5-Fluorouracil-induced Death of Jurkat T-Cells – A Role for Caspases and MCL-1

DINA M. ARESVIK[1], ROLF D. PETTERSEN[2], TORE G. ABRAHAMSEN[3] and MARIANNE S. WRIGHT[2]

[1]*Department of Paediatric Research, University of Oslo and Oslo University Hospital, Rikshospitalet, N-0027 Oslo, Norway;*
[2]*Department of Paediatric Research, Oslo University Hospital, Rikshospitalet, N-0027 Oslo, Norway;*
[3]*Department of Paediatrics, Oslo University Hospital, Rikshospitalet and University of Oslo, N-0027 Oslo, Norway*

Abstract. *5-Fluorouracil (5-FU) is frequently used in cancer treatment. Previous studies with 5-FU suggest that proapoptotic protein BAX and tumor suppressor protein TP53 are central factors in this process. As the leukemic T cell line Jurkat E6 has mutations in both these genes, we investigated a possible activation of alternative death pathways following 5-FU treatment. Here we show that 5-FU triggers apoptosis in Jurkat cells in a dose-dependent manner. Death responses were only moderately attenuated in the presence of a general caspase inhibitor. However, flow cytometric analysis showed activation of caspase 3 and a slight increase in ROS generation in a time- and dose-dependent manner. Furthermore, we observed 5-FU induced PARP cleavage and notably, reduced expression of antiapoptotic MCL-1L associated with the appearance of proapoptotic MCL-1S. Our results demonstrate the activation of alternative death pathways following treatment with 5-FU, despite mutations in the TP53 and BAX genes.*

Apoptosis, or programmed cell death, is essential for tissue development and homeostasis (1, 2), while deregulation of apoptosis may contribute to diseases such as cancer, autoimmunity and degenerative diseases (3). Apoptosis can be triggered by a number of factors, such as ligation of death receptors, growth factor withdrawal, ultraviolet- or γ-irradiation and chemotherapeutic drugs (4). Cysteine proteases or caspases, which play a central role in the regulation and execution of apoptotic cell death (4, 5), can be activated by two distinct, but interconnected signaling cascades: either the extrinsic receptor mediated pathway which is triggered by death receptor ligands or the intrinsic, mitochondrial pathway which is initiated by cellular stress (6-8).

Cellular stress, such as DNA damage, provokes alterations in mitochondrial functions (6, 7). These alterations are initiated by the breakdown of the mitochondrial membrane potential (MMP) and the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. Released cytochrome *c* then associates with the adaptor protein APAF1, procaspase-9 and dATP, forming the apoptosome. Apoptosome formation results in the activation of caspase-9, as well as other downstream caspases such as caspase-3, -6 and -7 (8-10).

The mitochondrial death pathway is regulated by pro- and antiapoptotic BCL-2-like as proteins. These proteins share one or more BCL-2 homology (BH) domains (8, 11). The proapoptotic multi-domain proteins BAX and BAK are required for the mitochondrial outer membrane permeabili-zation (MOMP) to occur during apoptosis, leading to cytochrome *c* release from mitochondria. BAX and/or BAK can also be activated by other BCL-2 family proteins, the BH3-only proteins, such as BID and BIM. In contrast, antiapoptotic BCL-2 family proteins, such as BCL-2, MCL-1 and BCL-xL, inhibit apoptosis. Other BH3-only proteins, such as PUMA, NOXA and BAD, antagonize the antiapoptotic proteins and sensitize cells towards cell death (6, 8, 11).

Most anticancer drugs target DNA (12), and in response to DNA damage the cellular tumor suppressor TP53 is activated (13, 14). This transcription factor binds then to specific target sequences on DNA and can thereby activate or repress the expression of a large number of proapoptotic genes, such as *BAX, NOXA* and *PUMA* (14). The anticancer drug 5-fluorouracil (5-FU) is a thymidylate synthase inhibitor, which can activate TP53 by several mechanisms (15).

Cancer cells, however, frequently harbor mutations in key apoptosis genes which make these cells resistant to cell death. The leukemic Jurkat E6 T-cell line has mutations in

Correspondence to: Dina M. Aresvik, Department of Paediatric Research, University of Oslo and Oslo University Hospital, Rikshospitalet, N-0027 Oslo, Norway. Tel: +47 23072986, Fax: +47 23072780, e-mail: Dina.Aresvik@rr-research.no

Key Words: Apoptosis, MCL-1, 5-fluorouracil, Jurkat, T cells.

both *TP53* and *BAX* genes. For *TP53*, Jurkat cells display four mutations in the DNA-binding domain (DBD), one of them giving a truncated protein (16). Mutations of *TP53* affecting the DBD may abolish the transactivation of TP53-responsive genes and affect the cytoplasmic functions of p53 (17-19). Mutant *TP53* can behave in a dominant-negative way when expressed together with wild-type *TP53* (19, 20). It has also been shown that some *TP53* mutations can lead to a gain of function in some pathways (20, 21). In hematological malignancies, *TP53* mutations are associated with unfavorable prognostic factors and resistance to chemo-therapy (22). Single base deletions and additions in a polyguanine tract located within the open reading frame of the *BAX* gene lead to a total absence of the protein (23). The absence/non-function of two defective crucial components, necessary for 5-FU-induced cell death, might suggest that Jurkat cells would be insensitive to 5-FU. Previous results on 5-FU-induced cell death in Jurkat cells are controversial. Guchelaar *et al*. showed that Jurkat cells, incubated with 5-FU, appeared to be relatively resistant and only very low numbers of apoptotic and necrotic cells could be detected when incubated with 10-100 μM 5-FU (24). Gruber *et al*., however, concluded that treatment with 10 μM 5-FU readily triggered apoptosis in Jurkat cells using other types of analysis (25). We wanted therefore to clarify if 5-FU can induce apoptosis in Jurkat E6 T-cells using a broad specter of methods and examine the impact of 5-FU on cell death mechanisms.

