Sabarubicin (MEN10755)-induced Apoptosis is Independent from mtDNA in A2780 Human Ovarian Tumor Cells

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Abstract. Background: The role of mitochondrial DNA (mtDNA) in anthracycline-induced apoptosis is controversial. Sabarubicin accumulates in the mitochondria of A2780 human ovarian tumor cells. The effects of this new anthracycline on the structure and the functionality of mtDNA, as well as on the apoptosis of mtDNA-depleted cells have been investigated. Materials and Methods: Sabarubicin-induced mtDNA cleavage was detected by Southern blotting and mitochondrial mRNA expression was analyzed by real-time PCR. Apoptosis was studied in mtDNA-depleted (q0) and parental (q+) A2780 cells detecting nuclear DNA fragmentation using ELISA and cytofluorimetrically using Annexin V/PI staining. Mitochondrial membrane potential was studied using the cyanine dye JC-1. Results: Sabarubicin induced mtDNA cleavage in the A2780 cells, but this damage did not affect mitochondrial mRNA expression. Apoptosis was induced by sabarubicin in q0 as well as in q+ cells. Conclusion: The results showed that mtDNA did not influence anthracycline-induced apoptosis in A2780 cells.

Anthracyclics are antitumoral drugs which mainly target nuclear DNA inducing both apoptotic and necrotic cell death and, depending on the cellular type, different signal transductions are triggered by anthracycline-induced DNA damage (1). It has also been demonstrated that mitochondrial DNA (mtDNA) is a target of anthracyclines and that they can cause mtDNA damage. In HeLa cells treated with doxorubicin, mitochondrial protein synthesis has been found to be inhibited indicating the formation of doxorubicin-adducts in mtDNA (2). The formation of intrastrand cross-linking in mtDNA following doxorubicin treatment had also been observed by Cullinane et al. after a long incubation time and at high drug concentrations (3).

Several studies have used mtDNA-depleted cells (q0) to investigate the role of mtDNA in anthracycline-induced apoptosis. The phenotype of such cells presents a non-functional respiratory chain, a compensatory up-regulation of glycolysis and slow growth. When apoptotic cell death has been analyzed, the resistance of q0 cells has been observed only in the presence of specific stimuli, such as the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (4). Other studies have described reduced sensitivity of mtDNA depleted cells to antitumoral drug-induced necrotic cell death (5). However no significant difference between parental (q+) and q0 cells has been reported in apoptosis triggered in vitro by anthracycline treatment (6). Thus, human q0 cell lines can still be induced to die by apoptosis suggesting that the apoptotic pathway does not depend on mitochondrial respiration (7-9).

Sabarubicin (MEN10755) is a new anthracycline which possesses a broader spectrum of antitumoral activity than doxorubicin in various human cancer cell lines. We have previously partially elucidated the importance of nuclear signalling in the apoptotic cascade triggered by this anthracycline in a model of ovarian cancer, cell line A2780 (10). In the present study, the role of mtDNA in apoptosis induced by sabarubicin in A2780 cells was investigated.

Materials and Methods

Chemicals. The sabarubicin was synthesized as previously described (11). Doxorubicin and teniposide (VM-26) were purchased from Sigma, MO, USA. Stock solutions were prepared in distilled water and stored at –20°C. The 5,5',6,6'-tetrachloro-1,1',3,3'-tetrakis(2-ethylbenzimidazolyl)carbocyanine iodide (JC-1) was purchased from Molecular Probes Inc., OR, USA.

Cell culture and derivation of q0 cells. The human ovarian carcinoma A2780 cell line was purchased from the European collection of cell cultures (ECACC), UK. The cells were cultured in standard...
medium (RPMI 1640 supplemented with 10% FCS and 2 mM glutamine) (Gibco, CA, USA) at 37°C in a humidified atmosphere with 5% CO₂. The mtDNA-depleted A2780 cells were selected by culturing the cells in the presence of etidium bromide (EtBr). Briefly, RPMI culture medium was supplemented with 0.1 μg/ml EtBr (Sigma), 100 μg/ml sodium pyruvate (Gibco), 50 μg/ml uridine (Sigma) and 2.5 mg/ml glucose. After 10 days, the absence of functional mtDNA was verified through quantitative amplification of the mitochondrial transcripts cytochrome c oxidase subunit I (COXI), cytochrome b (CYTB) and ATP synthase 6 (ATP6) (data not shown).

