is released from the mitochondria and translocates into the nucleus. It becomes an active executioner of the cells.¹¹ LA-induced PARP-1 expression may accelerate release of AIF from mitochondria and subsequent nuclear translocation. AIF localized in the nucleus initiates chromatin condensation and large-scale (50 kb) DNA fragmentation. Consequently, AIF plays a central role in the regulation of the caspase-independent apoptotic pathway.¹¹

The cytochrome *c*-mediated caspase activation pathway is well established.²² Upon sensing a variety of apoptotic stimuli, cytochrome *c* is released from mitochondria and triggers

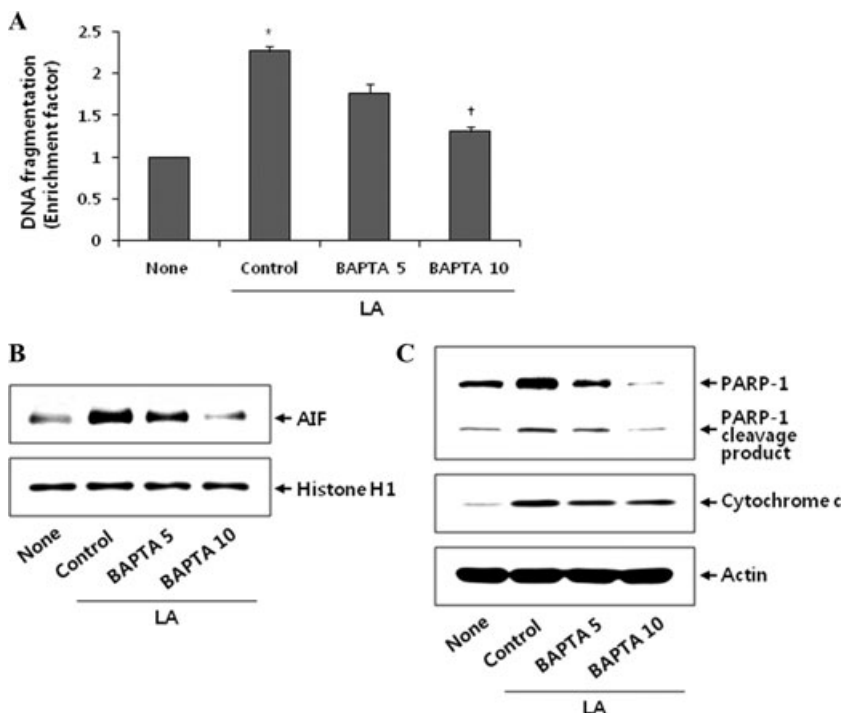


Figure 2. DNA fragmentation, nuclear AIF level, and levels of PARP-1, PARP-1 cleavage product, and cytochrome *c* in A549 cells treated with or without BAPTA-AM and cultured in the presence of LA. **(A)** The cells were pretreated with BAPTA-AM for 2 h. After removing BAPTA-AM from the medium, cells were treated with LA (1 mmol/L) for a further 24 h. Each bar represents mean \pm SE of three separate experiments. *, $P < 0.05$ versus none (cells treated without LA); †, $P < 0.05$ versus control (cells treated with LA without BAPTA-AM). **(B)** AIF in the nucleus was determined by Western blotting. Histone H1 served as a nuclear marker. The cells were pretreated with BAPTA-AM (5, 10 μ mol/L) for 2 h and then treated with LA (1 mmol/L) for 24 h after removing BAPTA-AM from the medium. **(C)** The levels of PARP-1, PARP-1 cleavage product, and cytochrome *c* in whole-cell extracts were determined in the cells pretreated with BAPTA-AM (5, 10 μ mol/L) for 2 h and treated with LA (1 mmol/L) for a further 24 h. Actin was used as a loading control.

formation of the apoptosome. The apoptosome subsequently forms an active holoenzyme with procaspase-9 and activates downstream executioner caspases, such as caspase-3, which eventually lead to programmed cell death.²² Activated caspase-3 then cleaves whole PARP-1 to prevent energy depletion and subsequent necrotic cell death.²³ Therefore, PARP-1 cleavage product is used as an activated caspase-3 marker. In the present study, LA increased the expression of cytochrome *c* and PARP-1 cleavage products, indicating that the caspase-dependent pathway is activated in LA-induced apoptosis of A549 cells. LA stimulates

mitochondrial Ca^{2+} release.⁸ Elevating intracellular Ca^{2+} concentration encourages mitochondrial membrane potential transition that releases cytochrome *c* from mitochondria to cytosol and triggers Ca^{2+} -modulated apoptosis.¹⁰ Accumulating evidence supports the present results showing that Ca^{2+} chelator inhibited LA-induced cytochrome *c* and PARP-1 cleavage products in lung cancer cells. In addition, lung cancer A549 cells are non-small-cell lung cancer cells, which are resistant to chemotherapy-induced apoptosis.²⁴ Therefore, LA may be beneficial for the treatment of lung cancer patients who show chemotherapy resistance.

