Cancer Chemother Pharmacol (2001) 48: 423–428
DOI 10.1007/s002800100375

ORIGINAL ARTICLE

**Abdelhadi Rebbaa · Pauline M. Chou
Mohammad Emran · Bernard L. Mirkin**

Doxorubicin-induced apoptosis in caspase-8-deficient neuroblastoma cells is mediated through direct action on mitochondria

Received: 2 May 2001 / Accepted: 10 August 2001 / Published online: 25 September 2001

Abstract The induction of p53 expression and stimulation of the Fas/caspase-8 pathway represent major mechanisms by which cytotoxic drugs induce apoptosis, but in neuroblastomas, the caspase-8 gene is often not expressed. *Purpose*: The aim of this study was to determine whether doxorubicin could induce apoptosis in caspase-8-deficient neuroblastoma cells and to define its mechanism of action. *Methods*: The caspase-8-deficient human neuroblastoma cell line, SKN-SH, was incubated with doxorubicin and the apoptotic response, as well as expression of apoptotic molecules in the p53/Fas/caspase-8 pathway, were determined. *Results*: SKN-SH cells incubated with doxorubicin readily underwent apoptosis in a concentration-dependent manner. Western blot analyses with specific antibodies demonstrated that both p53 and Fas ligand were endogenously expressed in SKN-SH cells, but their expression was not stimulated by doxorubicin. Fas receptor was not detected in these cells and caspase-8 was totally absent. Electron microscopic analyses of SKN-SH cells treated with doxorubicin revealed pronounced alterations in mitochondrial structure. This treatment also induced the release of cytochrome *c* from mitochondria and activated the downstream apoptotic intermediate, caspase-3. *Conclusion*: These results indicate that the p53/Fas/caspase-8 system does not play a role in mediating the apoptotic action of doxorubicin in the human neuroblastoma cell line SKN-SH. Thus, mitochondria and downstream apoptotic signaling intermediates may be considered as key targets for doxorubicin-induced apoptosis in neuroblastoma tumors having deficiencies in the Fas/caspase-8 system.

Keywords Doxorubicin · Apoptosis · Neuroblastoma · Caspase-8 · Mitochondria

A. Rebbaa (✉) · P.M. Chou
Department of Pediatrics,
Northwestern University Medical School,
Children's Memorial Institute for Education
and Research (CMIER), Cancer Biology and Chemotherapy,
Disease Pathogenesis Program, 2300 Children's Plaza M/C 224,
Chicago, IL 60614, USA
E-mail: arebbaa@childrensmemorial.org
Tel.: +1-773-8804014; Fax: +1-773-8808333

M. Emran
Department of Surgery, Northwestern University Medical School,
Children's Memorial Institute for Education
and Research (CMIER), Cancer Biology and Chemotherapy,
Disease Pathogenesis Program, 2300 Children's Plaza M/C 224,
Chicago, IL 60614, USA

B.L. Mirkin
Department of Molecular Pharmacology
and Biological Chemistry,
Northwestern University Medical School,
Children's Memorial Institute for Education
and Research (CMIER), Cancer Biology and Chemotherapy,
Disease Pathogenesis Program, 2300 Children's Plaza M/C 224,
Chicago, IL 60614, USA

Introduction

Recent advances in understanding the relationship between the action of chemotherapeutic drugs and altered cell signaling have demonstrated that drugs such as doxorubicin, methotrexate, cytarabine, etoposide and cisplatin induce apoptosis via activation of the Fas/caspase-8 system [1, 2, 3]. These drugs lead to increased transcriptional activity of the death ligand (Fas-L) which in turn activates the Fas receptor in an autocrine/paracrine manner. Activated Fas receptor links via its cytosolic domain to adapter proteins such as the Fas-associated death domain (FADD) and to the apoptosis initiator caspase-8 [4]. Activated caspase-8 cleaves Bid [5, 6] and when truncated Bid is incorporated into mitochondria, it leads to cytochrome *c* release. This activates caspase-9 which transmits apoptotic signals to the nucleus through activation of downstream targets such as caspase-3 and endonucleases, ultimately causing DNA fragmentation and cell death [4]. Mitochondria can also secrete an apoptosis-inducing factor (AIF) which acts directly at the nuclear level to induce apoptosis in a caspase-independent manner [7].

