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# Mapping of DNA Topoisomerase II Poisons (Etoposide, Clerocidin) and Catalytic Inhibitors (Aclarubicin, ICRF-187) to Four Distinct Steps in the Topoisomerase II Catalytic Cycle

*Maxwell Sehested*[1]* *and Peter Buhl Jensen*[2]

[1]Department of Pathology, Sundby Hospital, DK-2300 Copenhagen S, Denmark and [2]Department of Oncology, Rigshospitalet and Department of Internal Medicine, P, Bispebjerg Hospital, Copenhagen, Denmark

**ABSTRACT.** The complex catalytic cycle of topoisomerase II is the target of important antitumor agents. Topoisomerase II poisons, such as etoposide and daunorubicin, inhibit the resealing of DNA breaks created by the enzyme. This enzyme-coupled cell kill is susceptible to pharmacological regulation by drugs interfering with other steps in the enzyme's catalytic cycle (i.e. so-called catalytic inhibitors). From *in vitro* studies, it appears that there are 2 distinct sites in the cycle at which a complete antagonism of the toxicity of topoisomerase II poisons can be obtained. The first is the inhibition of the enzyme's binding to its DNA substrate as seen with intercalating drugs such as chloroquine and aclarubicin; a second, more specific, interaction is elicited by bisdioxopiperazines, which are thought to lock the homodimeric topoisomerase II in the form of a closed bracelet surrounding the DNA at the postreligation step. To investigate these *in vitro* findings in the more complex whole cell system, we studied enzyme-DNA binding in Western blots of 0.35 M NaCl nuclear extracts from human small cell lung cancer OC-NYH cells incubated with the bisdioxopiperazine ICRF-187 and aclarubicin. With ICRF-187, we found a reversible ATP dependent decrease in the extractable levels of both the α and the β isoforms of topoisomerase II. In contrast to ICRF-187, aclarubicin increased the amount of extractable enzyme from cells. Further, when using the terpenoid clerocidin, which differs from conventional topoisomerase II poisons by forming a salt- and heat-stable inhibition of DNA resealing, no antagonism was found by ICRF-187 on formation of DNA strand breaks and cytotoxicity. However, aclarubicin, which interferes early in the topoisomerase II catalytic cycle, was able to antagonize DNA breaks and cytotoxicity caused by clerocidin. The results indicate 4 different steps in the topoisomerase II cycle that can be uncoupled in the cell by different drug types: etoposide and clerocidin cause reversible and irreversible inhibition of DNA resealing, respectively, and DNA intercalating agents, such as aclarubicin, inhibit binding of topoisomerase II enzyme to its DNA substrate. Finally, bisdioxopiperazines as ICRF-187 partake in an energy dependent inappropriate binding of topoisomerase II to DNA after the resealing step. This knowledge may enable the design of rational combinations of topoisomerase II poisons and catalytic inhibitors to enhance the efficacy of anticancer therapy. BIOCHEM PHARMACOL 51;7:879–886, 1996.

**KEY WORDS.** topoisomerase II; bisdioxopiperazine; ICRF-187; aclarubicin; clerocidin; etoposide

The essential nuclear enzyme topoisomerase II allows the separation of intertwined DNA strands by creating a transient, double-stranded break in the DNA backbone. This catalytic cycle of topoisomerase II has been shown to be the cellular target of several clinically important anticancer agents [1]. Thus, drugs such as the anthracyclines daunorubicin and doxorubicin (Adriamycin) and the epipodophyllotoxins VP-16† and VM-26 cause DNA breaks by freezing a drug-enzyme-DNA complex in a so-called cleavable complex [1]. These cleavable complex forming drugs, also called topoisomerase poisons [2] have, as a consequence of their common mechanism of action, a common at-MDR phenotype [3, 4]. Recently, drugs that act on topoisomerase II without cleavable complex formation (i.e. so-called catalytic inhibitors) have attracted interest for 2 reasons. First, they appear to circumvent the at-MDR phenotype [5, 6] and may also act as antagonists to the topoisomerase II poisons. One prospective application of antagonists is to use them to improve the selectivity of the topoisomerase II poisons. Thus, we recently described how the antagonist chloroquine, due to its weak base properties, could protect normal cells and enable specific targeting of acidic tumors by VP-16 [7]. Therefore, more knowledge concerning different mechanisms of uncoupling the enzyme's catalytic cycle is needed to pursue the possibilities of pharmacological regulation of topoisomerase II-directed

* Corresponding author. Tel. (+45) 32343735; FAX (+45) 32343984.

† *Abbreviations:* at-MDR, altered topoisomerase II multidrug resistance; DPC, DNA protein cross-links; NB, nucleus buffer; SSB, single-strand breaks; VM-26, teniposide; VP-16, etoposide.

