Apoptosis is the most efficient death-pathway in tumor cells after topoisomerase II inhibition

Raafat A. El-Awady, PhD, MSc, Mahmoud M. Ali, BSc, Ekram M. Saleh, PhD, MSc, Fayek M. Ghaleb, MD.

ABSTRACT

الهدف: تؤدى معالجة الخلايا السرطانية بالأدوية المضادة للسرطان إلى موت تلك الخلايا بطرق مختلفة. الهدف من هذا البحث هو مقارنة فعالية الموت الإنتحاري المبرمج مع طرق الموت الأخرى في الخلايا السرطانية وذلك بعد إحداث كسور في الجديلة المزدوجة للحامض النووي منقوص الأوكسجين للخلايا عن طريق تأثير بعض الأدوية مثل عقار الأيتوبوسيد المثبط لأنزيم الطوبوايزوميريز–2

الطريقة: أجريت هذه الدراسة في قسم بيولوجيا الأورام–معهد الأورام القومي–جامعة القاهرة في الفترة من سبتمبر 2005م إلى أغسطس 2007م باستخدام ثلاثة أنواع مختلفة من الخلايا السرطانية: 1 – خلايا سرطان الثدي 2 – خلايا سرطان عنق الرحم 3 – خلايا سرطان المخ. لقد تمت دراسة حساسية هذه الخلايا لعقار الأيتوبوسيد باستخدام مادة صبغية قادرة علي التفريق بين الخلايا الحية والخلايا الميتة. كما تمت دراسة موت الخلايا بطريقة الموت الإنتحارى المبرمج بطريقتين هما طريقة الأنسياب الخلوي المتدفق وطريقة الفصل الكهربائي للحامض النووي منقوص الأوكسجين. وأخيراً تمت دراسة قدرة الخلايا علي إصلاح الكسر في الحامض النووي منقوص الأوكسجين بطريقة الفصل الكهربائي.

النتائج: بالنسبة لحساسية الخلايا لعقار الأيتوبوسيد كانت خلايا سرطان عنق الرحم هي الأكثر حساسية تلتها خلايا سرطان الثدي في حين كانت خلايا سرطان المخ هي الأقل حساسية لهذا العقار. النوع الوحيد من الخلايا الذي وجد أنه يموت بطريقة الموت الإنتحاري المبرمج هو خلايا سرطان عنق الرحم في حين أن النوعين الآخرين من الخلايا لم يستخدما هذه الطريقة للموت بعد العلاج بعقار الأيتوبوسيد. باستخدام جرعات من العقار تحدث نفس نسبة القتل في الثلاثة أنواع من الخلايا وجد أن خلايا سرطان عنق الرحم لديها أقل كمية من الكسور في الحامض النووي منقوص الأوكسجين تلتها خلايا سرطان المخ في حين كانت خلايا سرطان الثدي هي الأكثر من حيث كمية الكسور في الحامض النووي منقوص الأوكسجين والذي لا يمكن إصلاحه.

خاتمة: من هذه الدراسة يمكن إستنتاج ما يلي:– بالرغم من أن الخلايا السرطانية يمكن أن تموت بطرق مختلفة بعد علاجها بالأدوية المضادة للسرطان إلا أن طريقة الموت الإنتحارى المبرمج هي الأكثر فعالية وتحتاج إلي تركيزات منخفضة من الأدوية وكذلك كمية قليلة جدا من الكسور غير القابلة للإصلاح في جديلة الحامض النووي منقوص الأوكسجين لكي تعطي نفس مستوي القتل في الخلايا السرطانية. من الناحية التطبيقية هذا يعني إن الخلايا القادرة علي الموت بطريقة الموت الإنتحارى المبرمج قد تحتاج إلي تركيزات من الأدوية المضادة للسرطان أقل من تلك الخلايا التي لا تستطيع الموت بهذه الطريقة لكي تعطي مستوي معين من قتل تلك الخلايا السرطانية.

Objective: To compare the efficiency of apoptosis and other modes of cell death in killing tumor cells after the induction of DNA damage by topoisomerase inhibitors like etoposide.

