Apopotic Events in a Human Ovarian Cancer Cell Line Exposed to Anthracyclines

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Received July 12, 2000; accepted October 13, 2000

ABSTRACT

Cytotoxic drugs commonly used in cancer therapy promote tumor cell death by inducing apoptosis, but the cell death pathway(s) is likely dependent on the mechanism of drug action. In the present study, we investigated the mechanisms of cell death induced by doxorubicin (DXR) and the novel disaccharide anthracycline MEN 10755, in a human ovarian cancer cell line (A2780). Exposure to either anthracycline induced the up-regulation of several genes known to promote cell cycle arrest and DNA repair (WAF1/p21, GADD45) or apoptosis (bax, Fas). Although the expression of Fas was increased, an anti-Fas antibody ZB4 did not inhibit anthracycline-induced apoptosis, suggesting that the stimulation of the Fas receptor did not play a critical role in the induction of apoptosis in this cell line. We also observed that neither MEN 10755 nor DXR were able to induce apoptosis in A2780 cells deprived of the nucleus but retaining an intact mitochondrial function (cytoplasts) and that apoptosis induced by either anthracycline was inhibited by cycloheximide, indicating that it is an active process requiring new protein synthesis. Both the caspases inhibitors, ZVAD-fmk and DEVD-cho, inhibited at similar extent apoptosis induced by either DXR or MEN 10755, suggesting an involvement of caspase-3 in this response. We conclude that, in a tumor cell line of epithelial origin, the apoptosis following exposure to anthracyclines is an active process requiring protein synthesis and drug interaction with nuclear structures. The pathway was Fas-independent but likely involved bax and caspase-3 as effectors of the cascade culminating in apoptosis.

Different chemical classes of anticancer drugs can induce tumor cell death by multiple mechanisms. Although the initial intracellular targets of different cytotoxic drugs may be heterogeneous, there is increasing evidence indicating that drug-induced cytotoxicity commonly converges on the induction of programmed cell death (apoptosis). As an example, topoisomerase II inhibitors, such as doxorubicin (DXR), etoposide, or amsacrine, which induce DNA strand breaks, activate genes involved both in DNA repair and in the apoptotic process (Avramis et al., 1998; Wang et al., 1999). Some of the genes activated by DNA-damaging agents are p53-responsive and can modulate cell cycle progression, such as WAF1/p21, proliferating cell nuclear antigen, and GADD45 (Harper et al., 1993) or are directly involved in apoptosis as Fas-L (Mo and Beck, 1999; Wang et al., 1999).

In recent years there has been much interest in studying the role played by the Fas system in the induction of apoptosis of cancer cells following exposure to cytotoxic drugs [see Maggi (1998) for review]. Apoptotic cell death induced by activation of the Fas/Fas-L system requires a multistep cascade of biochemical events: the trimerization of Fas receptor induced by Fas-L stimulates the formation of a death-inducing signaling complex, which consists of the adapter protein FADD and the protease FLICE/caspase-8. The successive interaction of the FADD-death effector domain molecule with caspase-8 activates a caspase cascade, which ends with downstream activation of caspases (caspase-3 and -7) and cleavage of cellular substrates.

It has been reported that the exposure to cytotoxic agents as diverse as DXR, cis-platin, or methotrexate (Friesen et al., 1996; Herr et al., 1997) may promote apoptosis by inducing the expression of Fas/Fas-L in some cancer cell lines (e.g., leukemia), whereas in other instances the induction of apoptosis by cytotoxic drugs has been reported to be independent from the Fas system (Eischen et al., 1997; Gamen et al., 1997; Herr et al., 1997; Tolomeo et al., 1998). However, the final steps of Fas-independent apoptosis induced by cytotoxic drugs seem to proceed through the same downstream caspases (caspase-3 and...
-7) of the death receptors pathway (Eischen et al., 1997; Gamen et al., 1997; McGahon et al., 1998).