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drugs. Utilizing 3 different whole cells assays, we show that aclarubicin, VP-16, clerocidin, and ICRF-187 each have a distinct mechanism of action on the catalytic cycle of the topoisomerase II enzyme leading to several means of mutual antagonism

## MATERIALS AND METHODS

### *Drugs*

VP-16 was purchased from Bristol-Myers Squibb, Copenhagen, Denmark and ICRF-187 (dexrazoxane) from Eurocetus, Amsterdam, The Netherlands. Aclarubicin was purchased from Lundbeck, Copenhagen, Denmark, and clerocidin was a gift from Dr. Poul Rasmussen, Leo Pharmaceuticals, Ballerup, Denmark. VP-16 was in a formulation for clinical use and was further diluted in medium; ICRF-187 and aclarubicin were dissolved in water immediately prior to use, and clerocidin was dissolved in DMSO and stored in aliquots at –80°C. All other reagents were of analytical grade.

Affinity-purified rabbit polyclonal antibodies to the C terminal end of topoisomerase II α were obtained commercially (ICI, U.K.). Affinity-purified rabbit polyclonal antibodies to the N terminal end of topoisomerase II β were a generous gift from Dr. Fritz Boege, Würzburg University, Germany.

### *Cell Lines*

The human small cell lung cancer cell line OC-NYH [8] was used throughout the study. In addition, L1210 cells were used as internal controls in alkaline elution assays.

### *Clonogenic Assay*

A 3-week clonogenic assay was used as previously described [9]. Briefly, single cell suspensions ($2 \times 10^4$ cells/mL) in RPMI 1640 supplemented with 10% fetal calf serum were exposed to the drugs for 1 hr, washed twice in PBS at 37°C, and plated in triplicate in soft agar on top of a feeder layer containing sheep red blood cells. The number of cells was adjusted to obtain 2000 to 3000 colonies in the control dishes.

### *Western Blotting on Nuclear Extracts*

OC-NYH cells were incubated for 1 hr at 37°C in RPMI 1640 with 10% fetal calf serum with the desired drug(s), except for experiments including manipulation of cellular energy levels, where incubation was done in PBS, pH 7.4 containing 5% v/v fetal calf serum with either 10 mM glucose or 10 mM sodium azide and 10 mM 2-deoxyglucose. Thereafter, crude nuclear extracts were performed by a modification of a previously described method [10]. All steps were performed at 4°C. Exponentially growing cells were harvested and washed twice in NB (2 mM $KH_2PO_4$, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, and 0.2 mM DTT, 1 mM Phenylmethylsulfonylfluoride, pH 6.5). Cells were resuspended in 1 mL NB and lysed for 5 min by gently adding 9 mL of NB supplemented with 0.3% v/v Triton X-100. Nuclear pellets were spun down at 1000 × *g* for 10 min and washed with Triton X-100 free NB. Proteins were extracted for 30 min in NB with 0.35 M NaCl. Insoluble nuclear fragments were spun down at 17,000 × g and the supernatant collected. Extracts were dissolved in an equal volume of glycerol.

After heating the nuclear extracts for 5 min at 50°C, the sample was immediately loaded on a 7% SDS-PAGE gel containing 5% glycerol. Thereafter, all steps were performed at room temperature. The separated proteins were transferred to Trans Blot®, a PVDF-membrane (Bio-Rad) in a semi-dry electro-blot system (KemEnTec, Denmark) with 50 mM CAPS (3-[Cyclohexyl-amino]-1-propanesulfonic acid) pH 11.0 at 100 mA for 1 to 1½ hr. Membranes were blocked in 1% bovine serum albumin in TBS-T (10 mM Tris-HCl, pH 8.2, 150 mM NaCl, 0.05% Tween 20) for 1 hr and probed with either topoisomerase IIα (1:1000) or β antibodies (1:1500) for 1 hr. Alkaline phosphatase-conjugated swine-antirabbit antibodies (Dako, Copenhagen, Denmark) were used as secondary antibodies. The blots were developed using Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (both from Sigma, MO). The reaction was stopped in 20 mM EDTA. Total protein was measured by the Bradford assay [11] and simultaneously run Coomasie blue stained gels were analyzed to ensure that equal amounts of protein were loaded. Quantitation of immunoreactive bands were done by densitometric scanning.

### *Alkaline Elution*

Alkaline elution filter assays for DNA single-strand breaks (SSBs) and DNA protein cross-links (DPCs) were performed according to Kohn *et al.* [12] with minor modifications as in [5].

For measurement of SSBs, L1210 cells used as internal standard were exposed to 100 μM $H_2O_2$ for 60 min on ice, corresponding to an irradiation dose of 300 rad as described by Szmigiero and Studzian [13]. OC-NYH cells were incubated in medium supplemented with the indicated drug at 37°C for the specified periods, washed in 10 mL in ice-cold PBS and then lysed on the filter (Nucleopore, 2.0 μm pore size) with 5 mL of SDS-EDTA lysis solution (2% SDS, 0.1 glycine and 0.025 M $Na_2EDTA$) at pH 10, followed by addition of 1.5 mL SDS-EDTA lysis solution supplemented with 0.5 mg/mL proteinase K (Sigma). Mixing of standard and experimental cells was done immediately prior to lysis. DNA was eluted with tetrapropyl-ammoniumhydroxide-EDTA, pH 12.1 containing 0.1% SDS at a rate of 0.125 mL/min. Fractions were collected at 20-min intervals for 2 hr. Filters were treated with 400 μL 1 N HCl for 1 hr at 60°C, cooled, and 0.4 M NaOH was added prior to scintillation counting.