Materials and Methods

Cell cultures. Jurkat E6 cells obtained from American Type Culture Collection (ATCC, Mannassas, VA, USA) were maintained in RPMI-1640 medium (Cambrex Bio Science, Belgium), containing penicillin/streptomycin (Pen-Strep, Lonza, Switzerland) and 10% fetal bovine serum (Invitrogen Corp, USA). The cells were incubated at 37˚C in a humidified 5% CO_2 atmosphere. Cells were incubated with 5-FU (1, 10, 50 or 100 μM) (Flurablastin, Pfizer). Etoposide (ETO, 10 μM; Mayne Pharma Plc) and staurosporine (STS, 1 μM; Sigma Aldrich) were used as positive controls of apoptosis. In some experiments, cells were pretreated for 1 hour with Z-VAD-FMK (100 μM, Calbiochem), dissolved in 0.5% dimethylsulfoxide (DMSO).

Determination of cell death. Cell death was determined by staining with Annexin-V-Fluos (Roche, Germany) and propidium iodide (PI). Cells (2.5×10^5 cells/ml) were incubated in 24-well cell culture plates (Corning Inc, NY, USA) for 24, 48 or 72 hours, washed with phosphate-buffered saline (PBS) and binding buffer (10mM HEPES, 140 mM NaCl, 5mM $CaCl_2$, pH 7.4) and finally resuspended in binding buffer. Annexin-V-Fluos staining was performed according to the manufacturer's instructions. Cells were incubated for 10 min in darkness on ice followed by PI addition. Samples were then analyzed using a FACSCalibur (Becton Dickinson). Data were collected on 10,000 cells and analyzed using CXP Analysis software (Beckman Coulter).

Measurement of caspase-3 activity. Caspase-3 activity was measured by flow cytometry, using a phycoerythrin (PE)-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences, USA) according to the manufacturer's instruction. Briefly, cells were washed once in PBS, fixed and permeabilized using Cytofix/Cytoperm Fixation and Permeabilization solution for 20 min on ice, pelleted and finally washed with Perm/Wash buffer. Cells were then stained with PE-conjugated rabbit monoclonal antibody, raised against active caspase-3, for 30 min at room temperature in the dark. Subsequently, the cells were washed in Perm/Wash buffer, resuspended in Perm/Wash buffer and viable cells were analyzed as described above. Normal rabbit IgG-PE (Santa Cruz Biotechnology) was used as a negative control.

Measurement of mitochondrial membrane potential. 3, 3'-Dihexyloxacarbocyanine iodide ($DiOC_6$ (3), (Invitrogen, USA) was used to measure changes in the MMP. In brief, cells were incubated with $DiOC_6$ (3) (40 nM in PBS) for 15 min at 37˚C and then washed once with PBS and the viable cells were analyzed as described above.

Measurement of ROS generation. Dihydroethidium (HE) (Molecular Probes, USA) was used to measure mitochondrial ROS generation. Cells were incubated with 2 μM HE for 15 min at 37˚C, washed once with PBS, and the viable cells were analyzed as described above.

Western blot analysis. After the cells had been exposed to 5-FU, ETO or STS for the indicated time, they were collected, washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (25 μl/1×10^6 cells, (Cell Signaling Technology, USA)), including 1mM phenyl-methylsulfonic fluoride (PMSF) and Protease Inhibitor Cocktail (Complete Mini, Roche, Germany) for 30 min. Lysates were centrifuged for 10 min at 16000×*g*, the supernatants transferred to sterile vials and stored at –76˚C. Protein concentration of the samples was determined using a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, USA) and the Ascent Software for Multiscan Ascent. Equal amounts of proteins (30-40 μg) were separated by electrophoresis in a 15% polyacrylamide gel at 200 V for 60 min and blotted onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, UK) at 100 V for 2 h. After washing the membrane with tris buffered saline including 0.1% Tween-20 (TBS-T), non-specific binding was blocked during a 1 hour incubation period at room temperature with TBS-T containing 5% ECL blocking agent (Amersham Biosciences), washed with TBS-T and finally, the blots were incubated with primary antibodies against the proteins-of-interest overnight at 4˚C. The membranes were then washed and incubated with horseradish peroxidase-linked secondary antibody (Amersham Biosciences) for 1 h at room temperature. Protein bands were visualized by incubating the membrane in Western blotting detection reagents (Amersham Biosciences) for 30 seconds. Antibodies against Ezrin (Santa Cruz Biotechnology) and G3PDH (Trevigen, USA) were used as an internal loading control. The primary antibody against BAX was purchased from BD Pharmingen, antibody against MCL-1 (S-19) was obtained from Santa Cruz Biotechnology, and the antibody against cleaved PARP was purchased from Cell Signaling Technology.

Statistics. Data in graphs are expressed as the means+SD. For determination of statistical significance, Graph Pad Prism (Graph Pad Software, USA) was used for two-way ANOVA with Bonferroni post-test. Otherwise, representative data are shown.