Mitochondrial and total DNA extraction. For mtDNA extraction, the A2780 cells were treated with 2.5 μM doxorubicin, 5 μM sabarubicin or 25 μM VM-26 for 1 h at 37°C in 5% CO₂, then detached, centrifuged and resuspended in octylphenolphopoly (ethyleneglycolether) (Nonidet P-40) lysis buffer for 15 min at room temperature. Lysates were centrifuged at 7000 rpm for 5 min to pellet the nuclei and 10% SDS with 10 mg/ml proteinase K and 0.5 M ethylenediaminetetraacetic acid (EDTA) were added to the supernatants and incubated at 42°C overnight. After phenol/chloroform extraction, the mtDNA was precipitated for 1 h at –20°C and then centrifuged at 9000 rpm for 30 min at 4°C, resuspended in 70% ethanol, centrifuged again and finally resuspended in Tris-EDTA (TE) buffer. The mtDNA concentrations were determined spectrophotometrically by 260-280 nm reading. Total DNA was prepared by the standard procedure of phenol/chloroform extraction.

Southern blot analysis. The mtDNA from untreated and 2.5 μM doxorubicin, 5 μM sabarubicin or 25 μM VM-26 treated A2780 cells was digested with EcoR I restriction enzyme. For each sample, ten micrograms of EcoR I-digested mtDNA were size-fractionated by electrophoresis on 0.7% agarose gel in Tris-borate buffer (TBE), pH 8.0. Equal loading was visualized by ethidium bromide staining. Digested mtDNA was transferred overnight to a nylon membrane and probed with a 32P-labeled mtDNA fragment spanning the region 7441-8227 of human mtDNA. Hybridization, washing and labeling of the probe was carried out as described previously (12).

Probe specificity for mtDNA was tested by Southern blot comparing restriction patterns between total and mtDNA. Five micrograms of total DNA and 0.5 μg of mtDNA were restricted overnight at 37°C with BamH I, EcoR I and BstXI restriction enzymes in separate reactions. The digestions were size fractionated by gel electrophoresis on 0.7% TBE-agarose gel and Southern blot was performed.

Determination of anthracycline uptake in cytoplasmic and mitochondrial fractions of A2780 cells. The A2780 cells were incubated with sabarubicin or doxorubicin at the concentrations of 2.5 or 5 μM for 1 h at 37°C. After this time the cells were homogenized in ice-cold homogenizing buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 5 mM Hepes at pH 8.0. The homogenate was centrifuged at 700 xg for 10 min at 4°C to remove cell debris. The resulting supernatant was further centrifuged at 10,000 xg for 15 min. The final supernatant was separated as the cytosolic fraction while the pellet (mitochondrial fraction) was resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM sodium fluoride 10 μg/ml leupeptin and aprotinin) for 10 min at 4°C.

Anthracycline quantities in the fractions were determined by the high pressure liquid chromatography (HPLC) method and the HPLC data were normalized with the protein content of the samples. The protein concentrations in the fractions were determined using the Bio-Rad protein dye microassay according to the procedure of the kit. The purity of cellular fractions was verified by immunoblot with cytochrome c antibody. For each sample, the proteins were separated on 12% SDS-PAGE and transferred to poly(vinylidene difluoride) (PVDF)-membrane (Schleicher & Schuell, Dassel, Germany). After incubation with primary antibody to cytochrome c (Santa Cruz Biotechnology, CA, USA), detection was performed with horseradish peroxidase-conjugated IgG antibody and enhanced chemiluminescence (ECL) reagents (GE Healthare, Little Chalfont, UK).