For measurements of DPCs, 0.8 μm Metricel DM-800

filters (Gelman Sciences, Ann Arbor, MI) were used. OC-NYH cells were first incubated on ice with 5 mM $H_2O_2$ corresponding to 3000 rad [13], exposed to drug(s) for 1 hr at 37°C, and then lysis was performed with 5 mL sarcosyl-EDTA lysis solution (2.0 M NaCl, 0.2% sodium lauryl sarcosine and 0.04 M disodium EDTA, pH 10). Elution was carried out with tetrapropylammonium hydroxide-EDTA pH 12.1 without SDS at the same flow rate as for SSBs.

## RESULTS

### *Effect of Aclarubicin, VP-16, Clerocidin, and ICRF-187 on Extractable Topoisomerase II*

Incubation with the topoisomerase II poison VP-16 reduces the amount of extractable topoisomerase II α enzyme in cells [14]. Thus, it appears that by inhibiting religation, VP-16 traps the enzyme on its DNA substrate, leaving less freely available enzyme for salt extraction. We, therefore, performed a number of experiments with Western blotting on nuclear extracts subsequent to 1-hr incubations with the different topoisomerase II directed drugs. These showed for the first time that a catalytic inhibitor, ICRF-187, was able to induce a marked decrease in the extractable amount of both topoisomerase II α and β isoenzymes (Fig. 1, 2, and 4). In addition to VP-16, the terpenoid clerocidin also decreased the amount of extractable topoisomerase II, as would be expected for a topoisomerase II poison (Fig. 3). In contrast, aclarubicin, which had been shown to inhibit binding of topoisomerase II α to its DNA substrate in vitro [15], increased the amount of extractable topoisomerase II α (Fig. 4). Topoisomerase II mediated DNA cleavage *in vitro* by VP-16 has been described to be ATP stimulated [1, 2], and Fig. 3 demonstrates in a whole cell assay that depletion of cellular energy by coincubation with sodium azide and 2-deoxyglucose and omission of glucose inhibits the binding effect of both VP-16 and clerocidin on both

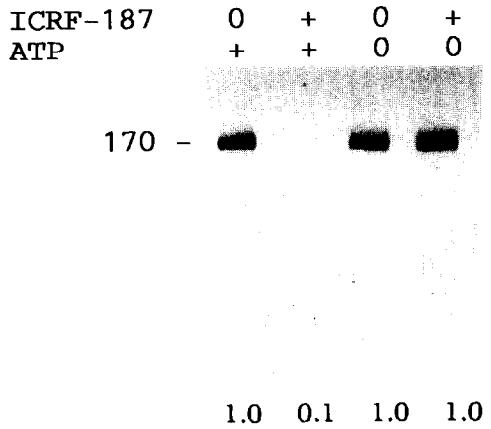


**FIG. 1. Western blot of 0.35 M NaCl nuclear extracts from OC-NYH cells, demonstrating energy dependence of ICRF-187 induced decrease in topoisomerase IIα. Note that changes in cellular energy do not affect enzyme levels in cells not treated with drug. A similar response was observed with the topoisomerase IIβ isoform (not shown). ICRF-187 +, Cells were incubated with 50 μM ICRF-187 for 1 hr at 37°C in PBS, pH 7.4 (lanes 2 and 4). ATP +, cells were incubated with 10 mM glucose (lanes 1 and 2); ATP 0, incubated with 10 mM 2-deoxyglucose and 10 mM sodium azide (lanes 3 and 4).**

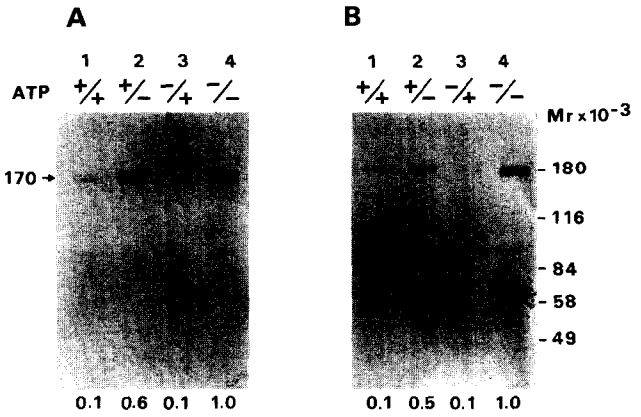


**FIG. 2. Western blot of 0.35 M NaCl nuclear extracts from OC-NYH cells, demonstrating reversible energy dependence of ICRF-187 induced decrease in both topoisomerase II isoforms. Cells were incubated with 50 μM ICRF-187 for 1 hr at 37°C in PBS, pH.7.4 with 10 mM glucose (lanes 1 and 2) or with 10 mM 2-deoxyglucose and 10 mM sodium azide (lanes 3 and 4), followed by a wash in PBS and incubation for 1 hr with 50 μM ICRF-187 in 10 mM glucose (lanes 1 and 3) or 10 mM 2-deoxyglucose and 10 mM sodium azide (lanes 2 and 4). Panel A, topoisomerase II α; Panel B, topoisomerase II β. ATP followed by + or −/+ or − refers to consecutive presence or absence of cellular energy. Numbers below lanes refer to results of relative densitometric scanning where lane 4 is set to 1.0.**