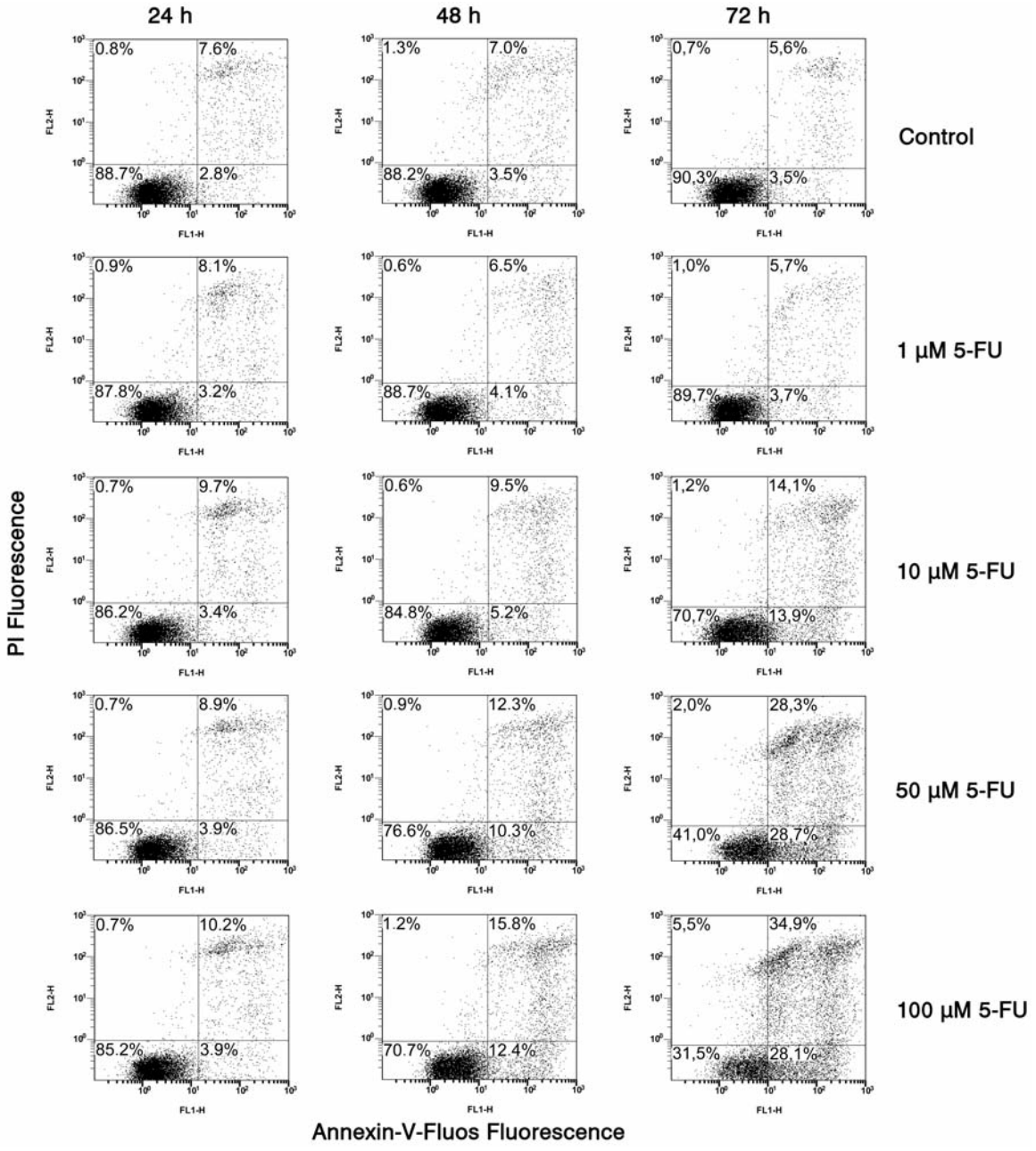
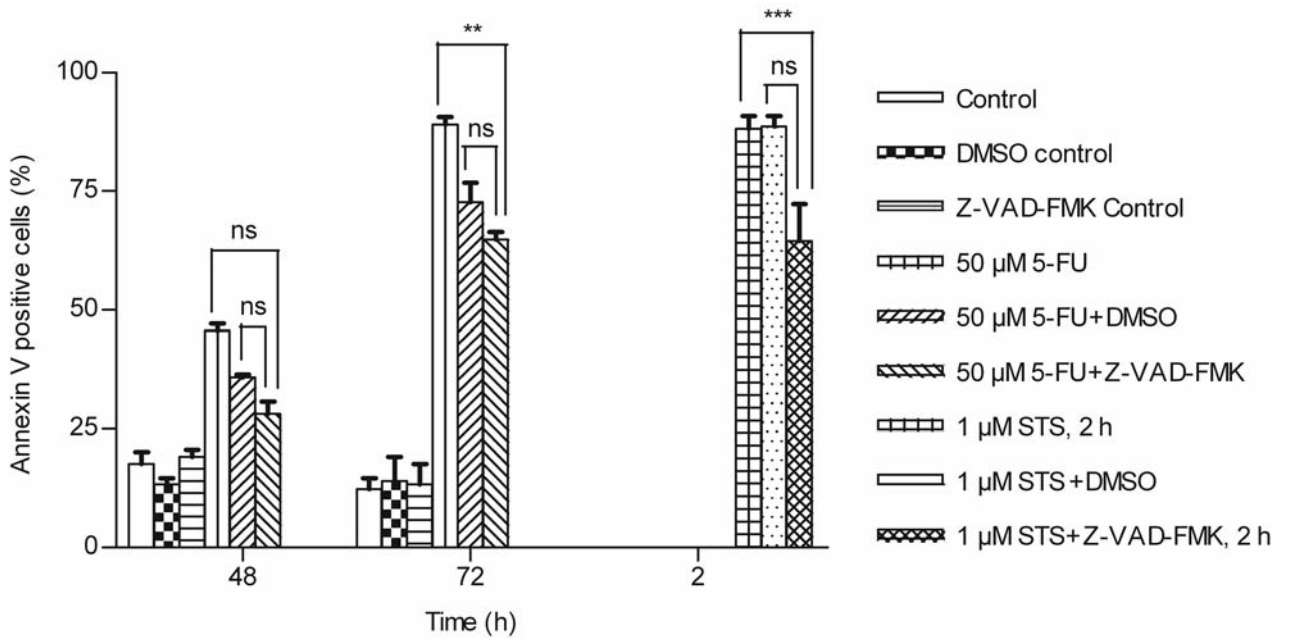


Figure 1. *Flow cytometry analysis of 5-FU induced apoptosis in Jurkat E6 cells. Cells were incubated with 5-FU (1-100 µM) for different time periods (24-72 h) and analyzed by flow cytometry. Living cells, early apoptotic cells and late apoptotic/necrotic cells are found in the bottom left quadrant, bottom right and top right quadrant respectively. One representative experiment out of 3 is shown.*

Results

Earlier investigations had shown that Jurkat E6 T-cells have mutations in *TP53* gene, resulting in a truncated protein and a mutation in a *BAX* gene, resulting in the total absence of the protein. To verify *BAX* expression in our cells, we first performed Western blot analysis, which could confirm that Jurkat cells do not express *BAX* (data not shown).