Methods: This study was carried out in the Tumor Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt, from September 2005 to August 2007. The breast cancer MCF7, the cervix carcinoma, human cervical adenocarcinoma (Hela), and the brain tumor U251 cell lines were exposed to etoposide. Apoptosis was detected using the flow cytometry and the DNA ladder formation methods. Cell viability was determined by a colorimetric assay, and the residual DNA double-strand breaks (dsb) were measured by gel electrophoresis.

Results: The Hela cells were the most, the MCF7's were moderately, whereas the U251's were the least sensitive to etoposide. Apoptosis was detected only in Hela cells whereas the other 2 cell lines showed a very low level of apoptosis (only 3% increase above the control cells). At equitoxic drug concentrations (namely IC50), the Hela cells showed the lowest amount of non-repaired DNA dsb, and the MCF7's showed the highest amount, whereas the U251 cells showed a moderate amount.

Conclusions: These results indicate that although other modes of cell death exist, apoptosis is the most efficient and requires lower drug concentrations and fewer numbers of non-repaired dsb to give the same killing effect. Clinically, this means that tumors that can execute apoptosis may require lower doses of topoisomerase inhibitors than those that lost the ability to exercise apoptosis.

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From the Department of Tumor Biology (El-Awady, Ali), Biochemistry Unit (Saleh), National Cancer Institute, Cairo University, and the Clinical Pathology Department, (Ghaleb), Ophthalmology Research Institute, Cairo, Egypt.

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Address correspondence and reprint request to: Dr. Raafat A. El-Awady Department of Tumor Biology, Pharmacology Unit, National Cancer Institute, Cairo, Egypt. Tel. +20 (2) 3664560. Fax. +20 (2) 3664720. E-mail: el_awady_raafat@hotmail.com

Etoposide, a topoisomerase II inhibitor, is commonly administered (orally or intravenously) to cancer patients. It is generally indicated, either alone or with other drugs, in the treatment of small cell lung cancer and germ cell neoplasms.[1] It is also active in a variety of tumor types, including acute leukemia, non-Hodgkin's lymphoma, Ewing's sarcoma, osteosarcoma, ovarian, gastric, and esophageal cancers,[2] as well as different pediatric tumors. Like most anticancer drugs, etoposide induces tumor cell death through the induction of different types of DNA lesions. Among these lesions, DNA double-strand breaks (dsb) is the most lethal in terms of cell death.[3] In addition, other types of DNA lesions (such as base damage, inter and intra strand crosslinks, and DNA single-strand breaks) were found to be converted into frank DNA dsb during their repair or during cellular replication. The fate of drug-induced DNA dsb depends on the genetic makeup of the treated cells. Thus, those breaks may: 1. repair correctly to reconstitute the integrity and continuity of the cellular genetic material (and this will lead to cellular resistance to the chemotherapy), 2. repair incorrectly or not repair at all to form the so called non-repaired (residual) dsb. Both, incorrectly, and non-repaired dsbs, are thought to be the initiators of cell death by different death mechanisms. This appears to be dependent on many cellular proteins such as p53, ataxia telagiectasia mutated, ataxia telagiectasia related, DNA-dependent protein kinase, RecA homolog, *E. coli*, breast cancer susceptibility gene, γ-H2AX, 53BP and Mre11 RAD51 and Nijmen breaking complex, which are involved in cell cycle checkpoints, signal transduction, DNA repair and apoptosis.[4-8] According to a widespread model, the anti-cancer chemotherapy will promote the tumor cells to undergo apoptosis by triggering the residual dsb, so apoptosis-resistant cells would be recalcitrant to such therapy.[9-11] However, in addition to apoptosis, which is mainly dependent on the activity of the tumor suppressor protein p53,[12] cells can be eliminated following DNA damage by other mechanisms. Until now, only 2 alternative mechanisms of chemotherapy induced cell death are known, the mitotic catastrophe (MC), and the terminal growth-arrest (TGA) through the accelerated senescence.[13] The MC, a form of cell death that results from abnormal mitosis, is described by many investigators as a mechanism of cell death after the exposure to many anticancer drugs.[14,15] While the term of MC has been used to describe a type of cell death that occurs during mitosis, there is still no broadly accepted definition. Occasionally, MC is used restrictively for the description of abnormal mitosis leading to cell death, which can occur through necrosis or apoptosis, rather than cell death itself.[16] Although different classes of cytotoxic agents will induce MC, the pathways of abnormal mitosis differ depending on the nature of the inducer and the status of cell-cycle checkpoints. Moreover, MC can develop due to the aberrant re-entry of tumor cells into the cell cycle after prolonged growth arrest.[16] Senescence (or TGA of cells) was shown to be readily induced in different tumor cell lines after treatment with a variety of anti-cancer agents. The strongest senescence induction was observed with the DNA-interactive agents such as doxorubicin, aphidicolin, and cisplatin. A somewhat weaker response was seen with ionizing radiation, cytarabine, and etoposide. The weakest effect, however, was observed with the microtubule-targeting drugs such as taxol and vincristine.[17] The contribution of these different death mechanisms (apoptosis, MC, and senescence) to the clinical response of tumors to chemotherapy remains uncertain. Elucidation of the factors that regulate the different aspects of chemotherapy induced tumor cell death and the different pathways of chemotherapy induced death should assist in improving the efficacy of the anti-cancer therapy, and may provide opportunities for the development of new cancer therapies. The aim of the present study is, therefore, to investigate the efficiency of different modes of tumor cell death and their dependence on the number of residual dsb after topoisomerase II inhibition.