Another set of important effectors of apoptosis, involved in drug-induced cell death, is the Bcl-2 family proteins, including a growing number of antiapoptotic (Bcl-2, Bcl-X<sub>L</sub>) and pro-apoptotic (Bax, Bak, Bad, Bid) factors (Chittenden et al., 1995; Yang et al., 1995; Hsu et al., 1997; Luo et al., 1998). Bcl-2-Bax or Bcl-X<sub>L</sub>-Bax complex forms a mitochondrial channel that regulates membrane permeability. Bax up-regulation described in human ovarian (Strobel et al., 1996), colon (Nita et al., 1998), and breast cancer cell lines (Sakakura et al., 1996) enhances the apoptotic response to antineoplastic drugs. The altered ratio between bax and the antiapoptotic components Bcl-2 or Bcl-X<sub>L</sub>, modulate the successive formation of the “apoptosoma” and the final apoptotic caspase cascade (Green and Kroemer, 1998).

We recently reported that the new anthracycline MEN 10755, a topoisomerase II inhibitor (Arcamone et al., 1997), possesses a broader spectrum of antitumor activity than DXR in various human cancer cell lines, which suggests its possible use for treatment of DXR-resistant tumors (Arcamone et al., 1997; Pratesi et al., 1998).

In this study we used a human carcinoma cell line (A2780) to investigate the expression of genes related to the induction of apoptosis, with particular reference to a possible involvement of the Fas system. The A2780 cell line seems particularly suited for this purpose, since it is sensitive to Fas induction following drug treatment (Uslu et al., 1996), showing a low basal expression of Fas protein and absence of Fas-L expression (our data, see below).

Because MEN 10755 exhibits a reduced steady-state accumulation compared with DXR, despite a comparable extent of DNA double-strand breaks and cytotoxicity induced by both drugs (Arcamone et al., 1997), we also investigated whether a cytoplasmic site of action could be the target for apoptosis induced by MEN 10755 or DXR in this cell line. For this purpose we studied whether the two anthracyclines can induce apoptosis in A2780 cells deprived of the nucleus but retaining an intact mitochondrial function (cytoplasts).

**Materials and Methods**

**Chemicals.** Doxorubicin was purchased from Sigma Aldrich Chemical Co. (Oakville, Ontario, Canada), and stock solutions (5 mM) were prepared in distilled water, aliquoted, and stored at −20°C. Propidium iodide (PI), Nonidet P-40, bovine pancreatic RNase A, and cycloheximide (CHX) were purchased from Sigma Chemical Co. (St. Louis, MO). MEN 10755 (batch MB 77/15) was synthesized as previously described (Arcamone et al., 1997), and stock solutions (5 mM) were prepared in distilled water, aliquoted, and stored at −20°C. Agarose was purchased from Life Technologies, Inc. (Gaithersburg, MD). Human recombinant soluble Fas-L (sFas-L) and caspase-3 substrate were purchased from Alexis Corp. (San Diego, CA); sFas-L was always used in combination with an enhancer IgG antibody at a concentration of 1 μg/ml (Alexis Corp., product no. 804-034-C050). Caspase inhibitors ZVAD-fmk and DEVD-cho were purchased from Bachem AG (Switzerland), dissolved in water, and stored at −20°C. Alamar Blue dye, supplied as a ready-to-use solution, was purchased from Biosource International (Camarillo, CA).

**Antibodies.** Monoclonal anti-Fas APO-1-3 was purchased from Alexis Corp., FITC-conjugated goat anti-mouse IgG was from Sigma, mouse IgG<sub>1</sub> isotype control was obtained from PharMingen (San Diego, CA), and ZB4 IgG1-blocking anti-Fas monoclonal antibody was from MBL (Nagoya, Japan).

**Cell Culture and Drug Treatment.** A2780 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS, Life Technologies, Inc.), 2 mM glutamine, 100 units of penicillin, and 100 μg of streptomycin at 37°C in 5% CO<sub>2</sub> and 95% humidified air. Cells were plated in 6-well plates (5 × 10<sup>5</sup> cells/well) and cultured for 16 to 18 h before treatment with various concentrations of drugs. Cells were detached from culture flasks by 0.05% trypsin and 0.02% EDTA treatment (Life Technologies, Inc.) for 5 min and processed for FACS analysis as described below. For experiments with caspase inhibitors, A2780 cells were preincubated with ZVAD-fmk (100 μM) or DEVD-cho (600 μM) for 3 h and then exposed to 1 μM MEN 10755 or DXR for an additional 48 h.