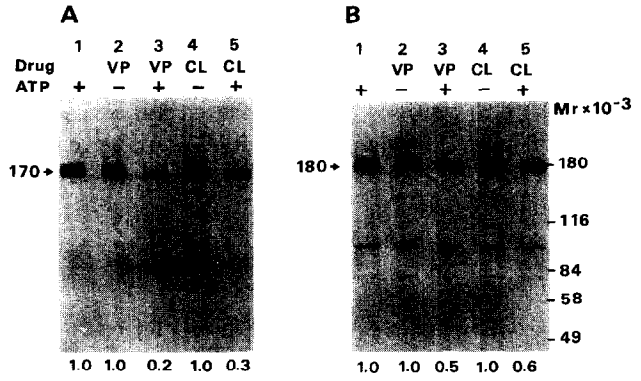


**FIG. 3. Both VP-16 and clerocidin decrease extractable topoisomerase II in an energy dependent manner. Western blot of 0.35 NaCl nuclear extracts of OC-NYH cells incubated for 1 hr at 37°C in PBS, pH 7.4 in 10 mM glucose (lane 1); in 10 mM 2-deoxyglucose, and 10 mM sodium azide with 100 μM VP-16 (lane 2); in 10 mM glucose with 100 μM VP-16 (lane 3); in 10 mM 2-deoxyglucose and 10 mM sodium azide with 100 μM clerocidin (lane 4); or in 10 mM glucose with 100 μM clerocidin (lane 5). Panel A, topoisomerase II α; Panel B, topoisomerase II β. VP, VP-16; CL, clerocidin. ATP followed by + or − refers to presence or absence of cellular energy. Numbers below lanes refer to results of relative densitometric scanning where lane 1 is set to 1.0.**

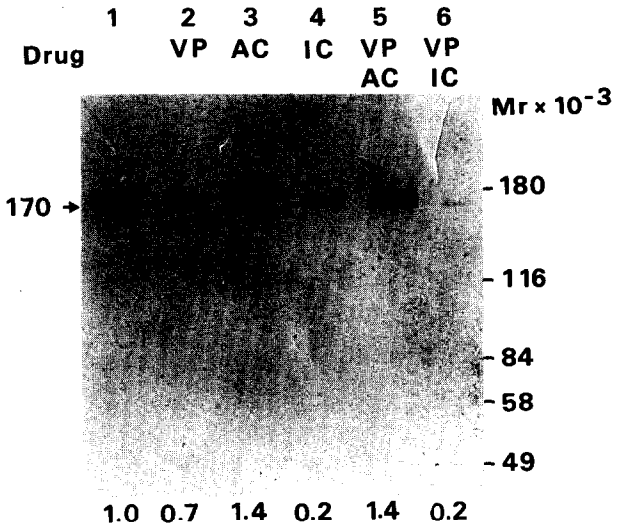


**FIG. 4. In contrast to ICRF-187, VP-16, and clerocidin, aclarubicin increases extractable topoisomerase II α. Western blot of 0.35 NaCl nuclear extracts of NYH cells probed with topoisomerase II α antibodies after incubation for 1 hr at 37°C in RPMI medium without drug (lane 1); with 20 μM VP-16 (lane 2); with 10 μM aclarubicin (lane 3); with 1 mM ICRF-187 (lane 4); with 20 μM VP-16 + 10 μM aclarubicin (lane 5); or with 20 μM VP-16 + 1 mM ICRF-187. VP, VP-16; IC, ICRF-187; AC, aclarubicin. Numbers below lanes refer to results of relative densitometric scanning where no drug is set to 1.0.**