Methods. This study was carried out in the Tumor Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt from September 2005 to August 2007. Approval was obtained from the local ethic committee prior to the commencement of the study. The 3 human tumor cell lines used in this study are the breast cancer cell line MCF-7, the cervix carcinoma cell line Hela, and the brain tumor cell line U251, all were described previously.[18] The 3 cell lines were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, pyruvate, and glutamate. All incubations were performed at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The anti-cancer drug etoposide was purchased from Calbiochem (Darmstadt Germany) and stock solution was obtained by dissolution in dimethyl sulfoxide (1mg/10ml) and stored frozen at 20°C to be diluted in growth culture medium in advance of an experiment. Cytotoxicity of etoposide against the cell lines was determined using the SulphoRhodamine-B (SRB) method as previously described by Skehan et al.[19] Cells were seeded in 96 well microtiter plates at a concentration of 5×10^4-10^5 cells/well in Roswell Park Memorial Institute 1640 supplemented medium. After 24 hours, cells were incubated for 48 hours with increasing concentrations of etoposide (8 wells/drug concentration). At the end of the treatment

period, the cells were fixed with 50% trichloroacetic acid (TCA) for one hour at 4°C. Wells were washed 5 times with water, stained for 30 minutes at room temperature with 0.4% SRB stain dissolved in one percent acetic acid. According to the standard procedure, the wells were then washed 4 times with one percent acetic acid and air dried and the dye was solubilized with 100 µl/well of 10 mM tris base (pH 10.5) at room temperature for 5 minutes on a shaker at 1600 rpm. The optical density (OD) in each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (Meter tech. ∑960, USA). The fraction of cells remaining survival at each drug concentration was determined by dividing the mean OD at the drug concentration by that obtained from control untreated cells after subtracting OD values from wells containing medium alone without cells. The IC50 values were calculated using sigmoidal concentration-response curve fitting models (Graph Pad, Prism Software, San Diego, CA). The DNA repair was estimated by measuring residual dsbs by constant-field gel electrophoresis (CFGE) as described by El-Awady et al.[18] Exponentially growing cells were treated with different concentrations of etoposide (5-100 µg/ml) at 37°C for 2 hours. The drug was then removed by washing the cells 2 times with drug-free medium followed by incubating the cells in drug-free medium for 22 hours to allow for the repair of etoposide-induced dsb. Monolayer cells were detached with trypsin, and the resulting cell suspension was mixed with an equal amount of 1.6% low melting point agarose (Bio-Rad, Munich, Germany) and pipetted into a 180 µl mould and left on ice to solidify to form agarose cell plugs. Plugs were incubated in lysis buffer (0.4 M ethylene diamine tetraacetic acid, EDTA, 2% N-Lauryl sarcosine, and one mg/ml proteinase-k, all Sigma, Deisenhofen, Germany) for 30 minutes on ice and then overnight at 37°C. Plugs were washed 3 times with Tris-EDTA buffer, and a piece of the plug containing approximately 10^5 cells was inserted into a preformed 0.8% agarose gel. Electrophoresis was run for 36 hours at 0.6 V/cm in 0.5x Tris-Borate-EDTA buffer. After completion of electrophoresis, the gel was stained overnight in 0.5 µg/ ml ethidium bromide solution, and then destained overnight in distilled water, and the fraction of DNA released (FDR) after each drug concentration was determined using a gel documentation system (Biometra, Goettingen, Germany). The FDR released into the gel, corresponding to fragmented DNA, was calculated according to the following equation:

FDR =FDR rel /(FDR plug +FDR rel),

FDR rel: fraction of DNA released outside the well.

FDR plug: fraction of DNA remaining in the well.

The FDR corresponding to IC50 values of the 3 cell lines were calculated using one site binding curve fitting models (Graph Pad, Prism Software, San Diego, CA). Induction of apoptosis by etoposide in the 3 cell lines was measured by Annexin V-FITC staining and ladder formation methods. (a) Annexin V-FITC staining method. Cells were treated as monolayer in T175 cm^2 tissue culture flasks with the corresponding IC50 (calculated from the survival curves, **Table 1**) for 2 hours at 37°c. The drug was removed by washing the cells twice with drug-free medium, and the cells were further incubated in drug-free medium and tested for annexin V staining at the indicated time intervals. Annexin V-FITC (BD Biosciences, San Jose USA) staining was performed according to the manufacturer's instructions. Briefly, the cells were washed twice with cold phosphate-buffered saline and then resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/ml. 100 µl of this cell suspension (1 x 10^5 cells) were transferred to polypropylene round-bottom tube before adding 5 µl of Annexin V-FITC and 5 µl of propidium iodide to them. The cells were gently vortexed and incubated for 15 minutes at room temperature (25°C) in the dark. Finally, 400 µl of 1X binding buffer were added to each tube and cells were analyzed by flow cytometry FACScan (Beckman Coulter, Epics XL Hialean) within one hour. (b) DNA ladder formation method. Cells were treated as monolayer with the indicated etoposide concentrations (5-100 µg/ml) at 37°C for 2 hours, the drug was washed and the cells were incubated in drug-free medium for 46 hours. The DNA ladder formation was tested by collecting the cells by trypsinization, putting them in agarose plugs as described above under dsb repair and the DNA was separated by electrophoresis for 6 hours at 1.5 V/cm in 0.8% agarose. The gel was then stained in ethidium bromide solution (1 µg/ml) for 3 hours and photographed.

Data are given as means (±SEM) of at least 3 independent experiments. Statistical analysis, data fitting and graphics were performed using the GraphPad prism program (San Diego, CA).

Results. The sensitivity of the 3 cell lines to different concentrations of etoposide was studied using the SRB assay after 48 hours incubation period with the drug. The survival of the 3 cell lines decreased proportionally with the drug concentration. At the highest drug concentration (15µg/ml) the survival were reduced to 33% for Hela, 42% for MCF7, and 58% for U251 of the control untreated cells (**Figure 1**). The cell survival was not reduced further by longer periods of incubation (72 and 96 hours) (data not shown). We further calculated the IC50 (concentration of the drug that reduces the survival to 50%) for the 3 cell lines (**Table 1**). According to this parameter, the Hela cells were the most sensitive to the action of etoposide followed by the MCF7 cells,

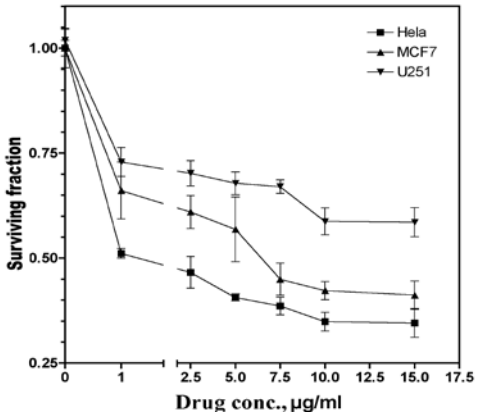

Figure 1. Survival of Hela, MCF7 and U251 cells after different concentrations of etoposide. Data represent 5 independent experiments.

