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In vivo inhibition of etoposide-mediated apoptosis, toxicity, and antitumor effect by the topoisomerase II-uncoupling anthracycline aclarubicin

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Abstract. A number of clinically important drugs such as the epipodophyllotoxins etoposide (VP-16) and teniposide (VM-26), the anthracyclines daunorubicin and doxorubicin (Adriamycin), and the aminoacridine amsacrine exert their cytotoxic action by stabilizing the cleavable complex formed between DNA and the nuclear enzyme topoisomerase II. We have previously demonstrated in several in vitro assays that the anthracycline aclarubicin (aclacinomycin A) inhibits cleavable-complex formation and thus antagonizes the action of drugs such as VP-16 and daunorubicin. The present study was performed to validate these in vitro data in an in vivo model. At nontoxic doses of 6 and 9 mg/kg, aclarubicin yielded a marked increase in the survival of non-tumor-bearing mice given high doses of VP-16 (80–90 mg/kg) in six separate experiments. In therapy experiments on mice inoculated with Ehrlich ascites tumor cells, aclarubicin given at 6 mg/kg roughly halved the increase in median life span induced by VP-16 at doses ranging from 22 to 33 mg/kg. An attempt to determine a more favorable combination of VP-16 and aclarubicin by increasing VP-16 doses failed, as the two drugs were always less effective than VP-16 alone. The way in which VP-16-induced DNA strand breaks lead to cell death remains unknown. However, VP-16 has been reported to cause apoptosis (programmed cell death) in several cell lines. To ascertain whether the protection given by aclarubicin could have a disruptive effect on the apoptotic process, we used the small intestine as an in vivo model. Whereas VP-16-induced apoptosis in crypt stem cells was detectable at a dose as low as 1.25 mg/kg, aclarubicin given at up to 20 mg/kg did not cause apoptosis. Indeed, aclarubicin caused a statistically significant reduction in the number of cells rendered apoptotic by VP-16. The present study thus confirms the previous in vitro experiments and indicates the value of including an in vivo model in a preclinical evaluation of drug combinations.

Key words: Etoposide – Topoisomerase II – Apoptosis

Introduction

The epipodophyllotoxin etoposide (VP-16) is a clinically important drug with activity in a variety of malignant diseases, notably small-cell lung cancer (SCLC), germ-cell tumors, and acute myeloid leukemia. VP-16 exerts its cytotoxic effect by stabilizing so-called cleavable complexes formed by DNA and the nuclear enzyme topoisomerase II [15, 17]. These stabilized cleavable complexes lead in a yet unknown way to cell death. Other drug types such as aminoacridines and anthracyclines also act in a similar fashion by inducing DNA breaks via interaction with topoisomerase II [23]. However, in investigating a number of anthracyclines, we discovered that aclarubicin differed in its cytotoxicity profile in a panel of SCLC cell lines [9]. This discovery led to several in vitro investigations in which we demonstrated that aclarubicin differed significantly from the classic anthracyclines such as daunorubicin and doxorubicin in that instead of inducing cleavable-complex formation, aclarubicin actually inhibited this process [10, 11]. Furthermore, aclarubicin was capable of inhibiting both the cleavable-complex formation induced by VP-16 and daunorubicin and the in vitro cytotoxicity induced by these two drugs in SCLC cell lines [10, 11].

Thus, we have demonstrated the in vitro antagonistic effect of a clinically used anthracycline on typical topoisomerase II-directed drugs. Accordingly, the present study was conducted to investigate whether these in vitro results would translate into the in vivo situation, especially as the clinical use of the combination of aclarubicin and VP-16 has been proposed [20, 27]. Furthermore we wished to

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examine the possible role of aclarubicin in the disruption of programmed cell death (apoptosis).