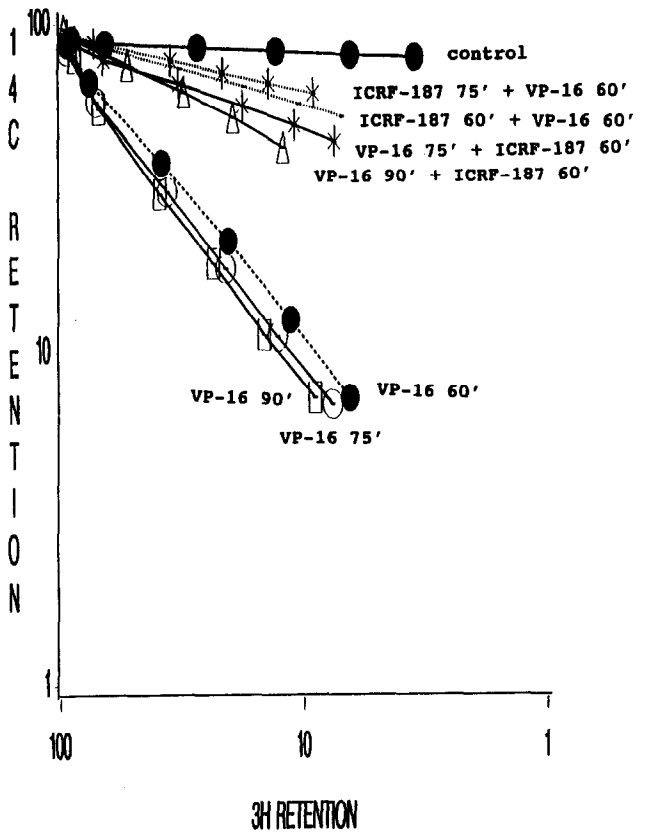


**FIG. 5. Alkaline elution assay showing reversal of VP-16 induced SSBs by ICRF-187. (●——●) no drug control; (●···●) 3 μM VP-16, 60 min; (*— —*) 100 μM ICRF-187 for 15 min followed by coincubation with 3 μM VP-16 for 60 min; (— — ——) 100 μM ICRF-187 + 3 μM VP-16 for 60 min; (*——*) 3 μM VP-16 for 15 min followed by coincubation with 100 μM ICRF-187 for 60 min; (○——○) 3 μM VP-16 for 75 min; (△——△) 3 μM VP-16 for 30 min followed by coincubation with 100 μM ICRF-187 for 60 min; (□——□) 3 μM VP-16 for 90 min.**

topoisomerase isoforms. Roca *et al.* have demonstrated *in vitro* that the presence of ATP is necessary for the formation of an ICRF-193-yeast topoisomerase II-plasmid DNA complex [16]; furthermore, as shown in Figs. 1 and 2, there is also a complete lack of ICRF-187 trapping effect on extractable topoisomerase II α and β in energy depleted cells. Using coincubation, aclarubicin was able to inhibit enzyme trapping on DNA induced by VP-16 (Fig. 4) as well as by ICRF-187 and clerocidin (not shown). However, when ICRF-187 and VP-16 were coincubated, no additive or inhibitory effects were observed (Fig. 4). Thus, the catalytic inhibitors aclarubicin and ICRF-187 have opposite effects on the levels of extractable topoisomerase II.

### *Effects of ICRF-187 and Aclarubicin on VP-16 and Clerocidin-Induced DNA Damage*

Drug effects were compared by alkaline elution assays for DNA breaks. Figure 5 shows that, as previously described, ICRF-187 is able to antagonize VP-16 induced SSBs when incubated simultaneously [17]. We, now, further demonstrate that ICRF-187 also antagonizes VP-16 induced SSBs when added up to 30 min after VP-16. Thus, VP-16 mediated DNA cleavage stimulation can be reversed by ICRF-187, which makes it an efficient antidote.

In contrast, Fig. 6 demonstrates the lack of effect of ICRF-187 on SSBs caused by clerocidin. ICRF-187, thereby, differs from aclarubicin, which effectively antagonizes clerocidin-induced SSBs (Fig. 6). A similar result was obtained for the effect of ICRF-187 and aclarubicin on DNA protein cross-links induced by clerocidin (data not shown). Thus, clerocidin is the first topoisomerase II poison that is not inhibited by ICRF-187 when incubated simultaneously. In Fig. 7, aclarubicin is shown to be able to antagonize VP-16 induced SSBs upon postincubation, as is also the case for ICRF-187 in Fig. 5. However, as is also shown in Fig. 7, aclarubicin is not able to inhibit clerocidin induced SSBs on postincubation, attesting to a difference between clerocidin and VP-16.

### *Effect of Aclarubicin and ICRF-187 on Cytotoxicity Induced by Clerocidin*

Figure 8 demonstrates that the results obtained by the alkaline elution experiments shown in Fig. 6 and 7 translate into cytotoxicity, because an inhibitory effect of aclarubicin is observed on clerocidin-induced cytotoxicity and ICRF-187 has no antagonistic effect. In fact, a slight additive cytotoxicity of ICRF-187 and clerocidin is seen. This is in