RNA extraction and quantitative real-time PCR detection. The g+ and g0 A2780 cells were seeded in 75 cm² flasks, at a density of 3x10⁴ cells/flask. After overnight incubation, the cells were treated with 2.5 μM doxorubicin, or with 5 μM sabarubicin, for 15 and 24 h. The cells were subsequently lysed with the lysis buffer of the “SV Total RNA Isolation System” kit (Promega, CA, USA) and the total RNA from the samples was prepared according to the procedure of the kit. The corresponding cDNAs were synthesized using the “Reverse Transcription System” kit (Promega). To determine the expression levels of the mitochondrial transcripts, duplicates of 1:100 dilution of the cDNA samples were amplified in 25 μl of TaqMan Universal PCR Master Mix, in the presence of primers and probes designed with the Primer Express software (Applied Biosystem, CA, USA). The selected sequences were: COXI: 5'-TCCGCTACCATAATCTATGCTAT-3' (upper primer), 5'GGAGTGTTGGCCGAGTCAGTAAT-3' (lower primer) and 5'-CCACCGGGGCTCA-3' (probe); CYTB: 5'-TCCACCGGCTCA-3' (upper primer), 5'-ATGGTGCTATGGGCTGC-3' (lower primer) and 5'-ATCCCTATCTATC-3' (probe); and ATPase6: 5'-GAGCGCGCGCATGTGATT-3' (upper primer), 5'-GAAGTGGGCTAGGGCATTTTTAA-3' (lower primer) and 5'-AGGCTTTGCTCATTAG-3' (probe). To calculate the expression levels, a standard curve of serial dilutions (from 1:10 to 1:1000) of cDNA from untreated cells was amplified in duplicate in the same experiment. As endogenous control, cDNA samples were amplified as described in the presence of the inventory transcript assay Hs9999901_s1 for the ribosomal RNA 18S (Applied Biosystem). The cycling conditions for all the amplifications were as follows: 95°C for 10 min, 45 cycles at 95°C for 15 sec and 60°C for 1 min. The amplification and quantitative real-time detection took place in a 7300 Real Time PCR system (Applied Biosystems).

Cell cycle analysis. The cell cycle phases were detected by flow cytometry using propidium iodide (PI) staining as previously described (13). The apoptosis and cell cycle phases were measured using CellQuest and Modfit analysis software (Macintosh, Facststation, Becton Dickinson, CA, USA).

Determination of apoptotic cell death by flow cytometry and annexin V/PI staining. Annexin V/PI staining was performed using the apoptosis detection kit purchased from R&D System (Minneapolis, MN, USA). After trypsinization (having assessed that the use of trypsin/EDTA did not interfere with annexin V
binding), the cells (10⁶ cells) were washed twice with cold phosphate buffered saline (PBS) pH 7.2, then resuspended in binding buffer (HEPES supplemented with 25 mM CaCl₂). The cells (1x10⁵) were incubated with 10 µl of fluorescein-conjugated annexin V (10 µg/ml) and 10 µl of PI (50 µg/ml) for 15 min at room temperature in the dark. At the end of the incubation time, 400 µl of binding buffer were added and the samples were analyzed on a FACSort flow cytometer using Lysis II software (Becton Dickinson).

**Detection of mitochondrial membrane potential by flow cytometry.** Changes in the mitochondrial membrane potential (ΔΨm) were analyzed with the membrane potential sensitive probe JC-1. This cyanine dye accumulates in the mitochondrial matrix under the influence of the ΔΨm and forms J aggregates which have characteristic absorption and emission spectra (14). Untreated controls and cells treated with 2.5 µM sabarubicin for 9, 18 and 72 h were incubated with 1 µM JC-1 for 15-20 min, then trypsinized, washed twice with PBS and analyzed on a Becton Dickinson FACSort instrument with an argon ion laser at 488 nm. The emission wavelength data were measured at 530 nm for the green or low membrane potential and 585 nm for the red or high membrane potential. The software used for data analysis was Cell-Quest (Becton-Dickinson).

**Detection of cellular DNA fragmentation.** In order to determine the cell death of the ϕ⁺ and ϕ⁰ A2780 cells treated with sabarubicin, the enrichment of oligonucleosomes in the cytoplasm of apoptotic cells was detected using the Cell Death Detection ELISAPLUS (Roche Molecular Biochemicals, IN, USA). The cells were cultured in 96-well plates (2x10⁵ cells per well) and treated with increasing concentrations of sabarubicin or doxorubicin for 21 h at 37°C. The cells were lysed and subjected to the immunoassay according to the instructions manual. Photometric analysis of ELISA plates was performed with Victor 1420 (Wallac, Turku, Finland).