**Cytotoxicity Assay.** The cytotoxicity of DXR and MEN 10755 was determined using the sulforhodamine B (SRB) assay (Skehan et al., 1990). A2780 cells were plated in 96-well microtiter plates in 200 μl of medium. After 24 h, the cells were exposed to drugs at the appropriate dilutions and allowed to incubate additional 1 or 24 h. After drug treatment, cells were washed with saline, incubated in drug-free medium for 72 h, then the cellular viability was measured by the SRB assay. The LC<sub>50</sub> (the concentration used to obtain 50% cellular mortality) was reported as a function of the drug concentration used.

**Determination of Apoptotic Cells by Flow Cytometry and Annexin V/FITC Staining.** Annexin V/FITC staining was performed using the apoptosis detection kit purchased from R&D Systems (Minneapolis, MN). After trypsinization (we assessed that the use of trypsin/EDTA does not interfere with annexin V binding), cells (10<sup>6</sup> cells) were washed twice with cold phosphate-buffered saline (PBS) pH 7.2, then resuspended in binding buffer (HEPES supplemented with 25 mM CaCl<sub>2</sub>). Cells (10<sup>5</sup>) 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, Mountain View, CA). We use bivariate flow cytometry to simultaneously measure log green fluorescence (FL1-height) versus log red fluorescence (FL2-height). Proper flow cytometric analysis was performed using the following controls: unstained cells, cells stained with annexin V-FITC only, and cells stained with PI only. To exclude any overlap of the two emission spectra, we analyzed singly stained cells to adjust electronic compensation. Setting quadrant statistics for determination of the frequency of cells undergoing apoptosis was achieved on untreated cells stained with both annexin V and PI or on untreated cells unstained. After appropriate setting, annexin V-positive cells were identified in the lower right quadrant of the dot plot, whereas annexin V-double-stained cells were identified in the upper right panel (see Vermes et al., 1995).

**Determination of Fas Expression by Flow Cytometry.** Analysis of Fas expression by flow cytometry was performed by standard procedures. Briefly, cells (10<sup>6</sup>) were washed twice with PBS containing FCS 1% (PBS/FCS 1%), centrifuged at 1200 rpm for 10 min and then incubated for 1 h at 0°C with anti-Fas APO-1 and -3 (1 μg/10<sup>6</sup> cells). The cells were washed twice with PBS/FCS 1% and stained for 1 h in the dark at 0°C with FITC-conjugated antibody. Cells were finally washed twice with PBS/FCS 1% and analyzed on a FACSort flow cytometer (Becton Dickinson). Fluorescence signals were collected in logarithmic mode while the relative cell numbers per channel were in linear mode.

**RNase Protection Assay.** Two human template sets, h-Apo-3 and h-Stress-1 (PharMingen), were used for the T7 polymerase-mediated synthesis of high specific activity, 32<sup>P</sup>-labeled, antisense RNA probes, which were hybridized with the target mRNAs (10 μg) and incubated at 30°C for 2 h. The labeled antisense probes were extracted from A2780 cells using the TRIzol reagent (Life Technologies, Inc.), according to the method of Chomezynski and Sacchi (1987). Free probe and other single-stranded RNA mol-
ecules were digested with RNases. The remaining “RNase-protected” probes were purified, resolved on 7 M urea, 5% polyacrylamide gels, and imaged by autoradiography. Two housekeeping gene products, L32 and GAPDH, have been used to assess the total RNA level for normalizing sampling and technical errors. Autoradiographic signals were quantified by densitometry using STORM 840 (Molecular Dynamics, Sunnyvale, CA).