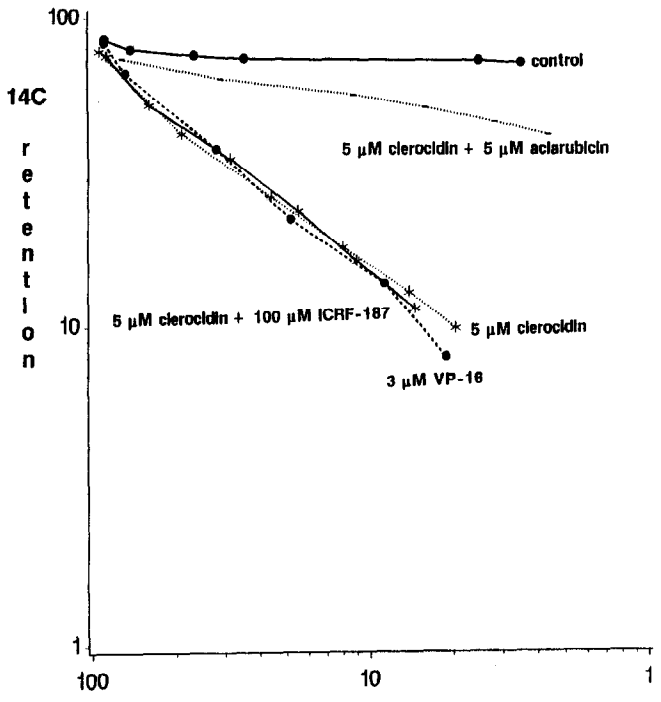


**FIG. 6. Alkaline elution assay showing inhibition of clerocidin induced SSBs by aclarubicin, but not by ICRF-187. All drug incubations and coincubations were for 60 min at 37°C.**

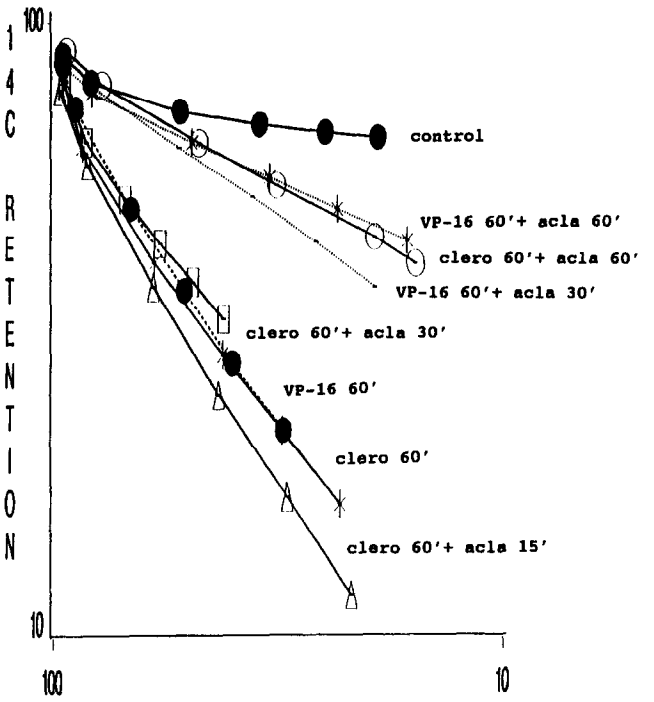


**FIG. 7. Irreversibility of clerocidin induced SSBs demonstrated by lack of inhibition of clerocidin induced SSBs by aclarubicin when postincubated 15–30 min after clerocidin. This is in contrast to VP-16 where postincubation of aclarubicin reverses VP-16 induced SSBs. (●——●) no drug control; (●— —●) 3 μM VP-16 for 60 min; (*— —*) 3 μM VP-16 + 5 μM aclarubicin both for 60 min (— — — —) 3 μM VP-16 for 30 min followed by coincubation with 5 μM aclarubicin for 30 min; (*——*) 5 μM clerocidin for 60 min; (○——○) 5 μM clerocidin + 5 μM aclarubicin both for 60 min; (□——□) 5 μM clerocidin for 30 min followed by coincubation with 5 μM aclarubicin for 30 min; (△——△) 5 μM clerocidin for 45 min followed by coincubation with 5 μM aclarubicin for 15 min.**

contrast to the inhibition of VP-16 induced cytotoxicity in the same cell line by ICRF-187 [17].

## DISCUSSION

Given that the nuclear enzyme DNA topoisomerase II is one of the most important targets in anticancer therapy, elucidation of various kinds of drug-enzyme interactions is of therapeutic interest. Until now, the clinically most important topoisomerase II directed drugs have acted by establishing a drug-enzyme-DNA cleavable complex [1, 18]. Because these drugs convert an essential enzyme to a lethal one, they are now commonly termed topoisomerase II poisons. Recently, however, drugs that act upon the enzyme without cleavable complex formation (i.e. so-called catalytic inhibitors) have attracted considerable attention both as a novel way of inducing tumor cell death and of regulating the activity of topoisomerase II poisons [7]. Two catalytic inhibitors, aclarubicin and ICRF-187, are included in this study.

Aclarubicin is in clinical use in many countries and showed activity similar to daunorubicin in a phase III trial in acute myeloid leukemia [19]. Aclarubicin inhibits VP-16 induced DNA breaks [20], and experiments using a band shift assay have indicated that it acts by inhibiting binding of enzyme to DNA [15], a mechanism of action similar to that of such other DNA intercalators as chloroquine [7], ethidium bromide [21], and 9-aminoacridine [22]. This mechanism is demonstrated for the first time in Western blots from whole cells in Fig. 4, where aclarubicin increases the level of extractable enzyme and inhibits the effect of VP-16 as well as clerocidin and ICRF-187 (not shown).