To investigate if 5-FU induces apoptosis in Jurkat E6 T-cells, we first performed FACS analysis using Annexin-V-Fluos and PI. During apoptosis, phosphatidylserine (PS) translocates from the inner part of the plasma membrane to the outer layer and becomes exposed on the external surface of the cell. Annexin-V has a high affinity for PS and is therefore suited to detect apoptotic cells. In conjunction with the permeability probe PI, a distinction can be made between



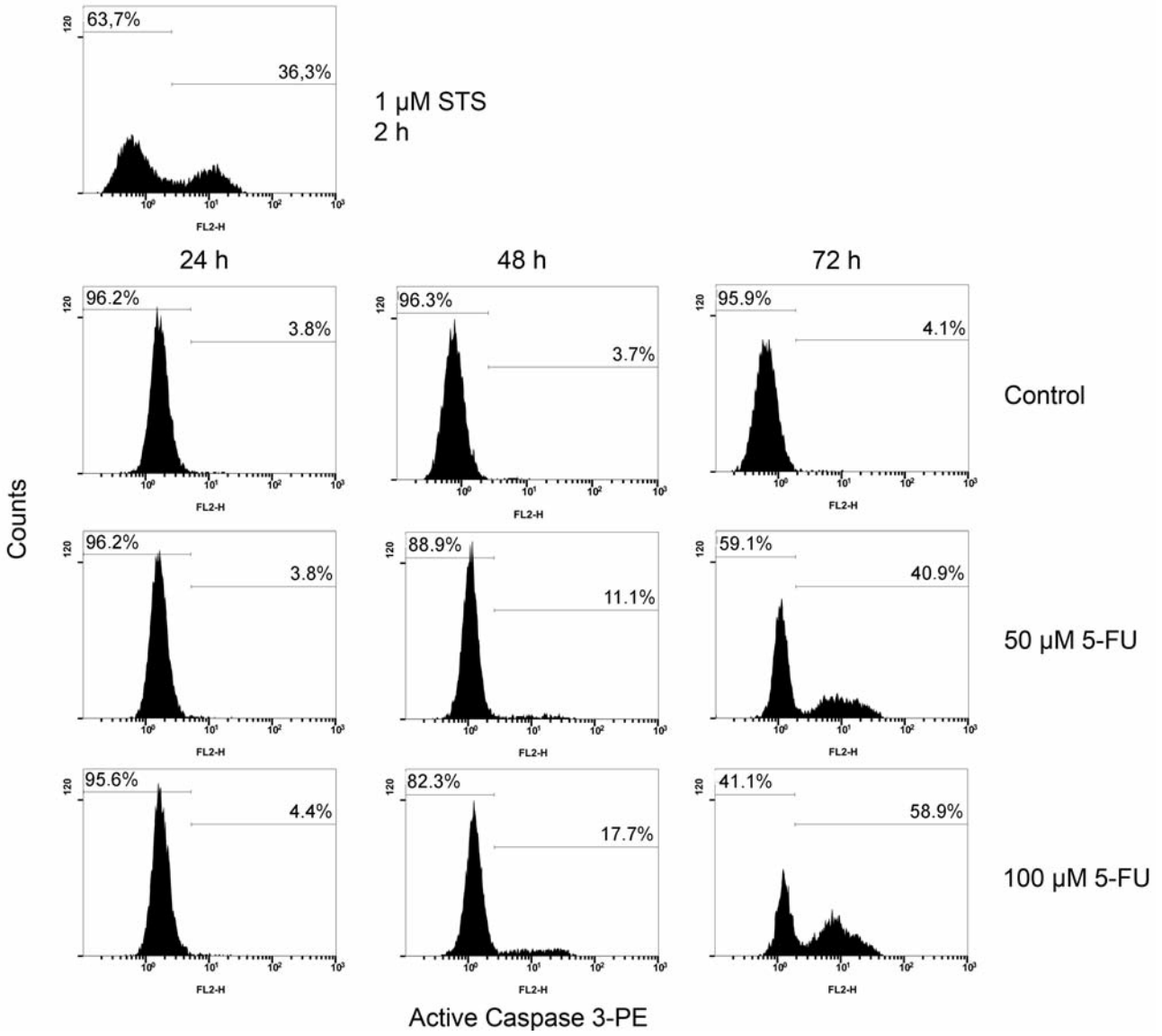
Figures 2. *Effect of Z-VAD-FMK on 5-FU induced cell death in Jurkat E6 cells. Cells were pretreated with 100 µM Z-VAD-FMK for 1 h before 5-FU treatment (50 µM) for the indicated time periods. A DMSO control (0.5%) was included. Cells were then stained with Annexin-V-Fluos and analyzed by flow cytometry. Each bar represents the mean+SD of 2 independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. (**) p<0.01 and (***) p<0.001.*

apoptotic cells with intact plasma-membrane integrity and late apoptotic/necrotic cells with leaky plasma membrane (26, 27). Thus, early apoptotic cells stain with Annexin-V but exclude PI, while late apoptotic and necrotic cells are both Annexin-V and PI positive (28). Assessments by flow cytometry showed that incubation with 5-FU did trigger cell death in Jurkat E6 T-cells in a dose- and time-dependent manner. Death responses were slow compared to STS-induced cell death. Maximal response to 5-FU was observed within 72 hours. The proportion of living cells after treatment with 100 µM 5-FU for 72 hours ranged from 3.6% to 31.5%, control cells showed a viability between 84.3% and 90.3%. No response was observed with 1 µM 5-FU (Figure 1).