Materials and methods

Drugs. Aclarubicin was obtained from Lundbeck (Copenhagen) and dissolved in sterile water to a concentration of 2 mg/ml. VP-16 was purchased from Bristol-Myers Squibb (Copenhagen) in solution for clinical use (20 mg/ml).

In vitro clonogenic assay. Drug toxicity in vitro was assessed by colony formation in soft agar with a feeder layer containing sheep red blood cells as described elsewhere [9]. Ehrlich ascites tumor cells of the EHR2 strain [5] were treated either with aclarubicin alone in increasing doses of up to 1.0 μg/ml or with a combination of aclarubicin and VP-16. The numbers of cells plated in control dishes were adjusted to obtain 2000–3000 colonies. Colonies were counted after 21 days.

In vivo experiments. First-generation hybrids of female random-bred Swiss mice and male inbred DBA mice were used as previously described [5]. The mice weighed between 19 and 21 g at the start of the experiments. The drugs were given i.p. on day 0 and deaths were observed for up to 60 days, after which the surviving mice were euthanized. In treatment studies, mice were inoculated i.p. with 15×10^6 EHR2 cells on day 0 and treated with drugs on day 4.

To get an indication of the site of the protection provided by aclarubicin, we performed a number of toxicology studies in non-tumor-bearing mice. For evaluation of hematologic toxicity, a 20-μl blood sample was drawn from a tail vein and stained with methyl violet for leukocyte counting. Blood samples were analyzed on days 3, 5, and 7, and every mouse was individually marked. The samples were counted in duplicate in a hemocytometer, and the variation between duplicates was always less than 15%. In addition, the spleen, sternum, lungs, liver, pancreas, lymph nodes, thymus, brain, kidneys, stomach, and small intestine were removed and immediately fixed in 4% buffered formalin. The organs were imbedded in paraffin and sections were stained with hematoxylin and eosin for light microscopy.

For evaluations of apoptosis [14, 26] in the stem cells in the crypts of the small intestine as described by Anilkumar et al. [2], animals were euthanized 3 h after they had received the drugs, as the yield of apoptotic cells is high at this time [2]. The piece of intestine fixed was the last 75% measured from the pyloric sphincter to the ileal/cecal junction [19]. The sections were blinded and apoptotic cells were counted in 50 random axially sectioned crypts. The results were statistically analyzed by Student's *t*-test.

Results

The results obtained in the clonogenic assay are presented in Fig. 1. VP-16 alone (60 μ*M*) reduced survival to less than 1% of the EHR2 cells. When the cells were preincubated with increasing doses of aclarubicin, the amount of surviving cells increased in a dose-dependent manner. Thus, as has previously been shown in the human SCLC cell line OC-NYH [10], aclarubicin antagonizes the cytotoxic effect of VP-16.

Using the log-probit method [5], we determined the dose that was lethal to 10% of the animals (LD_{10}) for VP-16 given as a single i.p. injection and found it to be 60 mg/kg and the LD_{90} to be 99 mg/kg. The LD_{10} for aclarubicin in the same strain of mice has been determined to be 16 mg/kg

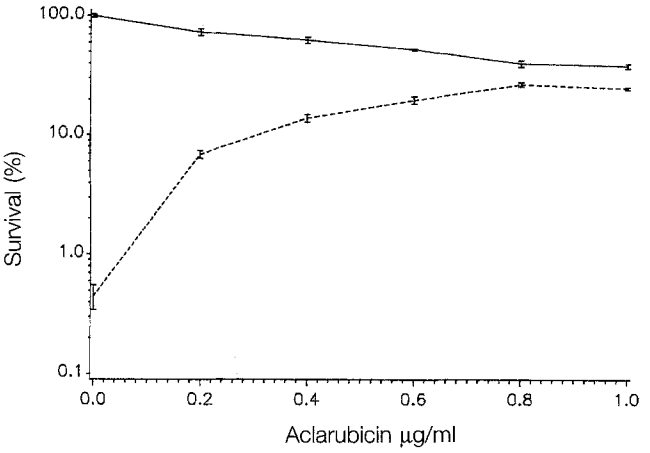

Fig. 1. Clonogenic assay using EHR2 cells, demonstrating that increasing concentrations of aclarubicin (*abscissa*) antagonize the cytotoxicity of VP-16. Drug incubation was carried out for 1 h. Bars equal the SEM of colony counts in obtained three petri dishes. ———, Aclarubicin alone; --------, 60 μ*M* VP-16 + aclarubicin