**Statistics.** All results were expressed as the mean±SD of data obtained from two to three separate experiments. The data were entered into Instat 2.03 GraphPad software (GraphPad Software Inc., CA, USA) to perform the Student’s t-test or the Tukey’s test. Means were considered significantly different at a confidence level of p<0.05.

**Results**

**Anthracycline accumulation in the mitochondrial fraction of A2780 cells.** As shown in Figure 1A-B, when the A2780 cells were incubated with sabarubicin or doxorubicin, a dose-related accumulation of the drugs was observed. Furthermore, sabarubicin concentration was significantly higher than doxorubicin in the mitochondrial fraction (p<0.05). The cytoplasmic and mitochondrial fractions were immunoblotted to detect cytochrome c in order to assess their purity (Figure 1C). As expected, cytochrome c was present in the mitochondrial fractions, while it was absent from the cytoplasmic fractions both in the untreated and in 1-hour sabarubicin treated cells.

![Figure 1](image-url) **Figure 1. Anthracycline uptake in cytoplasmic and mitochondrial fractions of A2780 cells.** Cells were incubated with 2.5 or 5 µM doxorubicin (Dxr) and sabarubicin (Saba) for 1 h. A: Anthracycline in mitochondrial fractions. B: Anthracycline in cytosolic fractions. Data are indicated as means±SD of two independent experiments. Asterisks indicate a significant difference between values (p<0.05). C: Cellular fractions were analysed for the presence of cytochrome c by Western blot; lanes 1, 2: mitochondrial fractions; lanes 3, 4: cytoplasmic fractions; lanes 5, 6 total fractions; cellular fractions were derived from both untreated (lanes 1, 3, 5) and sabarubicin-treated (lanes 2, 4, 6) cells.

**Anthracycline treatment and mitochondrial DNA cleavage.** Southern blot analysis using a mtDNA specific probe revealed high molecular weight fragments in the untreated control, while in the anthracycline treated samples smaller faint bands were also evident, which suggested the induction of mtDNA cleavage (Figure 2A). A similar pattern of DNA
cleavage was observed in the sample treated with the standard topoisomerase II poison teniposide (VM-26). The expected restriction fragments detected by the probe were the same for both total and mtDNA, indicating that spurious genomic sequences were not recognized (Figure 2B).

Anthracycline treatment and mitochondrial gene expression. Real-time RT-PCR data showed that, with respect to the untreated cells, the amount of mitochondrial transcripts for ATP6, CYTB and COX1 was not different ($p > 0.05$) from 0 to 24 h, in both the 5 $\mu$M sabarubicin and the 2.5 $\mu$M doxorubicin treated cells (Figure 3). Thus, in the presence of the anthracyclines, mtDNA retained its full functionality in the A2780 cells.

Nuclear DNA fragmentation in $q^+$ and $q^0$ A2780 cells. In the absence of drug treatment, nuclear DNA fragmentation in the A2780 $q^0$ cells was significantly higher than in the A2780 parental cells ($q^+$) ($p < 0.05$). In the presence of increasing concentrations of sabarubicin or doxorubicin, the absence of mtDNA did not inhibit anthracycline-induced apoptosis and the $q^0$ cells were as sensitive to apoptotic cell death as the $q^+$ cells (Figure 4).

Anthracycline-induced cell death. The percentages shown in Figure 5 represent the whole apoptotic/necrotic effect induced by drug treatment. Sabarubicin, at the highest concentration tested, induced apoptotic/necrotic cell death in A2780 $q^+$ and $q^0$ cells without statistically significant differences between the two cellular types ($p > 0.05$). Also staurosporine, used as positive control, induced similar responses both in A2780 $q^+$ and $q^0$ cells. The analysis of the cell cycle by PI staining assay showed a slight reduction of the S-phase in the A2780 $q^0$ cells (19% instead of 25%) but an increase of the dead cell fraction (22% instead of 5%) (Figure 6).
Mitochondrial membrane potential alterations induced by sabarubicin. The mitochondria maintained membrane potential both in the \( \Phi^+ \) and \( \Phi^0 \) A2780 cells, as shown by JC-1 uptake (Figure 7, panels A and E), with a slight increase in JC-1 aggregate formation (red fluorescence) in the \( \Phi^0 \) cells. In both cell types, sabarubicin triggered a transient hyperpolarization of mitochondria which started at 6-9 h post-treatment and peaked at 18 h (panels B, F). This event was followed by a progressive shift of fluorescence from red to green indicating an increase of JC-1 monomers related to membrane depolarization. This \( \Delta \Psi \)m drop culminated at 72 h (panels C, G) and showed kinetics superimposable with that of sabarubicin-induced apoptotic cell death (data not shown). The carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as depolarization control (panels D, H).