Caspases are central mediators of apoptosis (4). To determine if caspases were involved in 5-FU-induced cell death, we first examined the effect of Z-VAD-FMK, a pan caspase inhibitor, on death responses. Since Z-VAD-FMK was dissolved in 0.5% DMSO which may be toxic for cells, we also included a DMSO control. Using flow cytometry analysis, we observed no significant inhibition with Z-VAD-FMK compared to DMSO control of 5-FU-treated cells. However, compared to 5-FU-treated cells, the inhibition of cell death with Z-VAD-FMK was significant (Figure 2).

To further investigate caspase activity in 5-FU-induced cell death, we examined the activation of caspase-3. Active caspase 3, a marker for cells undergoing apoptosis, consists of a heterodimers of 17- and 12-kDa fragments, derived from the 32 kDa proenzyme (29). It is therefore possible to demonstrate caspase-3 activity by flow cytometry using antibodies raised against an epitope corresponding to active human caspase-3 (26). Our flow cytometry results show that 5-FU-activated caspase-3 in a time- and dose-dependent manner but considerably slower than in cultures with 1 µM STS (Figure 3). The response was first detected after 48 hours incubation with 50 µM 5-FU and maximal response was observed after incubation with 100 µM 5-FU for 72 hours (Figure 3). Normal rabbit IgG-PE was used as a negative control for caspase-3 staining (data not shown) and 1 µM STS was used as a positive control (Figure 3).

To demonstrate the functional consequence of activated caspase-3, we next analyzed the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP). PARP, a nuclear enzyme involved in DNA repair, is a target for caspases during apoptosis, and the specific cleavage by caspase-3 from its 116 kDa form to fragments of 89 and 24 kDa, is considered a marker of apoptosis (30, 31). Using Western blot analysis, we observed a clear PARP cleavage fragment of 89 kDa, occurring after 48 hours and much stronger at 72 hours after incubation with 50 µM 5-FU; similar results were observed after ETO stimulation for 16 hours and STS stimulation for 2 hours (Figure 4).

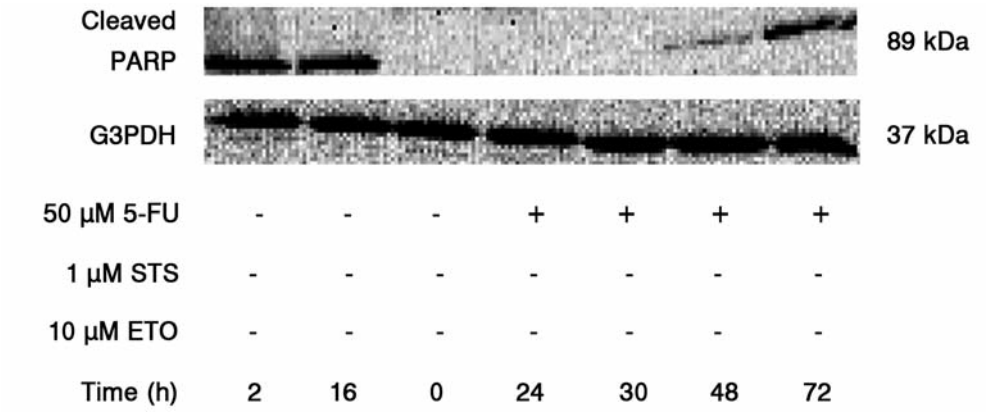


Figures 3. *Flow cytometry analysis of caspase-3 activity. Cells were permeabilized, fixed and stained for active caspase-3 and analyzed by flow cytometry. 1 μM STS was used as a positive control. One representative experiment out of three is shown.*

Since mitochondrial membrane permeabilization is an early event in the intrinsic apoptotic pathway (9, 32), we also analyzed the effect of 5-FU on the MMP using DiOC6(3) staining. However, following treatment of Jurkat cells with 5-FU (1, 10, 50 μM or 100 μM), we did not observe any changes in the MMP within 48 hours and when gaited forward and sideward scatters on viable, normal-sized cells (32, 33); 1 μM STS was used as a positive control (data not shown).

The respiratory chain in the mitochondria is the major site for superoxide formation (34). Increased production of reactive oxygen species (ROS) can cause cell dysfunction and death (9, 35). We analyzed the impact of 5-FU on ROS production using HE staining and flow cytometry. HE is oxidized by superoxide anions to ethidium bromide and emits a red fluorescence in the oxidized state (33). Jurkat cells were incubated with 5-FU (1, 10, 50 and 100 μM) for 24-48 hours and labeled with HE. Gating forward and sideward scatters on viable, normal-sized cells, we observed a slight increase in ROS production after 48 hours of incubation with 50 μM and 100 μM 5-FU (Figure 5).

MCL-1 is a BCL-2 family member that was identified as an early induction gene during differentiation of a myeloid leukemia cell line. Originally, MCL-1 was described as an antiapoptotic protein which promotes tumorigenesis, protects



Figures 4. *Western blot analysis of cleaved PARP was performed using total cell lysates of Jurkat E6 cells which were incubated with 50 μM 5-FU for the indicated time periods. 1 μM STS and 10 μM ETO were included as positive controls. G3PDH was used as a loading control. One representative experiment out of three is shown.*