Cytoplasts Preparation. A2780 cells were enucleated as previously described (Wigler and Weinstein, 1975) except for modifications. Cells were detached from tissue culture plates by 0.05% trypsin and 0.02% EDTA treatment (Life Technologies, Inc.) for 5 min and incubated (final concentration of 5 × 10^6 to 5 × 10^7 cells/ml) at 37°C for 45 min in RPMI medium containing 21 μM (10 μg/ml) cytochalasin B. The cell suspension was layered onto a previously prepared discontinuous Ficoll density gradient, consisting of 6 ml of 25%, 6 ml of 17%, 3 ml of 16%, 3 ml of 15%, and 6 ml of 12.5% Ficoll-400, all in RPMI medium. The gradients were centrifuged for 60 min in a prewarmed Beckman SW41 rotor at 25,000 rpm at 33°C. Cytoplasts were collected from the central interface between 12.5% and 15% Ficoll layers, diluted into RPMI 10% FCS, centrifuged, and plated. Enucleation efficiency was determined by staining with propidium iodide followed by evaluation under the fluorescence microscope. More than 90% of the cytochalasin-treated A2780 cells did not contain a nucleus.

Mitochondrial function of cytoplasts was assayed by the ability of cells and cytoplasts to change the oxidized (nonfluorescent) form of Alamar Blue dye to reduced (fluorescent) one. A2780 cells and cytoplasts were seeded in 96-well plates at 6 × 10^4 cells/ml. 2 h later, cells were incubated with the appropriate concentrations of tested compounds. After 18 to 20 h at 37°C, Alamar Blue was added to the medium in an amount equal to 10% of the culture volume and incubated for 20 h at 37°C. The fluorescence (530/590 excitation/emission wavelengths) was measured using a Victor microplate reader (Wallac, Turku, Finland).

Fluorogenic Substrate Assay for Caspase-3 Activity. A2780 cells were seeded in 6-well plates. After 18 to 20 h, DXR and MEN 10755 were added at a final concentration of 1 μM. In a set of experiments, after a 20-h period of incubation at 37°C, the cells were treated with Caspase-L for 1.5, 3, and 6 h. The peak of stimulation was observed at 3 h of incubation, and this time point was chosen for the final experiments.

Cytosolic extracts were prepared by lysing the cells in a buffer containing 1% Triton, 130 mM NaCl, 10 mM Na2HPO4, 10 mM NaF, and 100 μM phenylmethylsulfonyl fluoride. Caspase-3-like activity was determined by incubation of cell lysate with 25 μM the fluorogenic substrates acetyl-Asp(Om)-Glu(Om)-Val-Asp(Om)-aminomethylcoumarin (Ac-DEVD-amc) in a 200 μl of cell-free system buffer, comprising 20 mM HEPES, 10% glycerol, and 2 mM dithiothreitol. After 2 h at 37°C, the release of fluorescence was measured using a Victor microplate reader (Wallac Oy, Turku, Finland).

Results

Anthracycline-Induced Apoptosis in A2780 Cells. As a preliminary step for further studies on the involvement of the apoptotic pathway in anthracycline-induced cell death, we first compared the induction of apoptosis by MEN 10755 and DXR by using cytofluorimetry and annexin V/PI staining. Since the two anthracyclines have comparable cytotoxic potency on A2780 cell line (MEN 10755 LC50 = 27± 11 nM, DXR LC50 = 9 ± 2 nM after 24 h of treatment and successive 72 h in the absence of drug), we used equimolar concentrations of both compounds for further experiments. In particular the induction of apoptosis was measured at 1 and 0.1 μM, corresponding to the LC50 after 48 and 72 h, respectively, of continuous drug exposure. These concentrations are comparable with drug levels observed after a single administration of 7 mg/kg, i.e., of DXR or MEN 10755 in A2780 tumor xenografts on nude mice (data not shown). After 48 h of exposure, MEN 10755 and DXR (both at 1 μM) induced apoptosis in 35.7± 2.1% and 53.8± 3.5% of cells. At 0.1 μM, the percentage was not significantly different from untreated cells. At 72 h, apoptosis became detectable even at 0.1 μM and was comparable for both drugs (Fig. 1). We also observed that anthracycline-induced apoptosis is partially inhibited following CHX treatment (1 μg/ml for 48 h): a 54% reduction of apoptotic cells for both MEN 10755 or DXR (0.1 μM) was observed (data not shown), indicating that a novel synthesis of cytoplasmic or nuclear factors is mandatory for the apoptotic pathway.