The mechanism leading to increased expression of Fas-L by cytotoxic drugs is believed to be mediated by

the tumor suppressor molecule p53 [8]. Consistent with this model, p53 mutations are associated with reduced drug toxicity in panels of tumor cell lines including Burkitt's lymphoma [9], astrocytomas [10], gliomas [11] and melanomas [12]. They have also been linked with drug resistance in tumors such as breast cancer where these mutations represent a strong predictor of relapse and death [13], and have also been associated with resistance to several therapeutic regimens [14, 15, 16].

Neuroblastoma is the most prevalent solid tumor of the peripheral sympathetic nervous system in young children. One distinguishing feature of these tumors is that the caspase-8 gene is frequently silenced through DNA hypermethylation as well as by gene deletion [17, 18]. In light of these findings, it is critical to ascertain whether cytotoxic drugs such as doxorubicin can induce apoptosis in these tumors and, if so, by what mechanisms. In the present study the cytotoxic action of doxorubicin in the human neuroblastoma cell line, SKN-SH, was investigated by analyzing activation of the p53/Fas/caspase-8 system and induction of apoptosis in these cells. Our findings showed that doxorubicin elicits apoptosis in SKN-SH cells in a p53/Fas/caspase-8-independent manner and that mitochondria and the downstream apoptotic intermediate, caspase-3, appear to be the primary targets.

Materials and methods

Chemicals and reagents

The following items were purchased from the companies indicated: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from BioWhittaker (Walkersville, Md.), and doxorubicin and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (St. Louis, Mo.). Antibodies to p53, Fas-L, and Fas-R were from Upstate Biotechnology (Lake Placid, N.Y.). Caspase-8 antibody was generously provided by Dr. Honglin Li, Children's Memorial Hospital, Chicago. Cytochrome *c* antibody was purchased from Pharmingen (San Diego, Calif.). Specific antibody for cleaved caspase-3 was from Cell Signaling Technology (Beverly, Mass.). Beta actin, and AIF antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.); secondary antibodies conjugated to horseradish peroxidase and enhanced chemiluminescence reagents (ECL) were from Amersham (Arlington Heights, Ill.); and Immobilon-P transfer membrane for Western blotting was from Millipore (Bedford, Mass.).

Cell culture and cytotoxicity assay

Neuroblastoma (SKN-SH) cells were maintained in DMEM supplemented with 10% FBS at 37ºC in an atmosphere containing 5% CO_2. The cytotoxic activity of doxorubicin was quantitatively determined by a colorimetric assay utilizing MTT. Cells were seeded at 10^4 cells/well in 96-well plates and maintained in culture for 24 h at 37ºC in DMEM supplemented with 10% FBS. Doxorubicin (10^{-9} to 10^{-5} *M*) was then added to designated wells and the cells incubated for an additional 72 h. MTT (10 µl of 5 mg/ml solution) was added to each well followed by incubation for 4 h at 37ºC. The cells were solubilized in 100 µl 0.5 *N* HCl/isopropanol and incubated for 15 h at 37ºC. The optical density of this solution was measured at 570 nm and cell survival estimated by comparison with untreated control cells.

Measurement of DNA fragmentation

Cells were incubated with doxorubicin (0.5 µ*M*) for 24 h at 37°C and DNA prepared from Triton X-100 lysates for analysis of fragmentation. Briefly, cells were lysed in hypotonic solution containing 10 m*M* Tris HCl (pH 7.4), 1 m*M* EDTA, and 0.2% Triton X-100, and centrifuged at 1,1000 *g* for 5 min. Supernatants were electrophoresed on a 1% agarose gel and the DNA fragments visualized under UV light after staining with ethidium bromide.

Western blot analysis

The cells were seeded in 25-cm^2 flasks containing DMEM supplemented with 10% FBS and incubated at 37ºC. After incubation with doxorubicin, the cells were washed with cold PBS, and the monolayer was solubilized by the addition of 200 µl lysis buffer (50 m*M* HEPES, pH 7.4, 150 m*M* NaCl, 100 m*M* NaF, 1 m*M* $MgCl_2$, 1.5 m*M* EGTA, 10% glycerol, 1% Triton X100, 1 µg/ml leupeptin, and 1 m*M* phenylmethyl sulfonyl fluoride). Insoluble material was removed by centrifugation and the protein concentration of the supernatant determined. The soluble protein fraction (50 µg) was electrophoresed by SDS-PAGE and transferred to an Immobilon-P membrane. P53, Fas-L, Fas receptor, caspase-8, cleaved caspase-3, and β-actin were identified by incubation of the membrane with specific antibodies. Complexes were detected by sequential blotting with biotinylated secondary antibodies linked to peroxidase and reactive bands identified by ECL.