Table 1. Summary of 6 different experiments on VP-16 toxicity in non-tumor-bearing mice

Experiment number	Number of mice	VP-16 (mg/kg)	Survivors/ treated	ACLA + VP-16 (mg/kg)	Survivors/ treated
1	15	80	7/15 (47%)	9+80	13/15 (87%)
1	15	85	5/15 (33%)	9+85	11/15 (73%)
1	15	90	3/15 (20%)	9+90	12/15 (80%)
2	11	90	2/11 (18%)	6+90	9/11 (82%)
3	15	85	3/15 (20%)	6+85	12/15 (80%)
3	15	85	3/15 (20%)	6+85 (s)	11/15 (73%)
4	15	85	9/15 (60%)	9+85	14/15 (93%)
4	15	85	9/15 (60%)	9+85 (s)	13/15 (87%)
5	15	90	5/15 (33%)	9+90	14/15 (93%)
5	15	90	5/15 (33%)	9+90 (s)	9/15 (60%)
6	15	90	5/15 (33%)	9+90	11/15 (73%)
6	15	90	5/15 (33%)	9+90 (s)	10/15 (67%)

The protective effect of a low nontoxic dose of aclarubicin on VP-16 toxicity is demonstrated. ACLA, Aclarubicin; (s), simultaneous administration of aclarubicin and VP-16 (in all other experiments, aclarubicin was given i.p. 20 min before VP-16)

in similar single-injection i.p. therapy, and the LD_{90} has been found to be 30 mg/kg (Torben Skovsgaard, personal communication). As would therefore be expected, aclarubicin doses of 6 and 9 mg/kg were atoxic, and no death was registered in these groups. Table 1 shows that VP-16 given at doses ranging from 80 to 90 mg/kg resulted in a

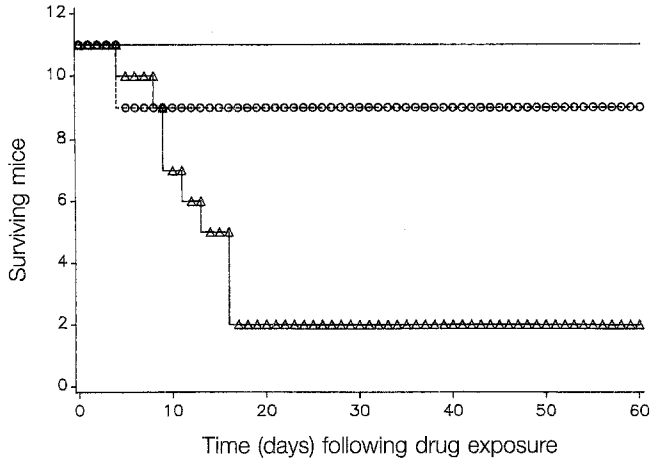

Fig. 2. Toxicity of VP-16 alone and of the combination of aclarubicin and VP-16 in non-tumor-bearing mice. The figure illustrates experiment 2 in Table 1. ———, 6 mg/kg aclarubicin; ΔΔΔΔΔΔ, 90 mg/kg VP-16; ΘΘΘΘΘΘ, 6 mg/kg aclarubicin + 90 mg/kg VP-16

