Abstract. The use of anthracyclines as antitumor drugs dates back to the 1970s, but the mechanism of the cytotoxicity of these compounds has long been a matter of debate. There is increasing evidence indicating that drug-induced cytotoxicity commonly converges on the induction of apoptosis. Many authors point to the fact that double-strand breaks, resulting from stabilization of cleavable complexes, are the signal for the initiation of the apoptotic cascade. In this work, the possible correlation between stabilization of topoisomerase II (topoII) - DNA complexes, apoptosis induction and cytotoxicity was studied. Parental human cervix carcinoma cells, HeLa, and its subline resistant to vinblastine, KB-V1, were exposed to doxorubicin (DOX) and the novel anthracyclines annamycin and WP903, given at the concentrations 0.2 and 2.0 μg/ml (DOX and annamycin) or 0.2 and 1.0 μg/ml (WP903). It was found that annamycin was the strongest topoII poison in HeLa cells at both concentrations used, whereas poly (ADP-ribose) polymerase (PARP) cleavage was observed dose-dependently in KB-V1 cells treated with annamycin or WP903. Simultaneously, apoptosis, observed as cell morphology or phosphatidylserine translocation, was evident in both cell types exposed to the novel anthracyclines, independent of concentration. DOX appeared to be the weakest apoptotic inducer. On the basis of these studies, it can be suggested that topoII poisoning is not the key process leading to apoptosis and seems to be cell specific. PARP cleavage is probably not an evident marker of anthracycline-induced apoptosis which, in turn, does not seem to be the determinant in the cytotoxic action of these compounds. The efficiency of anthracycline antibiotics, interpreted as cytotoxic action, was dependent on cell type.

Anthracyclines are potent, broad-spectrum chemotherapeutic agents, effective against solid tumors and malignant hematological disease (1, 2). Although the initial intracellular targets of this group of anticancer drugs may be heterogenous, there is increasing evidence indicating that drug-induced cytotoxicity commonly converges on the induction of programmed cell death (apoptosis). One of the most important routes resulting in the apoptotic cascade in cells treated with anthracyclines is the stabilization of the topoisomerase II (topoII) - DNA cleavable complex (3, 4). Cardoso et al. (5) note that topoIIα is the target of anthracyclines, and preliminary data suggest its promising role as a predictive marker of sensitivity to these drugs.

DNA topoisomerases are ubiquitous enzymes that can solve the topological problem associated with DNA generated during the key cellular processes such as replication, transcription, recombination, repair and chromatin assembly. TopoII introduce transient breaks in both DNA strands (6). Anthracyclines interrupt the breakage / reunion reaction of topoII resulting in accumulation of a topoII-DNA covalent intermediate, the cleavable complex (3, 4). This makes topoisomerases powerful poisons that cut up DNA and damage the genome. This action leads to activation of stress-associated signaling pathways, cell cycle arrest and activation of the biochemical cascade of apoptosis (3, 7). However, the details of the downstream signaling from cleavable complexes to cell death are poorly understood (3, 8).

Many authors point to the fact that double-strand breaks, resulting from stabilization of cleavable complexes and the
inability of the cells to repair DNA lesions, are the signal for apoptosis (9-11). However, the role of topoII and cleavable complexes in anthracycline cytotoxicity is still controversial (12, 13).

Another doubt discussed in many papers is the role of apoptotic processes in the death of tumor cells exposed to anthracyclines. Some authors have shown that anthracyclines induce apoptosis, but that this event is not directly related to cell death. Fedier et al. (14) found that DOX was a strong inducer of apoptotic processes in mouse lung fibroblasts, regardless of their susceptibility to that agent.

Due to the serious problem of multidrug resistance and high cardiotoxicity in chemotherapy and, in particular, anthracycline therapy, new anthracycline derivatives with better therapeutic indices are being sought.

As a part of a collaboration with the Priebe group, at the MD Anderson Cancer Center, Houston, Texas, USA, for the screening studies of a number of novel anthracyclines, WP903 and annamycin were chosen as the most promising drugs in anticancer therapy. DOX, as a known agent, was treated as the reference drug.

The aim of this work was to define a possible correlation between the topoII poison activity of the compounds, apoptosis intensity and cytotoxic activity.

Apoptotic events were morphologically evaluated using the fluorescence microscope and in PARP cleavage assay. Stabilization of cleavable complexes as well as PARP cleavage were studied using the Western blot technique.