MEN 10755 Increases Transcription of Fas, bax, WAF1/p21, and GADD45 mRNAs but Does Not Induce Fas-L mRNA. We used an RNase protection assay to analyze the role of a panel of genes involved in the apoptotic pathway in anthracycline-induced cell death in A2780 cells.

The time course of mRNA synthesis showed a significant increase in the transcription of genes involved in cell cycle arrest and DNA repair (WAF1/p21, GADD45) and of pro-apoptotic genes, such as bax and Fas, following incubation with 0.1 μM MEN 10755 for 6 to 48 h (Fig. 2). The same effect was observed with 0.1 μM DXR. Transcription of genes involved in TNF signaling (TNFR, RIP) and of Fas-L, FADD, and FLICE genes was unaffected by anthracycline treatment, whereas FAF mRNA was slightly down-regulated. Wild-type p53 and bcl-X mRNA levels were comparable with controls, whereas bcl-2 was undetectable in our system.

MEN 10755 Increases the Expression of a Functional Fas Receptor on A2780 Cell Membrane. The expression of Fas protein on the A2780 cell membrane was assessed through flow cytometry. We found that Fas expression correlates quite well with the increase in mRNA transcription. A peak of expression was observed at 14 to 24 h with 0.1 μM DXR and at 24 to 48 h with 0.1 μM MEN 10755 (Table 1). When anthracycline-induced Fas was triggered by exogenous Fas-L (100 ng/ml), an increase of apoptotic cell death occurred, indicating that the Fas signaling pathway is functionally active (Table 2). To assess whether activation of Fas could be involved in anthracycline-induced cell death in this
cell line, we further analyzed the effect of an antagonist ZB4 antibody on the anthracycline-induced apoptosis. ZB4 (2 μg/ml) potently blocked the increase of apoptosis following application of Fas-L, but it did not interfere with MEN 10755 or DXR (both 1 μM)-induced apoptosis, also using longer incubation times (48 h) (data not shown).

**Cytoplast Sensitivity to Anthracycline-Induced Cell Death.** It has been demonstrated that enucleated cells (cytoplasts) are sensitive to Fas-mediated cytotoxicity with the same kinetics and cytoplasmic changes as their nucleated counterparts (Nakajima et al., 1995). We tested cytoplasts to confirm the hypothesis that the nucleus is the prominent target of MEN 10755-induced cytotoxicity and to further verify that the Fas/Fas-L system is not the effector of MEN 10755-induced apoptosis.

As previously described, we confirmed that cytoplasts re-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>6 h</th>
<th>14 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.0 ± 3.5</td>
<td>13.6 ± 3.2</td>
<td>14 ± 2.3</td>
<td>10.9 ± 5.7</td>
<td>10.4 ± 1.7</td>
</tr>
<tr>
<td>DXR</td>
<td>2.7 ± 1.0</td>
<td>45.2 ± 5.4**</td>
<td>54.9 ± 6.5**</td>
<td>19.1 ± 9.0</td>
<td>23.3 ± 11.5</td>
</tr>
<tr>
<td>MEN 10755</td>
<td>3.2 ± 1.5</td>
<td>15.1 ± 0.8</td>
<td>30.6 ± 0.4*</td>
<td>26.8 ± 7.0</td>
<td>21.2 ± 9.6</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01.
MEN 10755 37.5 ± 4.3 41.6 ± 4.8 72.8 ± 10.9* 39.3 ± 5.9

* p < 0.05.

When anthracycline-regulated Fas receptor was stimulated with Fas-L (100 ng/ml) for 3 h, caspase-3 activity showed a consistent increase of caspase-3 activity after 18 to 24 h of treatment (Fig. 4), as previously described in other tumor cell lines (Datta et al., 1996; Los et al., 1997).

When anthracycline-regulated Fas receptor was stimulated with Fas-L (100 ng/ml) for 3 h, caspase-3 activity showed a more relevant increase than in the presence of MEN 10755 or DXR alone, indicating the existence of a distinct and additive pathway of caspase-3 activation for MEN 10755 or DXR treatment compared with CD95 triggering (Fig. 5).

