Journal of Biochemistry and Molecular Biology, Vol. 38, No. 5, September 2005, pp. 619-623

JOURNAL OF
Biochemistry and Molecular Biology

Release of Cytochrome *c* from Isolated Mitochondria by Etoposide

Jung-Hee Park[†] **and Tae-Hyoung Kim**[†,*]
[†]Department of Biochemistry, Chosun University School of Medicine, 375 Seosuk-Dong, Dong-Gu, Gwangju, 501-759, Korea

Received 15 February 2005, Accepted 19 August 2005

The efficacy of chemotherapeutic agents on tumor cells has been shown to be modulated by tumor suppressor gene p53 and its target genes such as Bcl-2 family members (Bax, Noxa, and PUMA). However, various chemotherapeutic agents can induce cell death in tumor cells that do not express the functional p53, suggesting that some chemotherapeutic agents may induce cell death in a p53-independent pathway. Here we showed that etoposide can induce the similar degree of cell death in p53-deficient HCT 116 cells, whereas 5'-FU-mediated cell death is strongly dependent on the existence of functional p53 in HCT 116 cells. Further, we provide the evidence that etoposide can induce the cytochrome *c* release from isolated mitochondria, and etoposide-induced cytochrome *c* release is not accompanied with the large amplitude swelling of mitochondria. These data suggest that etoposide can directly induce the mitochondrial dysfunction irrespective of p53 status, and it may, at least in part, account for the p53-independent pathway in cell death induced by chemotherapeutic agents.

Keywords: Cytochrome c release, Etoposide, Mitochondria, p53

Introduction

Cell killing activity of chemotherapeutic agents has been shown to be dependent on interactions between drugs and cellular proteins, and tumor suppressor gene p53 was suggested to be a key player in these cellular processes to induce cell death in tumor cells (Lowe *et al.*, 1993a). For example, tumor cells expressing p53 wild type protein are extremely susceptible to the induction of cell death by various chemotherapeutic agents such as 5'-fluoro-uracil (5'-FU), whereas various tumor cells having mutation in p53 are resistant to drug-induced cell death (Lowe *et al.*, 1993b; Lowe *et al.*, 1994; Bunz *et al.*, 1999). Importantly, cancers with p53 mutations are often resistant to chemotherapy (Lowe *et al.*, 1994; Carson and Lois, 1995; Galmarini *et al.*, 2003), and reintroduction of wild type p53 into p53 mutant tumors regains susceptibility to drug-treatment (Fujiwara *et al.*, 1993).

Chemotherapeutic drugs are believed to induce cell death through stabilization of p53 which regulates transcription of target genes. The mechanism whereby p53 induces cell death is to modulate mitochondrial function, at least in part, by either upregulating the transcription of proapoptotic genes or by downregulating the transcription of antiapoptotic genes. For instance, p53 has been shown to transactivated Noxa and PUMA that cause the efflux of mitochondrial death-promoting proteins including cytochrome *c* (Oda *et al.*, 2000; Nakano and Vousden, 2001; Yu *et al.*, 2001; Seo *et al.*, 2003). In addition, transcriptional repression of p53 target genes is likely to play an important role in p53-induced cell death; for example, RNAi-mediated knock-down of PLK and PPTG1 is sufficient to induce cell death (Kho *et al.*, 2004). Interestingly, recent studies demonstrate that p53 protein directly translocates to the mitochondria and permeabilizes the outer membrane of mitochondria by interacting with Bax and Bak (Leu *et al.*, 2004).

Although p53-dependent apoptosis plays a crucial role in chemotherapeutic agent-mediated cell death, the evidence that supports the existence of p53-independent cell death induced by chemotherapeutic agents has been accumulating (Lanni *et al.*, 1997; Marchini *et al.*, 1999; Abeysinghe *et al.*, 2001; Petit *et al.*, 2003). However, the mechanism by which chemotherapeutic agents induce cell death in a p53-independent way is not fully understood. In present study we showed that etoposide can directly induce the release of cytochrome *c* from isolated mitochondria, suggesting that etoposide may cause the mitochondrial dysfunction in a more direct way rather than indirect way through p53 function.