Table 2. Summary of experiments conducted on tumor-bearing mice

Number of experiments	Dose (mg/kg)	Variation in median survival (days)	Median ILS (%)
7	NaCl + NaCl	12–15	
7	ACLA + NaCl (6)	12–16	8
4	NaCl + VP-16 (11)	14–16	8
6	NaCl + VP-16 (22)	18–29	66
2	NaCl + VP-16 (33)	22–28	92
4	ACLA + VP-16 (6 + 11)	13–15	0
6	ACLA + VP-16 (6 + 22)	14–18	15
2	ACLA + VP-16 (6 + 33)	17–22	54

An antagonistic effect of aclarubicin on VP-16 therapy is demonstrated in mice with Ehrlich ascites tumor. In all, 15 mice were used in each treatment group in each experiment. ACLA, Aclarubicin; ILS, increase in life span

60-day survival of 18%–60% of the mice in the different experiments. When these high doses of VP-16 were combined with the nontoxic dose of aclarubicin, a marked increase in the percentage of survivors was seen in all six experiments. This finding is further illustrated graphically in Fig. 2.

In Table 2 the results of therapy experiments on mice bearing EHR2 cells are shown. The median survival of control mice treated with NaCl was 13 days. VP-16 given at doses ranging from 22 to 33 mg/kg had a marked antitumor effect, as the median life span was increased by 66% and 92%, respectively, in treated animals as compared with control mice, whereas a VP-16 dose of 11 mg/kg failed to produce any increase in median life span. The addition of aclarubicin to VP-16 resulted in a decrease of approximately 50% in the antitumor effect of VP-16 as is also shown in Fig. 3.

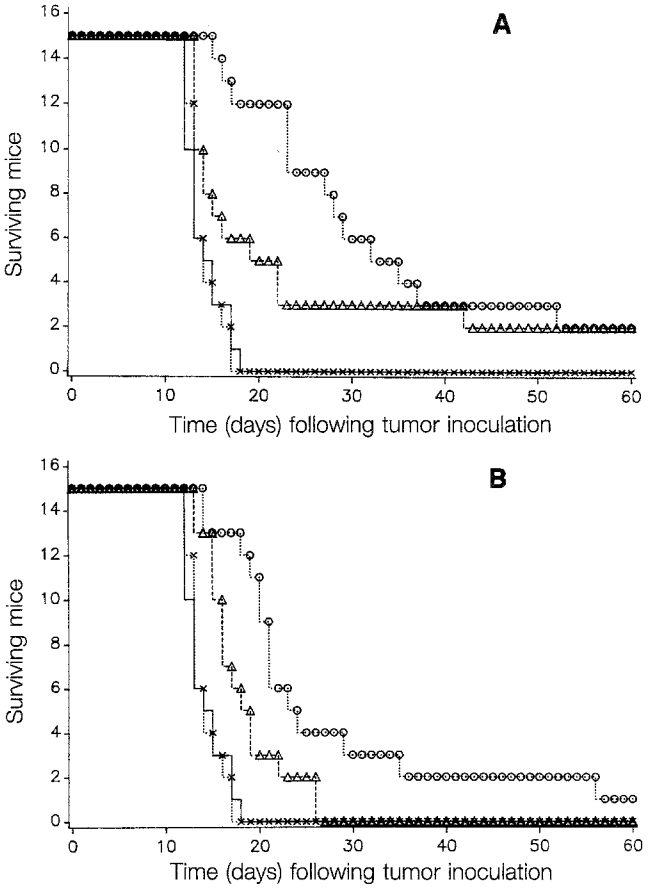

Fig. 3A, B. Treatment of mice bearing EHR2 tumors with VP-16. The antagonistic effect of aclarubicin on VP-16 therapy is shown. **A** ———, NaCl; ××××, 6 mg/kg aclarubicin; ΘΘΘΘ, 22 mg/kg VP-16; ΔΔΔΔ, 6 mg/kg aclarubicin + 22 mg/kg VP-16. **B** ———, NaCl; ××××, 6 mg/kg aclarubicin; ΘΘΘΘ, 33 mg/kg VP-16; ΔΔΔΔ, 6 mg/kg aclarubicin + 33 mg/kg VP-16