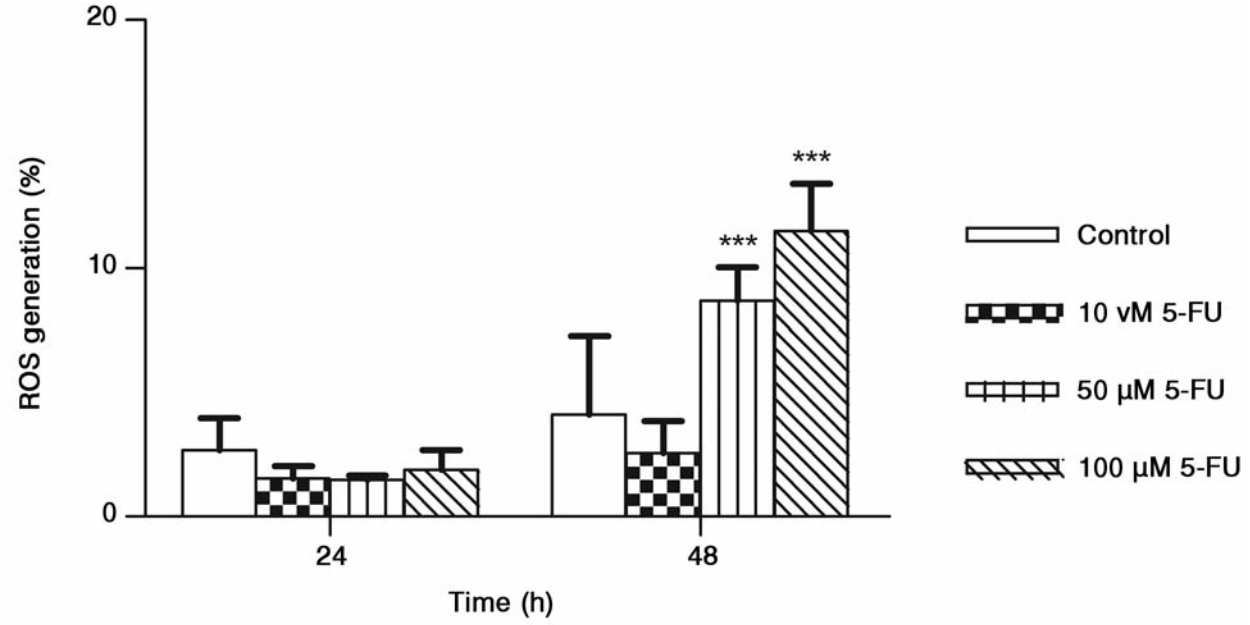


Figure 5. *5-FU-induced intracellular ROS production. Cells were incubated with 5-FU (10-100 μM) for indicated time periods and stained with 2 μM HE for 15 min and analyzed in FL-2. Each bar represents the mean+SD of three independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. ***p<0.001.*

against apoptosis, and confers drug resistance on malignant cells. However, MCL-1 undergoes a complex transcriptional, post-transcriptional and post-translational regulation process. This regulation not only modifies MCL-1 expression, but also its function (36-38). To investigate if 5-FU-induced cell death in Jurkat E6 T-cells was mediated by MCL-1, cells were treated with 50 μM 5-FU for 24-72 hours and analyzed by Western blot. Interestingly, we observed that MCL-1L expression was significantly reduced within 24-72 hours following the addition of 50 μM 5-FU. This decrease was associated with the appearance of a shorter form of MCL-1, an approximately 28 kDa protein, which was first detected at 48 hours and more abundantly at 72 hours. Similar processing of MCL-1 was also seen when apoptosis was induced with 1 μM STS (2 hours) or with 10 μM ETO (16 hours) (Figure 6).

Discussion

As Jurkat E6 T-cells have mutations in both *TP53* and *BAX* genes, it was of interest to determine their response to treatment with 5-FU and delineate the nature of death progression.

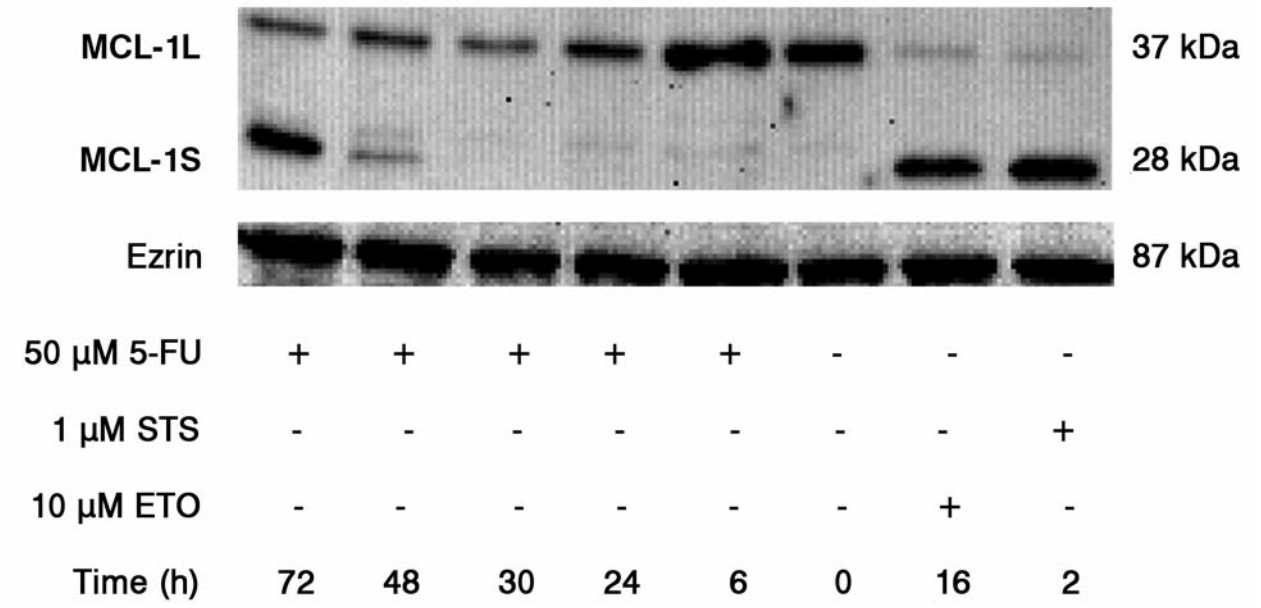


Figure 6. *Western blot analysis of MCL-1 was performed with total cell lysates of Jurkat E6 cells after incubation with 50 µM 5-FU for the indicated time periods. 1 µM STS and 10 µM ETO were included as positive controls. Ezrin was used as a loading control. One representative blot out of three experiments is shown.*

In the present study, we demonstrate the effects of 5-FU on the Jurkat cell line *via* Annexin-PI staining, caspase-3 activation and PARP cleavage. Using Annexin-PI, we observed that cells apparently proceeded through an apoptotic stage and died in a time- and dose-dependent manner, yet the response was much slower compared to STS.