Materials and Methods

Cell culture and reagents The HCT 116 parental cells derived

*To whom correspondence should be addressed.
Tel: 82-62-230-6294; Fax: 82-62-226-4165
E-mail: thkim65@mail.chosun.ac.kr

from human colorectal cancer, HCT 116 p53-deficient cells, and HCT 116 Bax-deficient cells were kind gifts from Dr. B. Vogelstein at the Johns Hopkins University, Baltimore, USA (Bunz *et al.*, 1999; Zhang *et al.*, 2000). The HCT 116 cells were cultured in McCoy's 5A medium containing 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. 10-*N*-nonyl acridine orange (NAO) were purchased from Molecular probes (Eugene, USA). All chemical reagents including Etoposide, 5'-FU, Cyclosoporin A (CsA), and $CaCl_2$ were purchased from Sigma (St. Louis, USA) unless it is indicated. Antibody against cytochrome *c* was purchased from PharMingen (San Diego, USA). Recombinant TRAIL protein was purified as previously described (Seol and Billiar, 2000).

Crystal violet assay The HCT 116 cells were plated on 24-well plates, and upon the treatment of indicated reagents the attached cells were stained with 0.4% crystal violet for 10 minutes at room temperature. Then the plates were washed with tap water and air-dried. The pictures were taken by Olympus Digital camera (Japan). Densities were measured using the imaging analysis program, AlphaEaseFC software (San Leandro, USA).

Isolation of mitochondria and cytochrome c release assay Murine liver mitochondria were isolated as previously described (Kim *et al.*, 2000). Liver of BALB/c mice was homogenized with a Douncer homogenizer in Buffer A containing 250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 5 mM HEPES-NaOH, pH 7.2, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 1,000 × *g* for 10 minutes at 4ºC to remove intact cells and nuclei, and the supernatants were further centrifuged at 10,000 × *g* at 4ºC for 10 minutes to precipitate the heavy membrane fractions (mitochondria). The mitochondrial pellets were resuspended in Buffer B containing 250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH_2PO_4, 5 mM sodium succinate, 25 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. Mitochondria were kept on ice and used within 2-4 hours after preparation. For cytochrome *c* release assay, 100 μl of mitochondria (0.5 mg/ml) were treated with indicated amounts of etoposide and then incubated for 1 hour at 30ºC. The mitochondria were pelleted by centrifugation at 10,000 × *g* for 10 minutes, and 30 μl of supernatants were analyzed by SDS-PAGE followed by immunoblot with antibody against cytochrome *c*.