Electron microscopy

SKN-SH cells incubated with doxorubicin (0.5 µ*M* for 24 h), and appropriate controls, were harvested in trypsin. Cell suspensions were centrifuged at 1000 *g* for 5 min at 4ºC, and the culture medium discarded. The cell pellet was immediately fixed in 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide and processed for electron microscopy using conventional techniques. Ultrathin sections stained with lead citrate and uranyl acetate were then examined with a Zeiss-10 A electron microscope (Carl Zeiss, Oberkochen, Germany).

Cytochrome *c* and AIF release

The cells were seeded in 25-cm^2 flasks at 5×10^5 cell/ml in DMEM with 10% FBS and incubated for 24 h. Doxorubicin was then added for an additional 24 h. Cells were harvested in trypsin and the cell suspension centrifuged at 1,000 *g* for 5 min at 4ºC. After washing with ice-cold phosphate-buffered saline, mitochondria were isolated by resuspending the cell pellets in five volumes of ice-cold buffer A (20 m*M* Hepes-KOH, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* sodium EDTA, 1 m*M* sodium EGTA, 1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 µg/ml pepstatin A) containing 250 m*M* sucrose. Cells were lysed by 15–20 passages through a 25-gauge needle, and homogenates centrifuged at 1000 *g* for 5 min at 4ºC. The supernatants were then centrifuged at 10,000 *g* for 15 min at 4ºC, and the resulting mitochondria pellets resuspended in 50 µl lysis buffer. Protein concentration was determined and 50 µg of each specimen were separated on 12% polyacrylamide gel, electrotransferred to Immobilon-P membrane and stained with anti-cytochrome *c* and AIF antibodies.

Results

Cytotoxic potency and apoptotic effects of doxorubicin in SKN-SH cells

Anthracycline derivatives such as doxorubicin are frequently used in the chemotherapy of numerous

human malignancies including neuroblastomas [19, 20]. The effect of doxorubicin on cell viability was determined by incubating SKN-SH cells with increasing concentrations of drug ranging from 10^{-9} to 10^{-5} *M* for 72 h, at which time the percentage of viable cells was determined by MTT assay. Cytotoxicity was concentration-dependent and doxorubicin had an IC_{50} of approximately 5×10^{-7} *M* (Fig. 1A).

The apoptotic response of SKN-SH cells to doxorubicin was determined by measuring DNA fragmentation following cell exposure to increasing concentrations of the drug for 24 h. This treatment caused a concentration-dependent enhancement of DNA fragmentation (Fig. 1B) which correlated with an increase in cytotoxicity (Fig. 1A). These findings suggest that apoptosis may account for doxorubicin-mediated cell death in SKN-SH cells.

Effect of doxorubicin on the p53/Fas/caspase-8 system in SKN-SH cells

Experiments were performed to verify that p53 and/or the Fas pathway were involved in the induction of apoptosis by doxorubicin in SKN-SH cells. Western blot analyses using specific antibodies to p53 and Fas-L revealed that while both molecules were expressed in these cells, their expression was not significantly altered by incubation with doxorubicin (Fig. 2). In contrast, expression of the Fas receptor was not detected in either untreated or doxorubicin-treated SKN-SH cells (Fig. 2). These findings are in agreement with previously reported findings showing that neuroblastoma tumors may express Fas-L but not the corresponding receptor [21].

Western blots performed to detect caspase-8 expression revealed that, as expected, this molecule was not present in SKN-SH cells incubated with or without the drug (Fig. 2). The lack of expression of these two key elements (Fas receptor and caspase-8) of the apoptotic pathway, indicates that the p53/Fas/caspase-8 system does not play a significant role in doxorubicin-induced apoptosis in these cells.