The bisdioxopiperazine ICRF-187 is licensed in several European countries and is in clinical trials in the U.S.A. as a cardioprotector in conjunction with anthracycline therapy. The raceme of ICRF-187, ICRF-159 (razoxane), has showed activity as an anticancer agent [23] and a ICRF-154 derivative, MST-16, is undergoing clinical trials in Japan [24]. Another possible clinical use of ICRF analogues is that of a "rescue" agent. Thus, we have shown that ICRF-187 can increase the *in vivo* $LD_{10}$ of etoposide nearly 4-fold in healthy mice [25]. There is, therefore, considerable interest in elucidating the mechanism of interaction of bisdioxopiperazines with the topoisomerase II catalytic cycle. Using Western blots on cells, we demonstrate that ICRF-187 markedly reduces the amount of 0.35 M NaCl extractable enzyme of both α and β isoforms in an energy dependent manner (Figs. 1 and 2) similar to that seen with topoisomerase II poisons such as VP-16 and clerocidin (Fig. 3). This can be attributed to these drugs causing more enzyme to bind to its DNA substrate, thereby leaving less freely available enzyme for salt extraction. Because bisdioxopip-

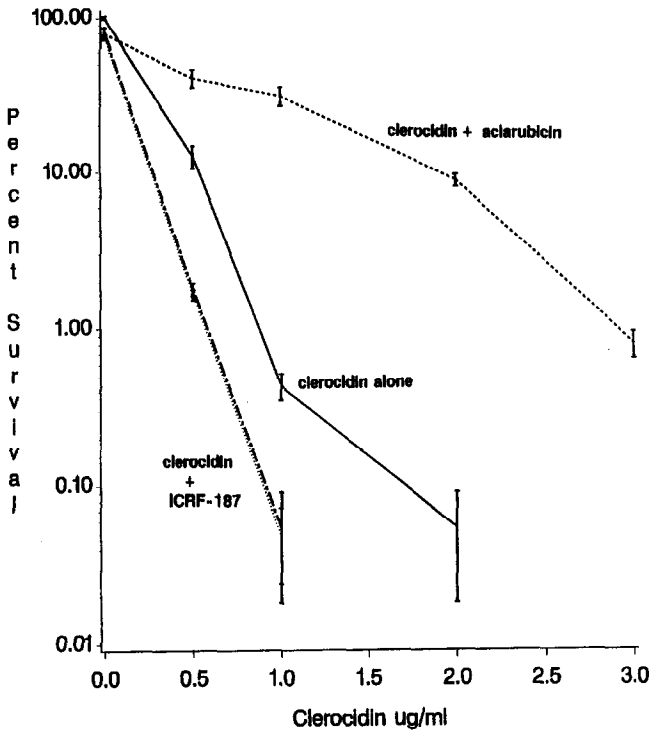


**FIG. 8. Clonogenic assay showing inhibition of clerocidin induced cytotoxicity by aclarubicin, but not by ICRF-187. Abcissa is clerocidin concentration in μg/mL. Aclarubicin was 0.5 μM and the two ICRF-187 doses, which gave identical results, were 150 and 300 μM.**

erazines are EDTA-like divalent cation chelators, one may hypothesize that they act at the magnesium/ATP binding site of topoisomerase II. Magnesium plays 2 distinct roles in the topoisomerase II-DNA interaction, namely by participating directly in topoisomerase II mediated cleavage and by providing the enzyme with a magnesium-ATP substrate for the topoisomerase II mediated ATPase reactions [26]. The findings that bisdioxopiperazines do not by themselves stimulate DNA cleavage [17, 26], that they markedly inhibit topoisomerase II ATPase activity in the presence of $MgCl_2$ [16], and that they need ATP to bind the enzyme to DNA both *in vitro* [16] and in whole cells (Figs. 1 and 2), support the hypothesis that they bind to magnesium in the DNA-enzyme-ATP-magnesium complex. On the other hand, the strongly metal ion binding opening form ICRF-198 was without effect on topoisomerase II activity in an *in vitro* assay [28], showing that the ability to bind magnesium alone is not enough to inhibit the enzyme.

Roca and Wang have, based on *in vitro* studies, recently proposed a two-gated topoisomerase II clamp model (Fig. 9), where ICRF-193 locks the closed-clamp N (entrance) gate form of the enzyme in the presence of ATP, thus preventing its conversion to the openclamp form (i.e. after reaction 4 in Fig. 9) [29]. To further investigate the interaction of aclarubicin and ICRF-187 with topoisomerase II in whole cell systems, we used not only the classical topoisomerase II poison VP-16, but also the terpenoid clerocidin.