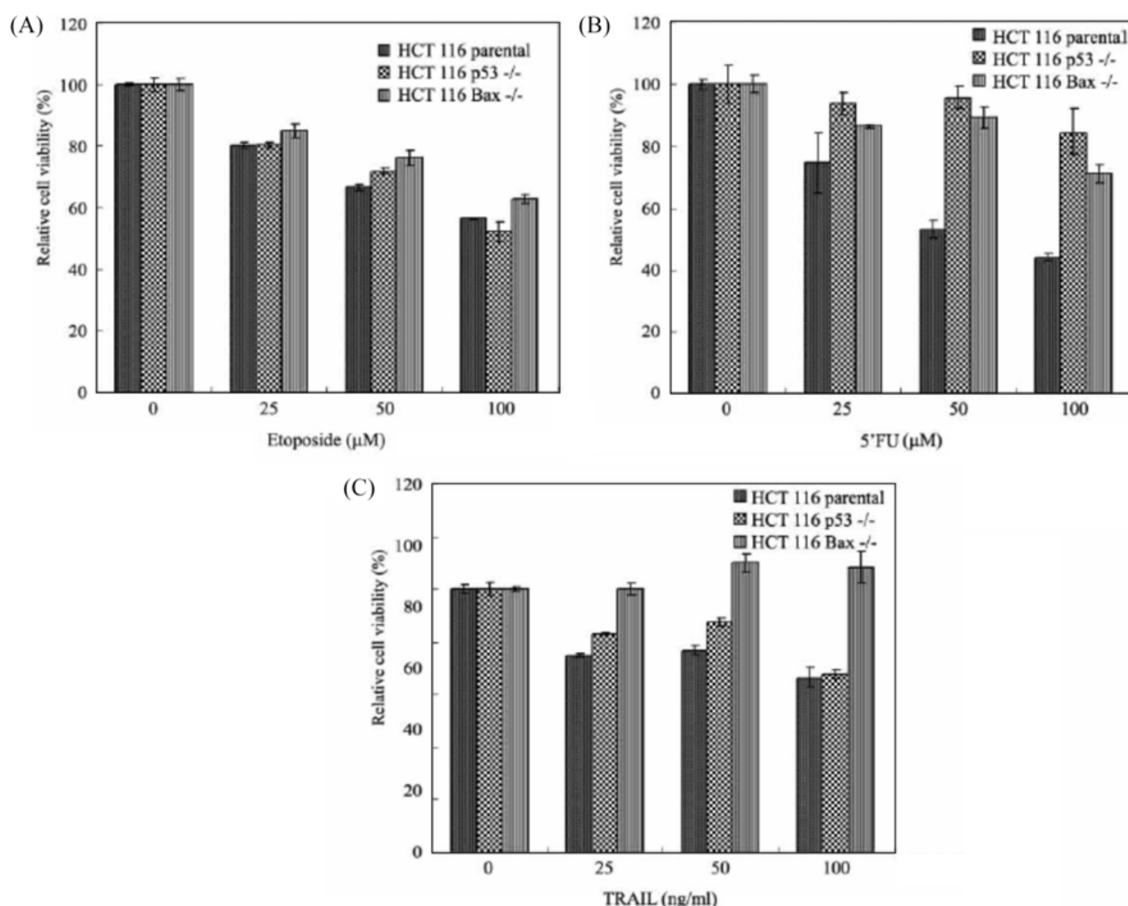

Fig. 1. Etoposide induces cell death in p53-deficient cells. HCT 116 parental cells, HCT 116 p53-deficienct cells, and HCT 116 Bax-deficient cells were treated with the indicated amount of etoposide (A), 5'-FU (B), and TRAIL (C) for 24 hours. The attached cells were stained with crystal violet for 10 minutes and then destained with tap water. The pictures were taken by Olympus Digital camera. Densities were measured using the imaging analysis program, AlphaEaseFC software (San Leandro, California, USA). Data are representative of five experiments individually carried out.

Mitochondrial swelling assay Mitochondrial swelling was measured as previously described (Kim *et al.*, 2000). In brief, the mitochondria were suspended in Buffer B at 1 mg/ml (800 mg total) to which different chemicals were added as indicated in the figure legends. Light absorbance at 540 nm was measured at the designated time points during the 75 minutes incubation period at 30ºC using a spectrophotometer (Amersham Pharmacia Biotech, Ultrospec Plus). A decreased light absorbance is consistent with an increase in mitochondrial volume (Petronilli *et al.*, 1993).

Results and Discussion

p53 has been known to play a key role in cell death induced by chemotherapeutic agents. To see whether p53 function is essential in cell death induced by etoposide or 5'-FU, HCT 116 parental cells derived from human colorectal cancer or HCT 116 p53-deficient cells that are deleted by homologous recombination were treated with etoposide or 5'-FU. HCT 116 parental cells were substantially killed by either etoposide or 5'-FU in a dose-dependent manner; however, HCT 116 p53-deficient cells showed differential responses to etoposide and 5'-FU in a way that HCT 116 p53-deficient cells were sensitive to etoposide, but resistant to 5'-FU (Fig. 1A and B). In addition, TRAIL which is a potent death-inducing ligand of TNF superfamily induces cell death in both HCT 116 parental cells and HCT 116 p53-deficient cells (Fig. 1C), consistent with previously report that TRAIL can induce cell death irrespective of p53 status (Ravi and Bedi, 2002; Song *et al.*, 2003). Whereas TRAIL failed to induce cell death in HCT 116 Bax-deficient cells that are deleted by homologous recombination (Fig. 1C), etoposide was able to induce cell death in HCT 116 Bax-deficient cells (Fig. 1A). However, HCT 116 Bax-deficient cells were partially resistant to 5'-FU. This suggests that 5'-FU induced cell death is dependent to p53 status in that Bax plays a crucial role as previously described. Etoposide, however, induces cell death irrespective of p53 status, suggesting that etoposide has another pathway to induce cell death in HCT 116 cells.