To examine whether a favorable combination of the two drugs could be found in therapy experiments, we raised the dose of VP-16 against a fixed dose of aclarubicin as shown in Fig. 4. The dose of VP-16 was raised to 90 mg/kg, and doses above 70 mg/kg had a diminishing effect on the therapy due to toxicity. At doses below 70 mg/kg the effect on tumor-bearing mice was enhanced as measured by the observed increase in life span. Aclarubicin given at 9 mg/kg prior to VP-16 diminished the life span as compared with that resulting from VP-16 treatment alone. High-dose aclarubicin (LD_{10}, LD_{50}) had no effect on the life span of tumor-bearing mice, and when it was used in combination with VP-16, drug-treated mice died before the saline-treated control animals. In conclusion, we found no combination of aclarubicin and VP-16 that gave a higher increase in life span than that produced by VP-16 alone.

The hematologic toxicity as examined by leukocyte counts showed a nadir on day 3 along with reductions of 57% in the VP-16 group and 79% in the group treated with both drugs (Table 3). On day 7 we observed full restitution of the granulocyte count in both groups. These results indicate that aclarubicin does not provide protection against VP-16-induced bone marrow toxicity. The animals usually

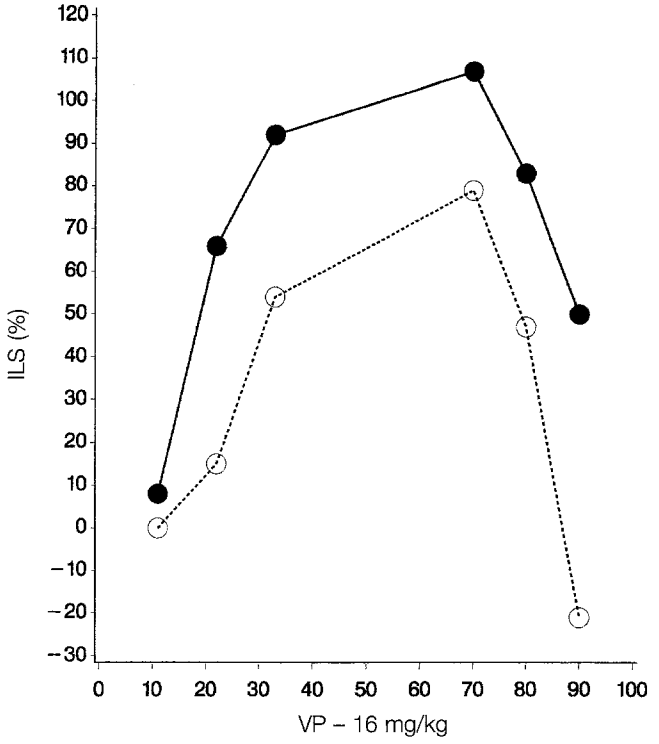

Fig. 4. Effect of increasing doses of VP-16 on tumor-bearing mice as measured by the observed increase in life span (*ILS*) and the effect of the drug combination VP-16 and 9 mg/kg aclarubicin on the life span. An antagonistic effect of aclarubicin is apparent at all doses. ●-●-●-●, VP-16 alone; ○---○---○, VP-16 + 9 mg/kg aclarubicin

Table 3. Lack of inhibition of VP-16-induced granulocytopenia by aclarubicin

	Median leukocyte count ($\times 10^9$)		
	Day 3	Day 5	Day 7
NaCl + NaCl	4.2	6.7	6.3
ACLA 6 mg/kg	4.1 (2%)	6.5 (3%)	8.7 (–38%)
VP-16 90 mg/kg	1.8 (57%)	3.9 (42%)	5.9 (6%)
ACLA + VP-16 6 mg/kg + 90 mg/kg	0.9 (79%)	3.1 (54%)	5.8 (8%)