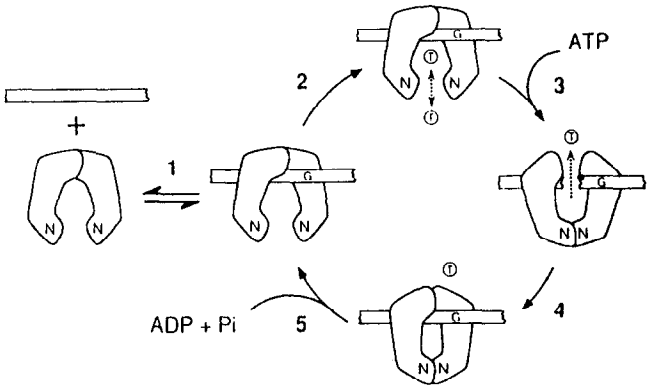


**FIG. 9. The two-gate model of eukaryotic DNA topoisomerase II proposed by Roca & Wang [29] (reprinted with permission). The results in the present study indicate that aclarubicin acts at reaction 1, VP-16 and clerocidin act after reaction 3 and before reaction 4, and ICRF-187 acts between reactions 4 and 5.**

Clerocidin has been reported to have the unique property of irreversibly stabilizing the normally transient intermediate formed between the enzyme and its DNA substrate, as clerocidin induced DNA breaks are salt and heat stable [30]. ICRF-187 is able to inhibit VP-16 induced SSBs, not only when incubated simultaneously, but also when added up to 30 min after VP-16 (Fig. 5). The drug is, thus, able to interfere with the established equilibrium between the topoisomerase II mediated DNA religation and the religation inhibition caused by VP-16. This result suggests that the effects of ICRF-187 on the DNA-enzyme complex is more stable than that obtained with VP-16. In accordance with this, VP-16-induced SSBs are known to be reversible within minutes after the drug has been removed from cells. This is not the case with clerocidin, and ICRF-187 cannot inhibit clerocidin mediated SSBs (Fig. 6), pointing to the possible covalent nature of clerocidin binding. In contrast to ICRF-187, aclarubicin is still able to inhibit SSBs (Fig. 7) and DPCs (not shown) induced by clerocidin, as long as the two drugs are incubated simultaneously. This is in agreement with aclarubicin inhibiting the enzyme's access to its DNA substrate (Fig. 4). The difference between clerocidin and a typical topoisomerase II poison such as VP-16 is also demonstrated in Fig. 7, where aclarubicin is unable to reverse SSBs induced by clerocidin although it readily reverses those provoked by VP-16. These results are summarized in Table 1.

The present study indicates that ICRF-187 interferes with the topoisomerase II-DNA catalytic cycle at a later stage than that of aclarubicin, which appears to act at the first stage of the catalytic cycle by inhibiting the enzyme's attachment to its DNA substrate (i.e. at reaction 1 in Fig. 9). Because ICRF-187 itself does not cause DNA breaks [17], we can conclude that it does not act before reaction 4 in Fig. 9, where ATP binds to topoisomerase II and the enzyme cleaves DNA and opens its second (exit) gate. However, because ICRF-187 inhibits VP-16 induced DNA breaks [17], it could act either early in the cycle (i.e. before

**TABLE 1. Summary of effects of drugs on topoisomerase II**

| | Acla | VP-16 | Cler | ICRF |
|---|---|---|---|---|
| Causes SSBs | 0 | + | + | 0 |
| Inhibits VP-16-induced SSBs and DPCs | + | nd | nd | + |
| Inhibits VP-16 induced SSBs postinc. | + | nd | nd | + |
| Inhibits cler-induced SSBs and DPCs | + | nd | nd | 0 |
| Inhibits cler-induced SSBs postinc. | 0 | nd | nd | 0 |
| Inhibits VP-16-induced cytotoxicity | + | nd | nd | + |
| Inhibits cler-induced cytotoxicity | + | nd | nd | 0 |
| Effect on extractable topo II | incr. | decr. | decr. | decr. |
| Rel. topo II uncoupl. power | +++ | + | ++++ | ++ |

Acla, aclarubicin; cler, clerocidin; decr., decrease; incr., increase; postinc., postincubation; rel., relative; topo II, topoisomerase II; +, effect, 0, no effect, nd, not done/not relevant.

DNA cleavage) or after the religation step. Two lines of evidence in the present study indicate that ICRF-187 acts at the postreligation step. First, ICRF-187 is not able to inhibit clerocidin induced DNA breaks (Fig. 6). Both clerocidin and VP-16 are expected to act by inhibiting religation of the DNA break. Where this is a quickly reversible process for VP-16, it is not for clerocidin, as explained above. Thus, clerocidin in this instance acts as a "marker" for the religation step. However, when ICRF-187 is given a long preincubation of 1 hr, then clerocidin induced SSBs are inhibited (not shown), which agrees with ICRF-187 keeping the postreligation closed clamp form of the enzyme shut. The second line of evidence is that ATP is necessary for ICRF-187 to bind topoisomerase II to DNA (Figs. 1 and 2).

In conclusion, our results indicate that drugs can interact with the DNA topoisomerase II catalytic cycle in at least 4 different ways, exemplified by: 1. aclarubicin, which inhibits its binding of topoisomerase II enzyme to its DNA substrate; 2. VP-16, which causes reversible inhibition of religation; 3. clerocidin, which causes irreversible inhibition of religation; and 4. ICRF-187, which binds the enzyme to DNA after the religation step.

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