**Materials and Methods**

**Drugs.** Doxorubicin (DOX) was purchased from Fluka, Germany; WP903 and annamycin were from the MD Anderson Cancer Center, Houston, TX, USA.

**Cells.** The HeLa human cervix carcinoma cell line and its subline resistant to vinblastine, KB-V1, were purchased from the American Tissue Culture Collection. Cells were grown in MEM supplemented with 10% fetal calf serum and antibiotics. KB-V1 cells were maintained in MEM with vinblastine, 0.5 μg/ml.

**Statistical analysis.** The statistical evaluation of the results was performed using Student’s t-test and Cochrane’s Cox test for unrelated samples.

**Results**

**Cytotoxic activity.** The cytotoxic activity of the drugs tested was determined by the MTT test. As shown in Table I, both cell types exhibited comparable sensitivity to all drugs tested.
However, it can be observed that annamycin is about 3-fold less cytotoxic than DOX or WP903. The cytotoxic activities of DOX and WP903 were comparable.

Stabilization of cleavable complexes. The effect of stabilization of cleavable complexes topoII-DNA was observed using the Western blot technique. Anthracyclines can block topoII in complexes with DNA, resulting in lower intensity quenching of the topoII bands. As shown in Figure 1b, the strongest effect of topoII blocking in cleavable complexes occurred in the case of annamycin given to HeLa cells at both concentrations, i.e., 0.2 and 2.0 µg/ml. Slight quenching of the topoII band was observed in KB-V1 cells exposed to annamycin at the 0.2 µg/ml concentration (Figure 1b). Neither DOX nor WP903 was noted as having any influence on topoII-DNA complexes (Figure 1a, c).

Apoptosis induction. Cell morphology was evaluated in the cell cultures treated with the drugs studied in comparison with the control cell cultures (untreated). The use of Hoechst 33342 allows for the monitoring of any structural changes in genetic material, due to intercalation into DNA, which results in a

Figure 1. Stabilization of cleavable complexes topo II-DNA by DOX (a); annamycin (b); WP903 (c). Cell cultures were treated according to the procedure given in Materials and Methods. Results represent the mean ± SEM.
blue fluorescence visible by fluorescence microscope. Typical late apoptotic symptoms appeared in HeLa and KB-V1 cells exposed only to new anthracyclines, i.e., annamycin or WP903, independently of the concentrations used. The treatment resulted in rings of condensed chromatin around the nuclear membranes and apoptotic body formation (data not shown). The same significant difference in apoptotic induction between DOX and WP903 was also confirmed in annexin V assay by flow cytometry (Gruber, unpublished).

**PARP cleavage.** Apoptosis observed from morphological features was confirmed with the PARP cleavage assay employing the Western blot technique. PARP, 116 kDa, is a nuclear enzyme. Its proteolytic cleavage, linked to the activation of caspase-3 and -7, is one of the apoptotic effects and results in the formation of fragments such as 86 kDa, which can be detected with specific antibodies.

As presented in Figure 2b, the strongest effects defined as PARP cleavage were noted in KB-V1 cells treated with annamycin, 2 µg/ml or with WP903, 0.2 or 1.0 µg/ml (Figure 2b, c). A slight PARP cleavage effect was observed in HeLa cells treated with: DOX, 2.0 µg/ml; annamycin, 2.0 µg/ml; or with WP903, 0.2 or 1.0 µg/ml (Figure 2a, b, c).

**Discussion**

The cytotoxic effect of anthracycline antibiotics is multidirectional. One of the mechanisms consists of blocking
the cleavable DNA-topoII complexes governing the super-
helicity of DNA strands. It is well established that DNA
double-strand breaks can trigger apoptotic cell death (9, 17, 
18). It is suggested that the phenomenon of blocking cleavable 
complexes leads to cell death by a mechanism not entirely 
elicited (13). A very important target site for anthracycline 
antibiotics are cell proliferation systems including apoptotic 
processes. The effect of this group of compounds as apoptotic 
stimulators on tumor cells is indirect, i.e., they influence the 
activity of specific genes and their products, regulatory 
proteins (19). As given by Li and Liu (9), the blocking of 
cleavable topoII-DNA complexes may be involved in the 
commitment step of apoptotic cell death.