Effect of doxorubicin on the structural integrity of mitochondria in SKN-SH cells

SKN-SH cells incubated with doxorubicin (0.5 μ*M*) for 24 h were analyzed by electron microscopy to determine the effects of this drug on mitochondrial structures. Marked changes consisting of increased matrix density, altered cristae and a reduction in mitochondrial size

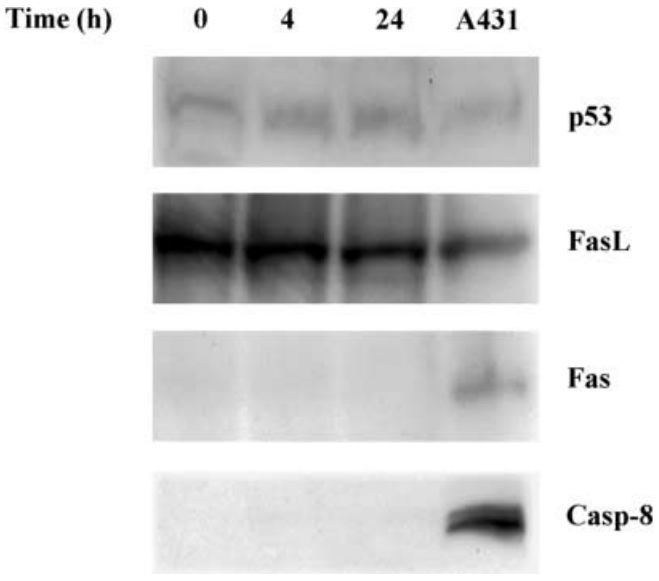

Fig. 2 Effect of doxorubicin on expression of p53, Fas ligand (*FasL*), Fas receptor (*Fas*), and caspase-8 (*Casp-8*) in SKN-SH cells. Cells were incubated with doxorubicin 0.5 μ*M* for the indicated times. Proteins were extracted and separated on 12% SDS-PAGE. Transferred to Immobilon-P membrane, and stained with specific antibodies. The epidermoid carcinoma cell line A431 served as control. Data are representative of three independent experiments

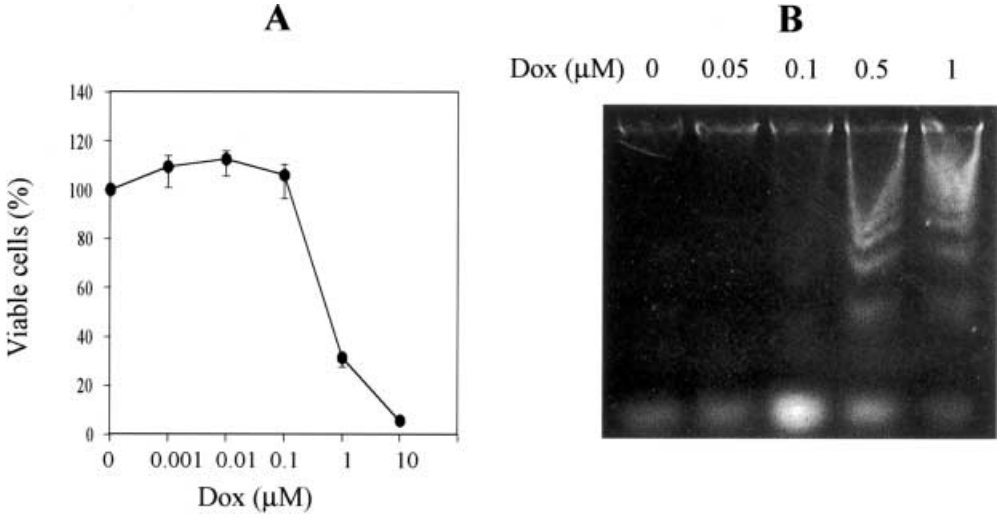

Fig. 1A, B Effect of doxorubicin on cell death and DNA fragmentation. **A** SKN-SH cells were incubated with incremental concentrations of doxorubicin for 72 h. Cell viability was determined by the MTT assay as described in Methods. **B** SKN-SH cells were incubated with doxorubicin for 24 h after which DNA was extracted and separated on 1% agarose gel, stained with ethidium bromide and visualized under UV light

(pyknosis) were observed (Fig. 3). Such structural abnormalities may lead to disruption of the mitochondrial membranes, increasing their permeability and causing the release of cytochrome *c* [22]. A similar type of mitochondrial injury has been observed in cardiomyocytes cultivated in the presence of doxorubicin [23]. These findings suggest that interference with mitochondrial function was the primary causative event in doxorubicin-induced cardiotoxicity. Our findings suggest that mitochondria may serve as an initiation site for doxorubicin-mediated apoptosis in SKN-SH cells.