An additive toxic effect of the two drugs can be seen. The numbers in parentheses represent the percentage of reduction in leukocyte counts of drug-treated mice relative to saline-treated controls

died around days 9–11 when they had been treated with 90 mg/kg VP-16; thus, we concentrated our studies on the parenchymal organs on day 7. The only organ displaying histologic signs of toxicity was the small intestine, which showed focal ulcerations of varying severity, with the deepest reaching the point of perforation of the peritoneum. Quantitation of these lesions was not possible due to their focal nature.

In line with results obtained by other investigators, we observed apoptosis in the stem cells of the crypts in the small intestine of the mouse [2, 19]. In Table 4 the results of drug-induced apoptosis are shown. The combination of aclarubicin and VP-16 yielded significantly less apoptosis than did VP-16 alone. It is also apparent that aclarubicin itself does not induce apoptosis to any significant degree. These results indicate that aclarubicin protects the stem cells in the small intestinal crypts from VP-16-induced apoptosis.

Table 4. Inhibition by aclarubicin of VP-16-induced apoptosis in crypt cells of the small intestine as determined in 2 separate experiments

	Number of apoptotic cells/50 crypts	
	Mean	Median
Experiment 1:		
NaCl + NaCl	9	8
NaCl + ACLA	8	9
NaCl + VP-16	145*	166
ACLA + VP-16	21*	19
Experiment 2:		
NaCl + NaCl	3	3
NaCl + ACLA	7	8
NaCl + VP-16	101**	101
ACLA + VP-16	31**	32

Groups of 7 mice were treated i.p. with either NaCl, 20 mg/kg ACLA, 1.25 mg/kg VP-16, or a combination of 20 mg/kg ACLA and 1.25 mg/kg VP-16 and were euthanized 3 h after treatment. The numbers of apoptotic cells were scored as described in Materials and methods
*, ** $P < 0.0001$ (Student's *t*-test)

Discussion

During the past decade there has been a major increase in our knowledge of the modes of action of drugs such as anthracyclines, which have been in clinical use for 30 years. It is to be hoped that this experimental knowledge will enable a more rational approach to the design of clinical trials.

Currently, cancer chemotherapy is mostly performed with multiple drugs so as to take advantage of the drugs' different modes of action and patterns of cross-resistance [6]. However, although a synergistic effect is obviously the goal, there are many instances in which different drugs can antagonize each other, leading to less than even an additive effect. It is obviously of great importance to identify such regimens in the preclinical setting. VP-16 and, to a lesser extent, aclarubicin are clinically used drugs, and combination therapy including these two drugs has been suggested [20, 27]. Despite consensus that VP-16 exerts its cytotoxicity via an interaction with topoisomerase II, leading to stabilization of the DNA-topoisomerase II cleavable complex [15, 17], the way in which aclarubicin is cytotoxic remains unknown.

We have demonstrated that the mechanism of action of aclarubicin is different from that of other anthracyclines such as doxorubicin and daunorubicin [10–12]. This could be an advantage in its combination with VP-16, as adding another topoisomerase II stimulator such as daunorubicin to a VP-16-containing regimen would be expected to give only an additive effect. However, it is apparent from our in vitro studies that instead of stimulating DNA breaks, aclarubicin actually inhibits cleavable-complex formation. Furthermore, there is evidence that this inhibition is due to the $COOCH_3$ group in the C-10 position of the anthraqui-

none preventing the initial noncovalent binding of the topoisomerase II enzyme to DNA [12, 21]. Despite compelling in vitro evidence, a preclinical evaluation should include the more complex in vivo situation. The present study validates the in vitro data, as aclarubicin antagonizes both VP-16 toxicity in non-tumor-bearing mice (Fig. 2, Table 1) as well as the VP-16-induced increase in life span in tumor-bearing mice (Fig. 3, Table 2).