A huge line of evidence showed that mitochondria play a crucial role in chemotherapeutic agent-induced cell death including etoposide such that cytochrome *c* is released from mitochondria in response to chemotherapeutic agents. This mitochondrial dysfunction caused by chemotherapeutic agents was believed to be an indirect process mediated through Bax, Noxa, or PUMA that are transactivated by p53 (Oda *et al.*, 2000; Nakano and Vousden, 2001; Yu *et al.*, 2001; Seo *et al.*, 2003); however, this transactivation mediated by p53 is likely to be true in case of 5'-FU but not of etoposide. Thus, we hypothesized that etoposide may cause mitochondrial dysfunction in a more direct way than an indirect way like transactivation. To test the possibility that etoposide can directly release cytochrome *c* from mitochondria, isolated mitochondria were treated with etoposide and then released cytochrome *c* was observed by Western blot analysis. As shown in Fig. 2A, etoposide was able to release cytochrome *c* from isolated mitochondria in a dose-dependent manner. Cardiolipin, a lipid exclusively localized in mitochondrial membrane, has been suggested to be a targeting site of tBid, the most potent protein that causes mitochondrial dysfunction (Lutter *et al.*, 2000; Kuwana *et al.*, 2002; Kim *et al.*, 2004). To investigate whether cardiolipin plays a role in etoposide-induced cytochrome *c* release, isolated mitochondria were treated with NAO, which has been shown to inhibit tBid targeting to mitochondria leading to reduction of cytochrome *c* release (Kim *et al.*, 2004). Etoposide-induced cytochrome *c* release was not affected with NAO treatment (Fig. 2B), suggesting that cardiolipin is not associated with etoposide-induced cytochrome *c* release.

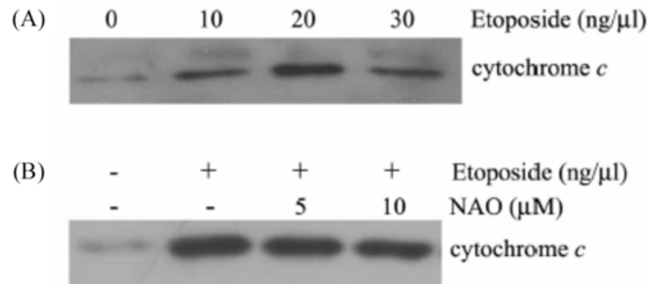

Fig. 2. Etoposide induces cytochrome c release from isolated mitochondria. (A) Murine liver mitochondria were isolated as mentioned in Materials and Methods. 100 μl of mitochondria (0.5 mg/ml) were incubated with indicated amounts of etoposide for 1 h at 30ºC. The mitochondria were precipitated by centrifugation at 10,000 × *g* for 10 min, and 30 μl of supernatants were analyzed by SDS-PAGE followed by immunoblot using antibody against cytochrome *c*. (B) Isolated mitochondria were pretreated with indicated amount of NAO for 15 min at room temperature, and then etoposide was added. After incubation for 1 h at 30ºC, the mitochondria were precipitated by centrifugation at 10,000 × *g* for 10 min. Supernatants were analyzed by SDS-PAGE followed by immunoblot using antibody against cytochrome *c*.

Many studies showed that chemotherapeutic agents can release the cytochrome *c* from mitochondria by opening permeability transition (PT) pore in cells, which is inhibited by CsA (Cassarino *et al.*, 1999; Brustovetsky *et al.*, 2002). To examine whether PT pore is opened by etoposide or not, we have checked the large amplitude mitochondrial swelling using isolated mitochondria by measuring light scattering at 540 nm. Isolated mitochondria are sensitive to 50 μM calcium ions to induce the large amplitude swelling which is completely suppressed by CsA as shown in Fig. 3A. Under this condition, etoposide was not able to induce the large amplitude swelling of mitochondria, suggesting that etoposide-induced cytochrome *c* release is not mediated by PT pore opening. Although etoposide is unlikely to induce the large amplitude swelling of mitochondria, we do not exclude the possibility that etoposide can induce the small amplitude swelling of the mitochondria. To further check that PT pore is not involved in etoposide-