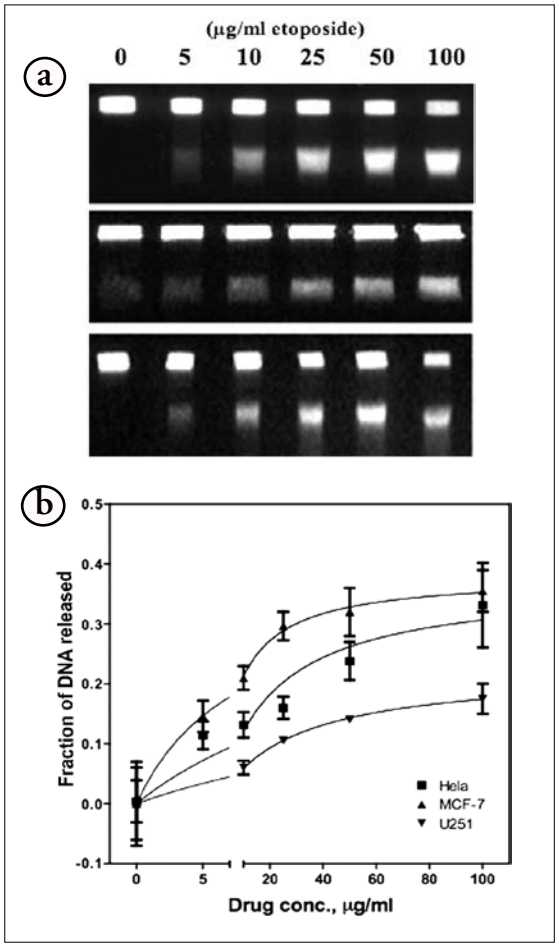

Figure 2 - Determination of non-repaired double-strand breaks in the 3 cell lines. (**a**) Ethidium bromide stained gels for non-repaired DNA fragments from MCF7 (upper), U251 (medium) and Hela (lower) cells. (**b**) dsb repair efficiency. The band intensities in ethidium bromide-stained gels were quantified, and the fraction of DNA released at each drug concentration was calculated. Data represent the results of at least 3 independent experiments.

Table 1 - Cell lines used in this study and their p53 status, and their etoposide-induced IC50, double-strand breaks repair efficiency and apoptosis induction.

Cell line	P53 status	IC50[1] (µg/ml)	FDR[2] (IC50)	Percent AnnexinV positive /PI negative cells	
				48h	72h
Hela	Null	1.35	0.021	55.6	69.3
MCF7	Wild-type	6.34	0.169	2.75	1.14
U251	Mutant	30.56	0.116	1.9	6.38

1. Drug concentration that reduces cell survival to 50% of the control untreated cells
2. Fraction of DNA released after treating the cells with the corresponding IC50