Experiments with the pan caspase inhibitor Z-VAD-FMK showed that cell death was not significantly blocked. A possible explanation for this is that an inhibition of the caspase cascade may not always prevent cell death. Z-VAD-FMK can modulate cell death in different ways, *e.g.* the addition of Z-VAD-FMK can block apoptotic cell death while sensitizing cells to necrotic or autophagic cell death (39, 40). However, caspase involvement was supported by the 5-FU-induced appearance of active caspase-3 followed by the appearance of an 89 kDa PARP cleavage product, shown by Western blot. These observations suggest that 5-FU may activate caspases in Jurkat E6 cells, but that cell death may also occur independent of caspase activation.

Contrary to a previous report (25), we were unable to observe any changes in MMP following 5-FU treatment using $DiOC_6$ (3). When viable cells are incubated with probes for measuring the MMP, the probes accumulate in mitochondria and the efflux of the fluorochrome, measured by the intensity of the cellular fluorescence, reflects changes in MMP (26). It has been reported that once released by depolarized mitochondria, $DiOC_6$ (3) can redistribute in other intracellular membranes such as those of the endoplasmatic reticulum. This may result in a small change in the total cellular fluorescence (41). However, the specificity of this fluorochrome as a mitochondrial probe is greater when it is used at low concentrations (28) and in order to investigate changes in MMP, we followed this recommendation.

Mitochondria can generate and release highly toxic ROS which can contribute to cell death (42). In our experiments, we observed that ROS production was slightly increased when gated the forward and sideward scatters on viable, normal-sized cells (32, 33).These results suggest that mitochondria can be involved to some extent in 5-FU- induced apoptosis in Jurkat cells, at least by producing ROS.

Following 5-FU treatment of Jurkat E6 cells, Western blots revealed a shorter form of MCL-1 48 hours after the onset of the treatment. In the positive apoptosis controls, STS and ETO, the same effect was seen but after a shorter time span.

Michels *et al*. (43) found that caspase-3 can cleave human MCL-1 at two aspartic acid residues, Asp^{127} and Asp^{157}. Cleaved forms of MCL-1 are missing parts of the *N*-terminal but contain still the BH3, BH1 and BH2 domains, as well as the transmembrane domain. In dying cells, the antiapoptotic function of MCL-1 was diminished by a caspase-mediated cleavage which generated a potent proapoptotic protein $MCL\text{-}1^{128\text{-}350}$ (43). For Western blots, we used an anti-MCL-1 (S-19) antibody that was raised against a peptide containing amino acid 121-139 from human Mcl-1; this antibody detects therefore both the uncleaved 37 kDa MCL-1L and the 28 kDa

Δ127-MCL-1 fragment. Amino acid 121-139 are lost in the Δ157-MCL-1 fragment (43) and the antibody was therefore not able to detect this fragment.

Moreover, MCL-1 can exist in the cell as two isoforms: again as a longer antiapoptotic (MCL-1L) and a shorter, alternatively spliced proapoptotic protein (36, 44, 45). The survival of a cell is apparently dependent on a delicate balance between the two forms. The spliced short variant of MCL-1 contains only a BH3 domain due to the elimination of exon 2 and a subsequent shift in the open reading frame (44, 45). The two spliced forms, form heterodimers and are able to neutralize each other (36, 44). Also this alternative spliced form is recognized by the anti-MCL-1 antibody (S-19).

Both the alternatively spliced form and the caspase 3-cleavage product of MCL-1 (MCL-1S) seem to have proapoptotic properties (36). Therefore, the appearance of a shorter form of MCL-1 during 5-FU treatment of Jurkat cells probably overcomes an early resistance to 5-FU, shifts the outcome towards cell death and resulting at the same time in a decreasing amount of full length MCL-1L protein. Since the appearance of a short form of MCL-1 coincides with caspase-3 activation and PARP cleavage, we assume that this MCL-1S protein is the cleaved form and not the alternatively splice variant, although both forms could be present. We did not perform experiments to check MCL-1 processing in the presence of a caspase-3 inhibitor.

In conclusion, 5-FU may induce cell death in Jurkat E6 T-cells independently of *TP53* and BAX, but involving caspase activation and MCL-1 processing. Further work will be necessary to identify the mechanism of 5-FU-induced apoptosis more precisely and determine in detail the role of MCL-1 in this process. Finally, it would be interesting to study the interplay between MCL-1 and other BCL-2 family proteins, especially BAK.

Acknowledgements

We wish to thank Monica Atneosen-Åsegg for collaboration and encouragement. The work was financed by the Norwegian Research Council.