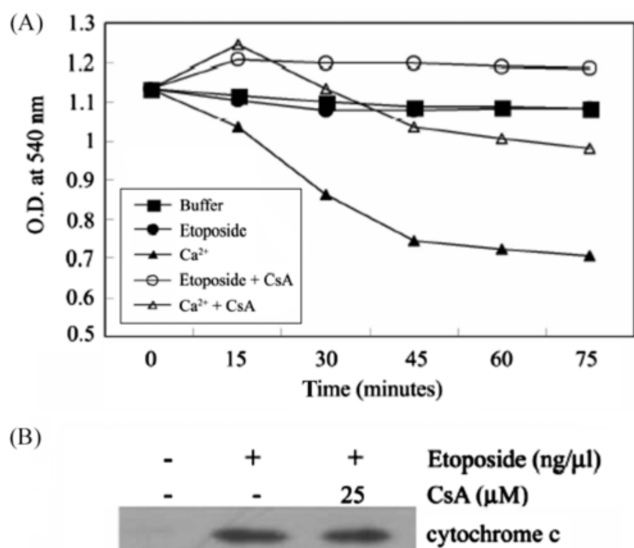

Fig. 3. Etoposide does not induce the large amplitude of mitochondrial swelling. (A) Mitochondrial swelling was measured as previously described (Kim *et al.*, 2000). The mitochondria were suspended in Buffer B at 1 mg/ml (800 mg in total) to which etoposide (30 ng/μl), $CaCl_2$ (50 μM), etoposide (30 ng/μl) plus CsA (50 μM) or $CaCl_2$ (50 μM) plus CsA (50 μM) were added. Light absorbance at 540 nm was measured at designated time points at 30°C using a spectrophotometer (Amersham Pharmacia Biotech Ultraspec Plus). Data are representative of five experiments individually carried out. (B) Murine liver mitochondria were isolated as mentioned in Fig. 2A. Mitochondria were incubated with indicated amounts of etoposide for 1 hour at 30°C in the presence or absence of CsA (25 μM). The mitochondria were precipitated by centrifugation at $10,000 \times g$ for 10 min, and supernatants were analyzed by SDS-PAGE followed by immunoblot using antibody against cytochrome *c*.

induced cytochrome *c* release, we examined whether CsA can affect the release of cytochrome *c* in response to etoposide. As expected, CsA failed to suppress the cytochrome *c* release induced by etoposide (Fig. 3B), indicating that there exists another pathway of cytochrome *c* release from isolated mitochondria in response to etoposide rather than PT pore.

In summary, we showed that etoposide can induce the cytochrome *c* release from isolated mitochondria *in vitro*, suggesting that etoposide may directly cause the mitochondrial damage in cell to activate the intrinsic death signaling pathway irrespective of p53 status. This process may not be mediated through PT opening or cardiolipin; however, the detailed mechanism in this process is still obscure, and further studies are needed.

Acknowledgments We thank Dr. Bert Vogelstein at Johns Hopkins University, USA, for the generous gifts of HCT 116 $p53^{+/+}$, HCT 116 $p53^{-/-}$, and HCT 116 $Bax^{-/-}$ cells. We also thank Dr. G.C. Kim of the Sungbukchoongang Hospital for helpful discussions. This work was supported by a grant (R13-2003-009-01002-0) from the Research Center for Resistant Cells (Medical and Engineering Research Center at Chosun University School of Medicine) funded by the Korea Science and Engineering Foundation (to T-H Kim).

References

Abeysinghe, R. D., Greene, B. T., Haynes, R., Willingham, M. C., Turner, J., Planalp, R. P., Brechbiel, M. W., Torti, F. M. and Torti, S. V. (2001) p53-independent apoptosis mediated by tachpyridine, an anti-cancer iron chelator. *Carcinogenesis* **22**, 1607-1614.

Brustovetsky, N., Brustovetsky, T., Jemmerson, R. and Dubinsky, J. M. (2002) Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. *J. Neurochem.* **80**, 207-218.

Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1999) Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J. Clin. Invest.* **104**, 263-269.

Carson, D. A. and Lois, A. (1995) Cancer progression and p53. *Lancet* **346**, 1009-1011.

Cassarino, D. S., Parks, J. K., Parker, W. D. Jr. and Bennett, J. P. Jr. (1999) The parkinsonian neurotoxin MPP+ opens the mitochondrial permeability transition pore and releases cytochrome c in isolated mitochondria via an oxidative mechanism. *Biochim. Biophys. Acta* **1453**, 49-62.

Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B. and Roth, J. A. (1993) A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res* **53**, 4129-4133.

Galmarini, C. M., Kamath, K., Vanier-Viornery, A., Hervieu, V., Peiller, E., Falette, N., Puisieux, A., Ann Jordan, M. and Dumontet, C. (2003) Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics. *Br. J. Cancer* **88**, 1793-1799.

Kho, P. S., Wang, Z., Zhuang, L., Li, Y., Chew, J. L., Ng, H. H., Liu, E. T. and Yu, Q. (2004) p53-regulated transcriptional program associated with genotoxic stress-induced apoptosis. *J. Biol. Chem.* **279**, 21183-21192.

Kim, T. H., Zhao, Y., Barber, M. J., Kuharsky, D. K. and Yin, X. M. (2000) Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. *J. Biol. Chem.* **275**, 39474-39481.

Kim, T. H., Zhao, Y., Ding, W. X., Shin, J. N., He, X., Seo, Y. W., Chen, J., Rabinowich, H., Amoscato, A. A. and Yin, X. M. (2004) Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. *Mol. Biol. Cell.* **15**, 3061-3072.

Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R. and Newmeyer, D. D. (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331-342.

Lanni, J. S., Lowe, S. W., Licitra, E. J., Liu, J. O. and Jacks, T. (1997) p53-independent apoptosis induced by paclitaxel through an indirect mechanism. *Proc. Natl. Acad. Sci. USA* **94**, 9679-9683.

Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E. and George, D. L. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat. Cell. Biol.* **6**, 443-450.

Lowe, S. W., Ruley, H. E., Jacks, T. and Housman, D. E. (1993a) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957-967.

Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. and Jacks, T. (1993b) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-849.

Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E. and Jacks, T. (1994) p53 status and the efficacy of cancer therapy in vivo. *Science* **266**, 807-810.

Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X. and Wang, X. (2000) Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat. Cell. Biol.* **2**, 754-761.

Marchini, S., Ciro, M. and Broggini, M. (1999) P53-independent caspase-mediated apoptosis in human leukaemic cells is induced by a DNA minor groove binder with antineoplastic activity. *Apoptosis* **4**, 39-45.

Nakano, K. and Vousden, K. H. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell.* **7**, 683-694.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T. and Tanaka, N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**, 1053-1058.

Petit, T., Bearss, D. J., Troyer, D. A., Munoz, R. M. and Windle, J. J. (2003) p53-independent response to cisplatin and oxaliplatin in MMTV-ras mouse salivary tumors. *Mol. Cancer Ther.* **2**, 165-171.

Petronilli, V., Cola, C., Massari, S., Colonna, R. and Bernardi, P. (1993) Physiological effectors modify voltage sensing by the cyclosporin A-sensitive permeability transition pore of mitochondria. *J. Biol. Chem.* **268**, 21939-21945.

Ravi, R. and Bedi, A. (2002) Requirement of BAX for TRAIL/Apo2L-induced apoptosis of colorectal cancers: synergism with sulindac-mediated inhibition of Bcl-x(L). *Cancer Res.* **62**, 1583-1587.

Seo, Y. W., Shin, J. N., Ko, K. H., Cha, J. H., Park, J. Y., Lee, B. R., Yun, C. W., Kim, Y. M., Seol, D. W., Kim, D. W., Yin, X. M. and Kim, T. H. (2003) The molecular mechanism of Noxa-induced mitochondrial dysfunction in p53-mediated cell death. *J. Biol. Chem.* **278**, 48292-48299.

Seol, D. W. and Billiar, T. R. (2000) Cysteine 230 modulates tumor necrosis factor-related apoptosis-inducing ligand activity. *Cancer Res.* **60**, 3152-3154.

Song, J. H., Song, D. K., Pyrzynska, B., Petruk, K. C., Van Meir, E. G. and Hao, C. (2003) TRAIL triggers apoptosis in human malignant glioma cells through extrinsic and intrinsic pathways. *Brain Pathol.* **13**, 539-553.

Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W. and Vogelstein, B. (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol. Cell.* **7**, 673-682.

Zhang, L., Yu, J., Park, B. H., Kinzler, K. W. and Vogelstein, B. (2000) Role of BAX in the apoptotic response to anticancer agents. *Science* **290**, 989-992.