Cytochrome *c* release and caspase-3 activation as determinants of doxorubicin cytotoxicity in SKN-SH cells

Apoptosis elicited by mitochondrial dysfunction has been shown to be initiated by an efflux of cytochrome *c* from the inter-membrane space of mitochondria into the cytosolic compartment [24, 25]. Incubation of SKN-SH cells with doxorubicin for 24 h induced a concentration-dependent release of cytochrome *c* from mitochondria (Fig. 4A). In contrast, doxorubicin treatment had no effect on the release of AIF from mitochondria, suggesting that the cytochrome *c*-related apoptotic pathway may be prevalent in this cellular model. Cytochrome *c* has been shown to activate caspase-9, which in turn cleaves and activates caspase-3, a downstream molecular intermediate in the apoptotic pathway. Caspase-3 activity in response to doxorubicin treatment was measured by detection of cleaved caspase-3 using a specific antibody. Doxorubicin treatment stimulated the activation of caspase-3 in a concentration-dependent manner implicating this apoptotic pathway in mediating doxorubicin action in these cells (Fig. 4B).

Discussion

The putative mechanism by which doxorubicin may induce apoptosis in the human neuroblastoma cell line SKN-SH, which lacks expression of caspase-8 but is sensitive to this drug, was investigated. Although incubation of SKN-SH cells with doxorubicin caused a strong cytotoxic effect (Fig. 1A) and DNA fragmentation (Fig. 1B), it did not alter the expression of p53 or

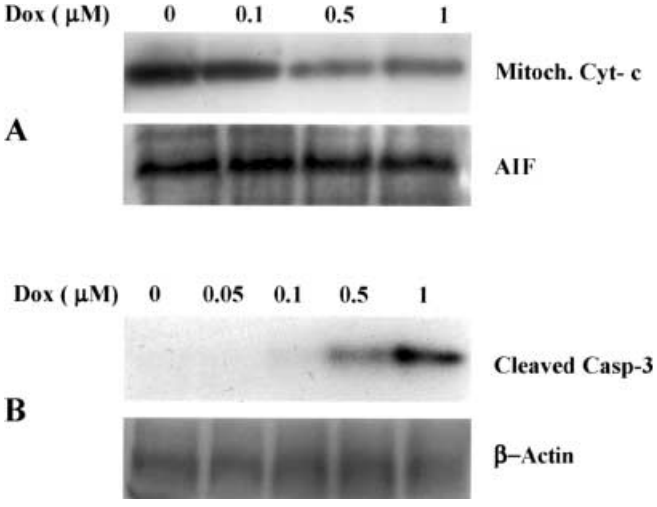

Fig. 4A, B Effect of doxorubicin treatment on cytochrome *c* and AIF release from mitochondria, and on caspase-3 activity. SKN-SH cells were exposed to doxorubicin at the indicated concentrations for 24 h. **A** Mitochondria were isolated and their content of cytochrome *c* (*Mitoch. Cyt-c*) and AIF (*AIF*) were analyzed by Western blotting using specific antibodies. **B** Expression of activated caspase-3 was measured in total cell lysate by Western blotting using specific antibody for the cleaved caspase-3. *β*-Actin was used as loading control