We further studied the role of other caspases in anthracycline-induced apoptosis using two well known caspase inhibitors, ZVAD-fmk, a caspase inhibitor of wide specificity and DEVD-cho, that inhibits more efficiently caspase-3 activity. As shown in Fig. 6, both 100 µM ZVAD-fmk and 600 µM DEVD-cho, incubated with 1 µM MEN 10755, reduced apoptosis from 31.9 ± 1.0% to 6.3 ± 1.1 and 11.9 ± 2.8%, respectively, suggesting that caspase-3 is one of the effector caspases involved in apoptotic cell death mediated by these anthracyclines.

**Discussion**

MEN 10755 is a recently discovered disaccharide anthracycline, characterized by a potent antitumoral activity against a large number of human tumor xenografts, and it is currently undergoing phase I clinical studies. Previous studies (Arcamone et al., 1997) indicate that the enzyme topoisomerase II is the main target for the cytotoxic action of MEN 10755: the drug stimulates topoisomerase II cleavage of DNA and displays a remarkable ability to elicit DNA single- and double-strand breaks, transforming the enzyme into a DNA-damaging agent.

Although the events interposed between the stabilization of the cleavage complex and the loss of cell viability have not yet been identified, the net result is the initiation of cell death, occurring through cell cycle arrest, and induction of necrosis or apoptosis. The results of the present study indicate that apoptosis initiated by exposure of A2780 cells to either MEN 10755 or DXR: 1) proceeds through an essentially similar pathway, requiring the presence of the nucleus (as demonstrated by experiments with cytoplasts) and active protein synthesis (inhibition by CHX); 2) occurs concomitantly through the expression of a panel of genes related to the induction of apoptosis; and 3) impinges on the caspase effector pathway, which is pharmacologically similar (possibly involving caspase-3) to that activated through the Fas/Fas-L pathway. In the A2780 cell line, exposure to anthracyclines induces Fas expression, and this response is linked to a functional Fas-dependent apoptosis (induced by Fas-L), yet the Fas/Fas-L pathway is not involved in anthracycline-induced apoptosis.

Overexpression of Bax mRNA fits well with the apoptosis kinetics observed in MEN 10755-treated A2780 cells and could be one of the molecular mechanisms responsible of MEN 10755-induced apoptosis (Figs. 1 and 2C). Bax, a member of Bcl-2 family of proteins, forms channels in mitochondrial membranes and is able to induce apoptosis in some cancer cell lines, inducing directly cytochrome c release (Sakakura et al., 1996; Strobel et al., 1996): this leads to the activation of downstream caspases and consequently to apoptosis.

WAF1/p21 and GADD45 are p53-dependent genes associated with growth control and cell cycle checkpoints following DNA damage, causing a G1 or G2/M arrest to permit DNA repair before replication or mitosis (Wang et al., 1997; Medema et al., 1998). When DNA repair fails, apoptosis can occur to eliminate irreparably damaged cells. The increase of WAF1/p21 and particularly GADD45 mRNA in A2780 cells, could explain a G2/M block that we observed by cytofluorimetric analysis of PI stained cells after 24 h of MEN 10755 treatment (data not shown).

Since a p53-sensitive region has been described in the gene promoter of the up-regulated pro-apoptotic factors (Chan and Owen-Schaub, 1995; Amundson et al., 1998), it is possible that, in this model of A2780 cells, the Fas up-regulation we observed was caused by the activation of p53 tumor suppressor protein, as already described (Ruiz-Ruiz and Lopez-Rivas, 1999). We did not detect any increase in mRNA p53 transcription following drug treatment, but it has been published that p53 activation is not always related to an increase of p53 mRNA or protein (Caelles et al., 1994; Wagner et al., 1994).