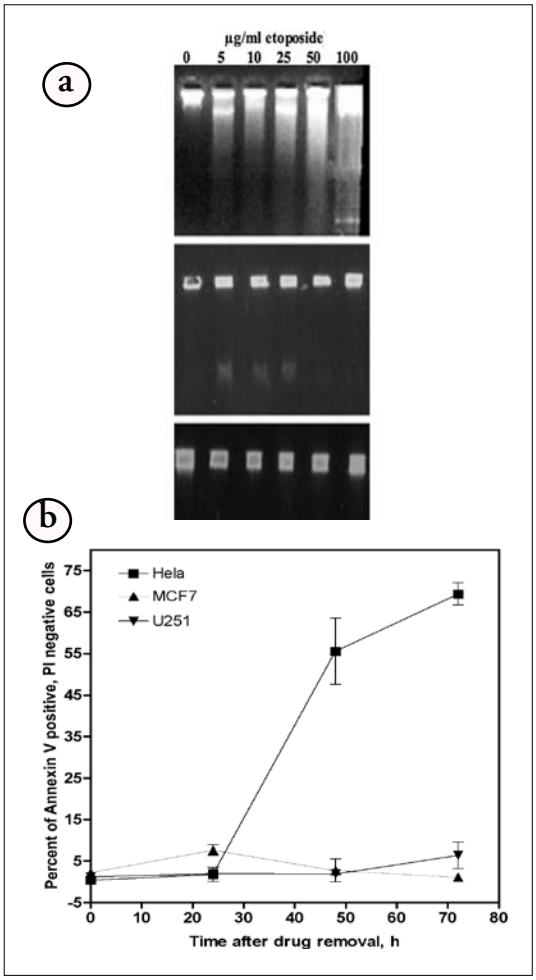

Figure 3 - Induction of apoptosis in the 3 cell lines after treatment with etoposide. (**a**) Ethidium bromide-stained gels for DNA ladder formation in Hela (upper), MCF7 (medium) and U251(lower) cells after treatment with etoposide. (**b**) Kinetics of apoptosis induction in the 3 cell lines after treatment with etoposide. Cells were treated with the corresponding IC50 (taken from the survival curves) for 2 hours. The drug was removed, and the cells were further incubated in drug-free medium and tested for annexin V staining at the indicated time intervals by flow cytometry cell sorting.

whereas the U251 cells were the most resistant. The Hela cells were 4.7 and 22.6 times more sensitive to etoposide than the MCF7, and the U251 cells. In an attempt to understand the reasons behind this clear different sensitivities to etoposide, we investigated the ability of the 3 cell types to repair the etoposide-induced DNA dsb. This was performed by measuring the ability of the cells to rejoin the broken DNA ends, and thus forming larger DNA fragments that are unable to migrate under the normal constant-field gel electrophoresis conditions. Cells were treated with different concentrations of etoposide (5-100 µg/ml) for 2 hours, then the drug was washed away, and the cells were incubated for another 22 hours in drug-free medium to repair the drug-induced dsbs and consequently the FDR (representing the fraction of residual dsb) was analyzed (**Figure 2a**). **Figures 2a & 2b** shows the increase in the residual DNA dsb in the 3 cell lines with the increased concentration of the drug. The 3 cell lines showed different abilities to repair the etoposide-induced dsb at all the tested drug concentrations. Comparing the FDR for the 3 cell lines at equitoxic drug concentrations (IC50) had revealed that the Hela cells were the most efficient in repairing the etoposide-induced dsb. They showed residual dsb that is 5.8 times lower than those shown by the U251, and 8.05 times lower than those shown by the MCF7 cells. To this point, it can be argued that the different dsb repair efficiencies cannot completely explain the different sensitivities of the 3 cell lines to etoposide. Etoposide is known to kill the cells by induction of apoptosis,[20,21] therefore, we examined the ability of the drug to induce apoptosis in the 3 cell lines. Using the annexinV assay it was found that etoposide was able to induce apoptosis only in the p53 null Hela cells and this was apparent only during the second 24 hours after removing the drug (**Figure 3b**). However, only a very small fraction of the other 2 cell lines; MCF7 (wild type-p53), and U251 (mutant p53) were annexinV positive even 72 hours after removing the drug, indicating lack of etoposide-induced apoptosis in these cells (**Figure 3b & Table 1**, last column). This result was confirmed when we tested etoposide-induced apoptosis in the 3 cell lines using the DNA ladder formation method (**Figure 3a**). Again, only the Hela cells showed DNA ladder 48 hours after incubation with different etoposide's concentrations.