In the present study, we demonstrate that LA induces apoptosis through the caspase-independent pathway (nuclear translocation of AIF and PARP-1 induction) and caspase-dependent pathway (cytochrome *c* release and PARP-1 cleavage), which is mediated by intracellular Ca²⁺.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Reed, L.J. 2001. A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes. *J. Biol. Chem.* **276**: 38329–38336.
2. Bilaska, A. & L. Wlodek. 2005. Lipoic acid – the drug of the future? *Pharmacol. Rep.* **57**: 570–577.
3. Simbula, G., A. Columbano, G.M. Ledda-Columbano, *et al.* 2007. Increased ROS generation and p53 activation in alpha-lipoic acid-induced apoptosis of hepatoma cells. *Apoptosis* **12**: 113–123.
4. Mounjaroen, J., U. Nimmannit, P.S. Callery, *et al.* 2006. Reactive oxygen species mediate caspase activation and apoptosis induced by lipoic acid in human lung epithelial cancer cells through Bcl-2 down-regulation. *J. Pharmacol. Exp. Ther.* **319**: 1062–1069.
5. Zheng, Y., H. Yamaguchi, C. Tian, *et al.* 2005. Arsenic trioxide (As₂O₃) induces apoptosis through activation of Bax in hematopoietic cells. *Oncogene* **24**: 3339–3347.
6. Singh, L.P., D.W. Cheng, R. Kowluru, *et al.* 2007. Hexosamine induction of oxidative stress, hypertrophy and laminin expression in renal mesangial cells: effect of the anti-oxidant alpha-lipoic acid. *Cell. Biochem. Funct.* **25**: 537–550.
7. Abdul, H.M. & D.A. Butterfield. 2007. Involvement of PI3K/PKG/ERK1/2 signaling pathways in cortical neurons to trigger protection by cotreatment of acetyl-L-carnitine and alpha-lipoic acid against HNE-mediated oxidative stress and neurotoxicity: implications for Alzheimer's disease. *Free Radic. Biol. Med.* **42**: 371–384.
8. Schweizer, M. & C. Richter. 1996. Stimulation of Ca²⁺ release from rat liver mitochondria by the dithiol reagent alpha-lipoic acid. *Biochem. Pharmacol.* **52**: 1815–1820.
9. Moini, H., L. Packer & N.E. Saris. 2002. Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. *Toxicol. Appl. Pharmacol.* **182**: 84–90.
10. Giacomello, M., I. Drago, P. Pizzo, *et al.* 2007. Mitochondrial Ca²⁺ as a key regulator of cell life and death. *Cell Death Differ.* **14**: 1267–1274.
11. Cregan, S.P., V.L. Dawson & R.S. Slack. 2004. Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* **23**: 2785–2796.
12. Yu, S.W., H. Wang, M.F. Poitras, *et al.* 2002. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**: 259–263.
13. Song, J.Y., J.W. Lim, H. Kim, *et al.* 2003. Oxidative stress induces nuclear loss of DNA repair proteins Ku70 and Ku80 and apoptosis in pancreatic acinar AR42J cells. *J. Biol. Chem.* **278**: 36676–36687.
14. Lipton, S.A. & E. Bossy-Wetzel. 2002. Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. *Cell* **111**: 147–150.
15. Otera, H., S. Ohsakaya, Z. Nagaura, *et al.* 2005. Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. *EMBO J.* **24**: 1375–1386.
16. Hong, S.J., T.M. Dawson & V.L. Dawson. 2004. Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. *Trends Pharmacol. Sci.* **25**: 259–264.
17. Li, G.Y. & N.N. Osborne. 2008. Oxidative-induced apoptosis to an immortalized ganglion cell line is caspase independent but involves the activation of poly(ADP-ribose)polymerase and apoptosis-inducing factor. *Brain Res.* **1188**: 35–43.
18. Homburg, S., L. Visocek, N. Moran, *et al.* 2000. A fast signal-induced activation of poly(ADP-ribose) polymerase: a novel downstream target of phospholipase c. *J. Cell Biol.* **150**: 293–307.
19. Barbouti, A., P.T. Doulias, B.Z. Zhu, *et al.* 2001. Intracellular iron, but not copper, plays a critical role in hydrogen peroxide-induced DNA damage. *Free Radic. Biol. Med.* **31**: 490–498.
20. Bentle, M.S., K.E. Reinicke, E.A. Bey, *et al.* 2006. Calcium-dependent modulation of poly(ADP-ribose) polymerase-1 alters cellular metabolism and DNA repair. *J. Biol. Chem.* **281**: 33684–33696.

21. Williams, B.L., M. Hornig, K. Yaddanapudi, *et al.* 2008. Hippocampal PARP-1 and caspase-3 activation in neonatal bornavirus infection. *J. Virol.* **82**: 1748–1758.
22. Jiang, X. & X. Wang. 2004. Cytochrome C-mediated apoptosis. *Annu. Rev. Biochem.* **73**: 87–106.
23. Soldani, C. & A.I. Scovassi. 2002. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* **7**: 321–328.
24. Gallego, M.A., C. Ballot, J. Kluza, *et al.* 2007. Overcoming chemoresistance of non-small cell lung carcinoma through restoration of an AIF-dependent apoptotic pathway. *Oncogene* **27**: 1981–1992.