**Discussion**

Anthracyclines act as topoisomerase II poisons, leading to the formation of double strand breaks in the genome as a whole. Anthracyclines can also interact with mtDNA in
cellular models forming DNA interstrand crosslinks (3). Since topoisomerase II activity has been reported in mammalian mitochondria (15, 16), the poisoning of topoisomerase II might participate in the development of mtDNA breaks. Other mechanisms have been described in in vivo experiments. Long-term doxorubicin exposure has been reported to cause mitochondrial injury in rats, which in turn may be responsible for the “dose memory” in
delayed manifestation of cardiomyopathy years after completion of therapy (17, 18). According to these studies, the most likely mechanism for generation and maintenance of mutated mtDNA is the production of free radicals (ROS), which doxorubicin induces directly by redox cycling and indirectly by binding cardiolipin. ROS might bring about mtDNA deletion with the passage of time.

Sabarubicin is a new anthracycline which has been previously shown to induce cell death and the activation of the nuclear factor-kappaB (19). In this study, sabarubicin accumulated in the mitochondrial fraction of the A2780 cells and double-strand breaks were detected in the mtDNA. Nevertheless, real time RT-PCR data showed no reduction in the amount of mitochondrial transcripts in sabarubicin-treated cells compared to untreated cells, indicating that mtDNA expression was not affected. Since the assayed mitochondrial mRNAs should derive from the processing of an intact polycistronic RNA (20), these data seem to suggest that the effects due to the anthracyclines on mtDNA might be overcome by recombinational repair, reversibility of breaks or by the transcription of mitochondrial genes from undamaged copies (21).

The role of mtDNA in anthracycline-induced apoptosis in the A2780 cells was investigated by comparing the apoptotic responses to sabarubicin in the qSq and qS+ cells. Both nuclear DNA fragmentation and the apoptotic exposure of phosphatidylserine on the outer cell membrane leaflet were detected in the two cell types. There was also similar sensitivity of both cell types to drug-induced cell death which was not caused by different replicative capacity as shown by cell cycle phase analysis. Consistently, the loss of mitochondrial membrane potential (ΔΨm) was preserved in the sabarubicin-treated qSq A2780 cells, as assessed by JC-1 staining, suggesting that the release of apoptogenic mitochondrial proteins, such as cytochrome c, persisted and that it was sufficient to trigger apoptosis as reported in previous studies (22). Thus, the mitochondrial pathway for apoptosis was maintained in the qSq A2780 cells.

The mitochondria of many tumor cell types present differences in number, size, shape, mtDNA rearrangements, membrane composition, membrane potential and glycolytic metabolism (23). The qSq cells resemble tumor cells in their dependence on glycolysis, but the functionality of their mitochondrial membrane was preserved because the membrane potential was maintained (8) and the apoptogenic factors on the mitochondrial membrane were retained (22). Such differences between the mitochondrial phenotype of qSq cells and tumor cells should be considered in the use of mtDNA-depleted cells as a model.

Independently of the competency of qSq cells to undergo apoptosis, several studies have reported loss of sensitivity to cell death in mtDNA depleted cells. HeLa cells lacking mtDNA were resistant to doxorubicin compared to the qS+ cells suggesting that cell death was dependant on ROS (6). In the A549 non-small cell lung cancer cell line, the qS+ cell line showed increased sensitivity to daunorubicin when compared with the qSq line (5). SK-Hep1 qSq hepatoma cells have been reported to be resistant to several drugs including doxorubicin; in these qSq cells, an adaptive increase of manganese superoxide dismutase expression was detected, indicating that this activity equilibrated ROS production due to the disruption of mitochondrial electron transfer as well as to p53 activation by cytotoxic drugs (24). We have discovered that A2780 qSq cells do not show any resistance to anthracyclines, suggesting that the functional state of the mitochondrial membranes is more important than mtDNA integrity.

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