References

1 Cory S and Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer *2*: 647-656, 2002.
2 Degterev A and Yuan J: Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol *9*: 378-390, 2008.
3 Danial NN and Korsmeyer SJ: Cell death: critical control points. Cell *116*: 205-219, 2004.
4 Lavrik IN, Golks A and Krammer PH: Caspases: pharmacological manipulation of cell death. J Clin Invest *115*: 2665-2672, 2005.
5 Riedl SJ and Shi Y: Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol *5*: 897-907, 2004.
6 Youle RJ and Strasser A: The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol *9*: 47-59, 2008.
7 Chipuk JE and Green DR: Dissecting *p*53-dependent apoptosis. Cell Death Differ *13*: 994-1002, 2006.
8 Green DR and Kroemer G: Pharmacological manipulation of cell death: clinical applications in sight? J Clin Invest *115*: 2610-2617, 2005.
9 Ow YP, Green DR, Hao Z and Mak TW: Cytochrome c: functions beyond respiration. Nat Rev Mol Cell Biol *9*: 532-542, 2008.
10 Kroemer G, Galluzzi L and Brenner C: Mitochondrial membrane permeabilization in cell death. Physiol Rev *87*: 99-163, 2007.
11 Chipuk JE and Green DR: How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol *18*: 157-164, 2008.
12 Roos WP and Kaina B: DNA damage-induced cell death by apoptosis. Trends Mol Med *12*: 440-450, 2006.
13 Vousden KH and Lane DP: *p*53 in health and disease. Nat Rev Mol Cell Biol *8*: 275-283, 2007.
14 Michalak E, Villunger A, Erlacher M and Strasser A: Death squads enlisted by the tumour suppressor *p*53. Biochem Biophys Res Comm *331*: 786-798, 2005.
15 Longley DB, Harkin DP and Johnston PG: 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer *3*: 330-338, 2003.
16 Cheng J and Haas M: Frequent mutations in the *p53* tumor suppressor gene in human leukemia T-cell lines. Mol Cell Biol *10*: 5502-5509, 1990.
17 Bode AM and Dong Z: Post-translational modification of *p53* in tumorigenesis. Nat Rev Cancer *4*: 793-805, 2004.
18 Bassett EA, Wang W, Rastinejad F and El-Deiry WS: Structural and functional basis for therapeutic modulation of *p53* signaling. Clin Cancer Res *14*: 6376-6386, 2008.
19 Green DR and Kroemer G: Cytoplasmic functions of the tumour suppressor *p*53. Nature *458*: 1127-1130, 2009.
20 Soussi T and Beroud C: Assessing *TP53* status in human tumours to evaluate clinical outcome. Nat Rev Cancer *1*: 233-240, 2001.
21 Strano S, Dell'Orso S, Di AS, Fontemaggi G, Sacchi A and Blandino G: Mutant *p53*: an oncogenic transcription factor. Oncogene *26*: 2212-2219, 2007.
22 Peller S and Rotter V: *TP53* in hematological cancer: low incidence of mutations with significant clinical relevance. Human Mutation *21*: 277-284, 2003.
23 Brimmell M, Mendiola R, Mangion J, and Packham G: *BAX* frameshift mutations in cell lines derived from human haemopoietic malignancies are associated with resistance to apoptosis and microsatellite instability. Oncogene *16*: 1803-1812, 1998.
24 Guchelaar HJ, Vermes I, Koopmans RP, Reutelingsperger CP and Haanen C: Apoptosis- and necrosis-inducing potential of cladribine, cytarabine, cisplatin, and 5-fluorouracil *in vitro*: a quantitative pharmacodynamic model. Cancer Chemother Pharmacol *42*: 77-83, 1998.
25 Gruber C, Henkel M, Budach W, Belka C and Jendrossek V: Involvement of tyrosine kinase p56/Lck in apoptosis induction by anticancer drugs. Biocheml Pharmacol *67*: 1859-1872, 2004.
26 Vermes I, Haanen C and Reutelingsperger C: Flow cytometry of apoptotic cell death. J Immunol Meth *243*: 167-190, 2000.
27 van GH, Kenis H, Lux P, Ungeth L, Maassen C, Deckers N, Narula J, Hofstra L and Reutelingsperger C: *In vitro* measurement of cell death with the annexin A5 affinity assay. Nat Protoc *1*: 363-367, 2006.

28 Bedner E, Li X, Gorczyca W, Melamed MR and Darzynkiewicz Z: Analysis of apoptosis by laser scanning cytometry. Cytometry *35*: 181-195, 1999.
29 Kumar S: The apoptotic cysteine protease CPP32. Int J Biochem Cell Biol *29*: 393-396, 1997.
30 Li X and Darzynkiewicz Z: Cleavage of poly(ADP-ribose) polymerase measured *in situ* in individual cells: relationship to DNA fragmentation and cell cycle position during apoptosis. Exp Cell Res *255*: 125-132, 2000.
31 Soldani C and Scovassi AI: Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis *7*: 321-328, 2002.
32 Zamzami N, Maisse C, Metivier D and Kroemer G: Measurement of membrane permeability and the permeability transition of mitochondria. Meth Cell Biol *80*: 327-340, 2007.
33 Castedo M, Ferri K, Roumier T, Metivier D, Zamzami N and Kroemer G: Quantitation of mitochondrial alterations associated with apoptosis. J Immunol Meth *265*: 39-47, 2002.
34 Gogvadze V and Orrenius S: Mitochondrial regulation of apoptotic cell death. Chem Biol Inter *163*: 4-14, 2006.
35 Gogvadze V, Orrenius S and Zhivotovsky B: Mitochondria in cancer cells: What is so special about them? Trends Cell Biol *18*: 165-173, 2008.
36 Le GS, Podar K, Harousseau JL and Anderson KC: Mcl-1 regulation and its role in multiple myeloma. Cell Cycle *3*: 1259-1262, 2004.
37 Michels J, Johnson PW and Packham G: Mcl-1. Int J Biochem Cell Biol *37*: 267-271, 2005.
38 Warr MR and Shore GC: Unique biology of Mcl-1: therapeutic opportunities in cancer. Curr Mol Med *8*: 138-147, 2008.
39 Vandenabeele P, Vanden BT and Festjens N: Caspase inhibitors promote alternative cell death pathways. Sci STKE *2006*: e44, 2006.
40 Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B and Melino G: Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ *16*: 3-11, 2009.
41 Salvioli S, Ardizzoni A, Franceschi C and Cossarizza A: JC-1, but not $DiOC_6$ (3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett *411*: 77-82, 1997.
42 Orrenius S, Gogvadze V and Zhivotovsky B: Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol *47*: 143-183, 2007.
43 Michels J, O'Neill JW, Dallman CL, Mouzakiti A, Habens F, Brimmell M, Zhang KY, Craig RW, Marcusson EG, Johnson PW and Packham G: Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. Oncogene *23*: 4818-4827, 2004.
44 Bae J, Leo CP, Hsu SY and Hsueh AJ: MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. J Biol Chem *275*: 25255-25261, 2000.
45 Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P and Whyte MK: Exon skipping in *Mcl-1* results in a *bcl-2* homology domain 3 only gene product that promotes cell death. J Biol Chem *275*: 22136-22146, 2000.

Received May 17, 2010
Revised May 17, 2010
Accepted July 7, 2010