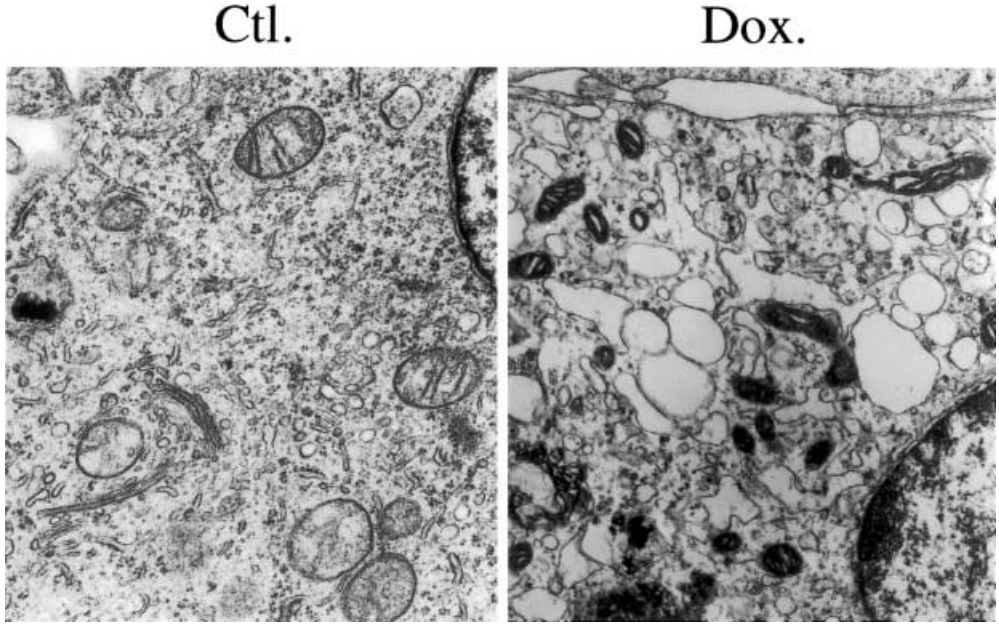

Fig. 3 Effect of doxorubicin on mitochondrial structure. Representative electron micrographs show structural alterations at the cytoplasmic and the mitochondrial level following exposure of SKN-SH cells to doxorubicin (*Dox.*) as compared to control cells (*Ctl.*). Note that the mitochondrial structure is changed after drug treatment. Both photographs were taken under the same magnification (×7000)

Fas ligand (Fig. 2). More significantly, Fas receptor, like caspase-8, was not detected in these cells, nor did incubation with doxorubicin modify this lack of expression. These findings suggest that an alternative mechanism to the Fas/caspase-8 system must be functional in mediating doxorubicin-induced apoptosis in these cells.

Although the majority of cytotoxic drugs are believed to induce cellular apoptosis through activation of death receptor signaling pathways, evidence has recently emerged indicating that cytotoxic drug-mediated apoptosis may not depend solely on the Fas pathway. Doxorubicin-induced apoptosis in human T-cell leukemia has been found to be mediated by caspase-3 activation in a Fas-independent manner [26]. Human glioma cells selected for resistance to Fas-L do not acquire resistance to chemotherapy [27]. However, the general caspase inhibitor, ZVAD-fmk, inhibits drug-induced cell death suggesting a role for caspases but not Fas, in drug-induced apoptosis in glioma cells [27].

The roles of mitochondria and downstream apoptotic signaling molecules as putative transducers of doxorubicin's cytotoxic activity were evaluated in this study. Electron microscopic analysis revealed that exposure of SKN-SH cells to doxorubicin caused extensive structural damage to mitochondria (Fig. 3). Further, we demonstrated that cytochrome *c* was released from mitochondria following incubation of SKN-SH cells with doxorubicin (Fig. 4). It is not known whether doxorubicin-induced cytochrome *c* release occurs via a direct action on the mitochondrial transporter or on the respiratory pathway leading to disruption of mitochondrial permeability as reported previously [28]. The effect of doxorubicin on caspase-3, a downstream signaling intermediate of cytochrome *c* [29], was investigated. Doxorubicin treatment of SKN-SH cells induced activation of caspase-3 in a concentration-dependent manner.

Collectively, these results indicate that doxorubicin induces apoptosis in SKN-SH cells by initially acting at the mitochondrial level leading to subsequent activation of the caspase cascade. Determination of molecular alterations caused by chemotherapeutic drugs at the cellular level represents a crucial step in identifying new signaling targets that may be useful in the design of a new generation of drugs to enhance the efficacy of classical cytotoxic drugs. Understanding these signaling perturbations may help predict potential cellular adaptation mechanisms to chemotherapeutic agents. The findings of this study suggest that targeting mitochondria or downstream apoptotic molecules such as caspase-9 or caspase-3 may enhance the activity of doxorubicin in neuroblastomas.

Acknowledgements The authors would like to thank Ms Roberta Gerard for her assistance in editing the manuscript. This study was supported by grants from the Illinois Public Aid (A.R.), the Anderson Foundation (B.L.M.), and the Medical Research Institute Council (B.L.M.).