Thus, as judged from the present study and previous preclinical studies, we would not recommend a clinical regimen including simultaneous VP-16 and aclarubicin administration. However, Rowe et al. [20] found that this combination was efficacious in relapsed acute myeloid leukemia. This discrepancy can be explained by a number of possibilities. First, the difference between humans and mice might be so great as to invalidate the preclinical in vivo data. In our opinion, this is not a feasible explanation, as both aclarubicin and VP-16 are lipophilic drugs that easily cross biomembranes and are active without further modification. Also, the in vitro evidence of the antagonistic effect of aclarubicin on VP-16-induced DNA breaks in human cancer cells makes it unlikely that the same effect would not occur in the patient. However, a major difference in drug metabolism and excretion between humans and mice cannot be excluded.

Second, all but one of the patients evaluated in the study by Rowe et al. [20] had been pretreated with the topoisomerase II-directed drug daunorubicin. This would presumably lead to the leukemia cells at relapse having either the classic P-glycoprotein-associated phenotype, the altered topoisomerase II multidrug-resistance (MDR) phenotype, or both [3]. Aclarubicin lacks cross-resistance in the altered topoisomerase II MDR phenotype [12] and has only a relatively low level of cross-resistance in cells exhibiting P-glycoprotein-mediated MDR [4, 9]. As the study by Rowe et al. [20] included only patients treated with both aclarubicin and VP-16, it is conceivable that the beneficial effect of the combination was due to the action of aclarubicin by itself. Thus, several studies have described remission rates for aclarubicin in relapsed acute leukemia, which although slightly lower than those reported by Rowe et al. [20], are within the same range [16, 18, 25]. However, although simultaneous treatment with VP-16 and aclarubicin would from our experimental evidence be expected to lead to a diminished effect of VP-16, it is possible that sequential treatment with VP-16 followed by aclarubicin would be advantageous, as VP-16 would be expected to induce the altered topoisomerase II MDR phenotype, which is fully sensitive to aclarubicin [12], thus leading to an alternating non-cross-resistant chemotherapy as suggested by Goldie et al. [7].

Furthermore, the present study shows that VP-16 causes apoptosis in the stem cells of the mouse small intestine, a result that is hardly surprising, considering that VP-16 is known to cause apoptosis in nonneoplastic [24] as well as in neoplastic cells [22]. However, in contrast to the reported induction of apoptosis by a "classic" anthracycline such as doxorubicin, which induces cleavable-complex formation [2, 19], we found that the anthracycline aclarubicin did not cause apoptosis in the small intestine, even at a dose of 20 mg/kg (Table 4), which is above the LD_{10} for this drug. To our knowledge, this is the first time that this distinction in modes of action between the different anthracycline classes has been reported. Moreover, the action of aclarubicin on apoptosis contrasts with that of two other non-cleavable-complex-forming topoisomerase II-active drugs that are both reported to induce apoptosis, namely, fostriecin [8] and novobiocin [1]. In rat thymocytes, VP-16-induced formation of cleavable complexes is followed by endonuclease cleavage leading to apoptosis as shown by Walker et al. [24]. As aclarubicin provides protection against the formation of cleavable complexes induced by VP-16 [10], it is reasonable to assume that the antagonistic effect of aclarubicin on VP-16-induced apoptosis is exerted during the early phase of VP-16-induced cell damage and thus contrasts with the effect of bcl-2 protein, which inhibits VP-16-induced apoptosis after the formation of DNA breaks [13].

In conclusion, the present study presents a preclinical in vivo validation of previous in vitro data demonstrating the inhibitory effect of the anthracycline aclarubicin on drugs such as VP-16, which stimulate DNA-topoisomerase II cleavable-complex formation. The study also presents an indication that the site of the protection provided by aclarubin against VP-16-induced toxicity in mice is in the stem-cell area of the small intestine.

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