Discussion. Etoposide exerts its cytotoxic effects by interference with the normal metabolism of DNA. This is accomplished through stabilizing the cleavable complex formed between the enzyme topoisomerase II and the cellular DNA during DNA replication, transcription, repair or recombination. This effect leads to the conversion of the reversible cleavable complexes into permanent protein-bound DNA dsb. Although most etoposide-induced DNA dsbs are rapidly repaired after drug removal, residual or incorrectly repaired breaks lead to genetic instability and increased mutation frequency and chromosomal aberrations, leading to activation of the cell cycle checkpoints and cellular death.[22,23] Despite the efficacy of topoisomerase inhibitors in the clinic, the presence of drug-resistant tumor cells remains a major barrier to the successful management of the majority of solid tumors. Mechanisms of resistance to the inhibitors of topoisomerases are variable and include: a) Alterations in the activity, cellular content or distribution of the enzymes b) Over expression of membrane efflux pumps that leads to reduction in the intracellular accumulation of the drugs,[24,25] and c) Efficient repair of drug-induced dsb in treated cells. All these mechanisms will lead, finally, to the reduction in the number of drug-induced dsb, which is the initiator of cell death. However, the contribution of these mechanisms to the clinical drug-resistance remains uncertain. The present study is designed to investigate the relationship between the number of etoposide-induced residual dsb, mode of cell death and sensitivity of cells to etoposide. We tested this purposed relationship in different p53 backgrounds by using 3 human tumor cell lines expressing either wild-type (MCF7), mutant (U251) or null (Hela) p53.[26] Survival results showed that only a concentration of 1.35 µg/ml etoposide was required to reduce the survival of p53 null Hela cells to 50% of the control untreated cells, whereas a concentration of 6.34 were required to give the same effect in the p53 wild-type MCF7, and 30.56 µg/ml were required to give the same effect in the p53 mutant U251 cells. However, the residual dsbs in the 3 cell lines were in the opposite direction for the survival results. The residual dsbs induced by IC50 were MCF7 the highest, U251 the intermediate, and Hela cells the lowest. To this point, our results show that neither the p53 status nor the number of residual dsb can explain the differences in the sensitivity of the 3 cell lines to etoposide. This finding agrees with several previous reports suggesting that p53 status is not a determinant of cells sensitivity to the DNA damaging agents.[27-30] In addition, it has been demonstrated that inactivation of p53 can enhance sensitivity to the chemotherapeutic agents in some tumor cells.[31,32] Another recent study showed that inactivation of p53 in 4 prostate tumor cell lines enhanced sensitivity of those cells to etoposide, vinblastine, and estramustine compared to the p53 wild-type cells,[33] a finding that totally agrees with our finding. However reports on the effect of p53 status on chemosensitivity are conflicting and it is thought that different chemotherapeutic agents may have different p53-dependent effects in different tumor cells.[34]

Our present data reports on the relationship

between residual DNA dsb and sensitivity to etoposide. Until recent time, apoptosis was considered the main pathway for cellular death after DNA damage induced by chemo or radiotherapy.[35,36] However, evidence emerged indicating the existence of other pathways that can be used by the cells to act efficiently with apoptosis or substitute it as a mode of cell death.[13] Support for this finding was obtained from the observations that many apoptosis defective cells are still capable of experiencing cell death after DNA damage induced by anticancer agents.[13,25] Accumulating evidence from previous studies indicate that non-repaired dsb are the main trigger of cell death by activating ATM-ATR pathways.[4-8]

In the present study we found that 55% of Hela cells showed signs of apoptosis (AnnexinV positive and DNA ladder) 48 hours after treatment with IC50 etoposide, whereas only less than 3% of the MCF7, and U251 cells were apoptotic after the same treatment with the respective IC50. This indicates that apoptosis is the pathway exclusively responsible for reducing survival of Hela cells to 50% after treatment with IC50. This was not the case for the other 2 cell lines in whom apoptosis can only account for the death of less than 3% of the cells, whereas other death pathways are responsible for killing the remaining 47% of the cells after their treatment with IC50 etoposide. Factors that determine whether the cells will die by apoptosis or other death pathways after DNA damage are still unclear. At least we can claim that p53 status is not an important determinant as, in our study, apoptosis was induced only in p53 null Hela cells but not in the p53 wild-type MCF7, or in the p53 mutant U251 cells. This is in the same line with many recent studies, which mentioned the existence of p53-independent DNA damage-induced apoptosis and the existence of death pathways other than apoptosis that also plays a significant role in the sensitivity of tumors to DNA-damaging agents.[26,37,38] Many p53-independent DNA-damage-induced apoptosis pathways exist such as the p63, p73, PUMA, and BAX pathway[39] and the NF-κB, FAS pathway.[40] Despite the availability of this array of apoptotic pathways (p53 dependent and independent), some tumor cells (such as MCF7, and U251) are still apoptosis resistant. It is difficult to expect that all these pathways are defective in such tumor cells but until now it is not clear how the cells decide to execute cell death through apoptosis or through other death pathways.

In conclusion, our results confirm previous observations for the existence of death pathways other than apoptosis. However, we report here that these pathways are far less efficient than apoptosis and require higher drug concentrations and higher number of residual dsb than those required by apoptosis to induce the same killing effect.

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