References

1. Friesen C, Herr I, Krammer PH, Debatin KM (1996) Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 2:574
2. Friesen C, Fulda S, Debatin KM (1997) Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. Leukemia 11:1833
3. Friesen C, Fulda S, Debatin KM (1999) Cytotoxic drugs and the CD95 pathway. Leukemia 13:1854
4. Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem J 326(1):1
5. Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491
6. Kim TH, Zhao Y, Barber MJ, Kuharsky DK, Yin XM (2000) Bid-induced cytochrome *c* release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. J Biol Chem 275:39474
7. Lorenzo HK, Susin SS, Penninger J, Kroemer G (1999) Apoptosis inducing factor (AIF): a philogenetically old, caspase-independent effector of cell death. Cell Death Differ 6:515
8. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR (1998) DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell 1:543
9. Fan S, el Deiry WS, Bae I, Freeman J, Jondle D, Bhatia K, Fornace AJ Jr, Magrath I, Kohn KW, O'Connor PM (1994) p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res 54:5824
10. Iwadate Y, Tagawa M, Fujimoto S, Hirose M, Namba H, Sueyoshi K, Sakiyama S, Yamaura A (1998) Mutation of the p53 gene in human astrocytic tumours correlates with increased resistance to DNA-damaging agents but not to anti-microtubule anti-cancer agents. Br J Cancer 77:547
11. Iwadate, Y, Fujimoto S, Tagawa M, Namba H, Sueyoshi K, Hirose M, Sakiyama S (1996) Association of p53 gene mutation with decreased chemosensitivity in human malignant gliomas. Int J Cancer 69:236
12. Li G, Tang L, Zhou X, Tron V, Ho V (1998) Chemotherapy-induced apoptosis in melanoma cells is p53 dependent. Melanoma Res 8:17
13. Kovach JS, Hartmann A, Blaszyk H, Cunningham J, Schaid D, Sommer SS (1996) Mutation detection by highly sensitive methods indicates that p53 gene mutations in breast cancer can have important prognostic value. Proc Natl Acad Sci U S A 93:1093
14. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L (1995) Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med 1:1029
15. Aas T, Borresen AL, Geisler S, Smith-Sorensen B, Johnsen H, Varhaug JE, Akslen LA, Lonning PE (1996) Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. Nat Med 2:811
16. Clahsen PC, van de Velde CJ, Duval C, Pallud C, Mandard AM, Delobelle-Deroide A, van den BL, Sahmoud TM, van de Vijver MJ (1998) p53 protein accumulation and response to adjuvant chemotherapy in premenopausal women with node-negative early breast cancer. J Clin Oncol 16:470
17. Teitz T, Wei T, Valentine MB, Vanin EF, Grenet J, Valentine VA, Behm FG, Look AT, Lahti JM, Kidd VJ (2000) Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 6:529
18. Takita J, Yang HW, Bessho F, Hanada R, Yamamoto K, Kidd V, Teitz T, Wei T, Hayashi Y (2000) Absent or reduced expression of the caspase 8 gene occurs frequently in neuroblastoma, but not commonly in Ewing sarcoma or rhabdomyosarcoma. Med Pediatr Oncol 35:541

19. Carter SK (1975) Adriamycin – a review. J Natl Cancer Inst 55:1265
20. Cheung NV, Heller G (1991) Chemotherapy dose intensity correlates strongly with response, median survival, and median progression-free survival in metastatic neuroblastoma. J Clin Oncol 9:1050
21. Koizumi H, Ohkawa I, Tsukahara T, Momoi T, Nakada K, Uchikoshi T (1999) Apoptosis in favourable neuroblastomas is not dependent on Fas (CD95/APO-1) expression but on activated caspase-3 (CPP32). J Pathol 189:410
22. Praet M, Pollakis G, Goormaghtigh E, Ruysschaert JM (1984) Damages of the mitochondrial membrane in Adriamycin treated mice. Cancer Lett 25:89
23. Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB (2001) Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. Cancer Res 61:771
24. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 185:1481
25. Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281:1309
26. Gamen S, Anel A, Lasierra P, Alava MA, Martinez-Lorenzo MJ, Pineiro A, Naval J (1997) Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way. FEBS Lett 417:360
27. Glaser T, Wagenknecht B, Groscurth P, Krammer PH, Weller M (1999) Death ligand/receptor-independent caspase activation mediates drug- induced cytotoxic cell death in human malignant glioma cells. Oncogene 18:5044
28. Doroshow JH (1983) Anthracycline antibiotic-stimulated superoxide, hydrogen peroxide, and hydroxyl radical production by NADH dehydrogenase. Cancer Res 43:4543
29. Reed JC, Paternostro G (1999) Postmitochondrial regulation of apoptosis during heart failure. Proc Natl Acad Sci U S A 96:7614