In our study bcl-X was expressed at a steady-state level both in controls and MEN 10755-treated cells, in the absence of Bcl-2 expression. Given that Bcl-X may suppress cell death in the same way as Bcl-2, the functional redundancy between these apoptosis inhibitors may compensate for the absence of Bcl-2 (Nita et al., 1998).
The possibility of targets additional to nuclear topoisomerase II was suggested by the findings of Serafino et al. (1999), sustaining that anthracyclines may also act through mechanism(s) involving the cytoplasmic compartment. The reduced cellular uptake of MEN 10755 as compared with DXR (Arcamone et al., 1997), despite comparable cytotoxic effects, allowed us to investigate this hypothesis, further supported by the observation of a more relevant cytoplasmic localization of MEN 10755 (data not shown). In the present article, we demonstrated that enucleated cells were not sensitive to anthracycline treatment, suggesting that “cytoplasmic” mechanisms could not be “per se” sufficient to induce cell death, although we cannot exclude the possibility of there being additional targets to nuclear topoisomerase II.

On the basis of our data we hypothesize that MEN 10755, after induction of DNA damage-responsive genes, alters mitochondrial membrane through the synthesis of pro-apoptotic factors such as Bax (Chittenden et al., 1995; Yang et al., 1995; Hsu et al., 1997; Luo et al., 1998), which induce cytochrome c release, procaspase-9-Apaf-1 triggering, and finally, effector caspases activation. We also observed that CHX treatment blocks anthracycline-induced apoptosis, further supporting the hypothesis that new protein synthesis is required for MEN 10755 or DXR apoptotic activity. When we evaluated the caspase inhibitors ZVAD-fmk and DEVD-cho, we found MEN 10755 or DXR apoptosis inhibited by both of them. Although it has been clearly demonstrated that the broad-spectrum caspase inhibitor ZVAD-fmk prevented drug-induced apoptosis (Tolomeo et al., 1998; Sun et al., 1999; Wesselborg et al., 1999), the effect of the inhibitor DEVD-cho, more specific for caspase-3, is not so clear (Tolomeo et al., 1998). In our hands, both peptides displayed a similar inhibitory effect on anthracycline-induced apoptosis, suggesting an important role for caspase-3 activation.

In conclusion, we demonstrated that MEN 10755-induced
cells were statistically compared with untreated cells (*p < 0.01). References

Binaschi for helpful discussion, and Simona Bozzitelli for typing and Mauro Piacentini (University of Rome Tor Vergata), Dr. Monica Acknowledgments and induction of apoptosis.
tive differences in cellular targets relevant for cytotoxicity al., 1996), cannot be explained on the basis of major qualita-
toward cancers, which are not responsive to DXR (Pratesi et
is fully functional and the engagement of the receptor by
receptor triggering. However, the anthracycline-induced Fas
centrations clinically significant, but is independent of Fas
ment of efficacy in vivo.
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Chan H and Owen-Schaub L (1995) Identification and analysis of a p53 binding

Fig. 6. Apoptosis inhibition following treatment with caspase inhibitors ZVAD-fmk or DEVD-cho. A2780 cells were preincubated for 3 h with 100 μM ZVAD-fmk or 600 μM DEVD-cho and then incubated also with 1 μM MEN 10755 or DXR for additional 48 h. The percentage of apoptotic cells was evaluated by cytofluorimetry using annexin V/PI staining. DXR + ZVAD-fmk or DXR + DEVD-cho, MEN 10755 + ZVAD-fmk, and MEN 10755 + DEVD-treated cells were statistically compared with untreated cells (p < 0.05, **p < 0.01).

apoptosis is correlated to bax up-regulation in a cellular model of human ovarian cancer cells wild-type p53, at concentrations clinically significant, but is independent of Fas receptor triggering. However, the anthracycline-induced Fas is fully functional and the engagement of the receptor by exogenous Fas-L determines a further increase in the apoptotic response induced by drugs, thus enhancing the total cytotoxic effect.

On the basis of the findings presented in this article, the broad spectrum of antitumor activity exerted by MEN 10755 toward cancers, which are not responsive to DXR (Pratesi et al., 1996), cannot be explained on the basis of major qualitative differences in cellular targets relevant for cytotoxicity and induction of apoptosis.

Acknowledgments

We acknowledge the expert review and comments from Dr. Franco Zunino (Istituto Nazionale Tumori, Milan, Italy) and from Prof. Mauro Piacentini (University of Rome Tor Vergata), Dr. Monica Binaschi for helpful discussion, and Simona Bozzitelli for typing and editing the manuscript.

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