The results obtained in this study do not confirm any 
correlation between stabilization of topoII-DNA complexes and 
apoptosis induction observed from specific morphology (data 
not shown) and PARP cleavage, confirmed with membrane 
phosphatidylserine translocation assay (Gruber, unpublished). 
In addition, the cytotoxic activity of the drugs tested does not 
seem to be dependent on these apoptotic events. These results 
do not confirm the observations made by some authors that 
reduced levels of topoII proteins or gene expression are 
correlated with cell sensitivity to anthracyclines. Such a 
conclusion was drawn by Evans et al. (20), testing small-cell lung 
cancer cell lines H69AR resistant to DOX and its parenteral H69 
cell line. Park et al. (21) have noted that the response of breast 
cancers to DOX chemotherapy was increased in the cases with 
topoII amplification, whereas the response rate was significantly 
decreased in the case without topoII amplification. De Jong et 
al. (22) have suggested that resistance of the human small cell 
lung carcinoma cell line, GLC4, to DOX was, in part, due to 
the reduced drug-induced formation of the cleavage complexes.
However, Den Boer et al. (10) have noted that neither topoIIa 
expression, nor activity correlated with the anthracycline 
cytotoxicity in leukemic cells. According to the authors, topoIIa 
may be an indicator of the proliferative status of cells without a 
direct relationship with anthracycline resistance in acute 
leukemia. Some authors point to the fact that the cytotoxicity 
of lesions generated by stabilization of cleavable complexes 
depends on the locus of the interactions: drug-topoII-DNA (7).

Apoptosis, a carefully regulated process of cell death, 
may proceed through mechanisms varying according to cell 
type (23). The results obtained by Ling et al. (24) have 
demonstrated that the cell-killing effect observed in murine 
leukemia cells, P388, is mediated, at least in part, by the 
induction of apoptosis. Such correlation was not exhibited in 
the current study with human cervix carcinoma cells, HeLa 
and KB-V1, and is in agreement with our earlier work on 
human melanoma cells treated with DOX (16), as well as that 
of Gariboldi et al. (25) who studied non-small cell lung 
carcinoma cells exposed to DOX and found no relationship 
between drug response and expression and/or subcellular 
localization of apoptosis-related proteins.

As shown by many authors, apoptosis induced by 
anthracyclines results in caspase activation, especially 
that of caspase-3, which cleaves PARP (26, 27). Faderl et 
al. (28) have noted that apoptosis induced by WP744, a 
novel anthracycline, in human acute myeloid leukemia 
cell lines K562, KBM-3 and OCIM-2 appeared to be 
mediated by caspase-3. WP744 induced cleavage of 
PARP. Caspase activation, in association with PARP 
cleavage and protein kinase C delta, has been confirmed 
by Huigsloot et al. (29) in rat mammary adenocarcinoma 
MTLn3 cells exposed to DOX.

Our results point to the fact that PARP cleavage is not 
such an evident marker of anthracycline-induced apoptosis, 
and that such a route of apoptosis may depend on the cell 
type or time of exposure to the drug. Yeung et al. (30) have 
noted that 18-h-treatment with DOX did not induce PARP 
cleavage in human anaplastic thyroid cell lines. Holleman 
et al. (31) did not observe the correlation between caspase-
3 activation or PARP cleavage and resistance to 
daunorubicin in human acute lymphoblastic leukemia 
cell lines. According to the authors, it seems that caspase-3 
activation and PARP inactivation are not essential for 
daunorubicin-induced apoptosis. Cell specificity of that 
apoptotic event has also been shown by Hopkins-
Donaldson et al. (32) in neuroblastoma cells, invasive 
(N-type cells) and non-invasive (S-type cells), treated with 
DOX. DOX was shown to induce caspase-3, -7, -8 and -9 
avtivation in S-type cells whereas N-type cells were killed 
by a caspase-independent mechanism.

The different cell responses and cytotoxic efficiencies of 
the drugs reported here suggest that the mechanisms 
responsible for the cytotoxic activity of anthracyclines are 
cell specific. This observation was confirmed for 
annamycin, which was 3-fold less cytotoxic than DOX for 
HeLa and KB-V1 cells. Ling et al. (24) have reported 
annamycin as 50-fold more cytotoxic than DOX against 
P388 cells resistant to DOX.

The results obtained here allow for the following 
conclusions to be drawn:
1. Stabilization of cleavable topoII-DNA complexes by 
anthracyclines seems to be a cell-specific process.
2. Stabilization of cleavable complexes is not condition 
sine qua non for apoptosis induction by anthracyclines.
3. PARP cleavage is not an evident marker of 
anthracycline-induced apoptosis, and this event depends on the cell type.
4. Apoptotic events do not seem to be the determinants in the 
cytotoxic action of anthracyclines.
5. The cytotoxic activity of anthracyclines depends on their 
chemical structure.

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References


