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ANTAGONISTIC EFFECT OF THE CARDIOPROTECTOR (+)-1,2-BIS(3,5-DIOXOPIPERAZINYL-1-YL)PROPANE (ICRF-187) ON DNA BREAKS AND CYTOTOXICITY INDUCED BY THE TOPOISOMERASE II DIRECTED DRUGS DAUNORUBICIN AND ETOPOSIDE (VP-16)

MAXWELL SEHESTED,* PETER BUHL JENSEN,† BOE SANDAHL SØRENSEN,‡ BENTE HOLM, ELLEN FRICHE§ and ERLAND J. F. DEMANT‖

Department of Pathology, Sundby Hospital, Copenhagen S, Departments of †Oncology and §Hematology, Rigshospitalet, Copenhagen Ø, ‡Institute of Molecular Biology, Aarhus University, Aarhus, and ‖Department of Biochemistry C, The Panum Institute, Copenhagen, Denmark

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Abstract—The effect of the bisdioxopiperazine cardioprotector ICRF-187 (ADR-529, dexrazoxan) on drug-induced DNA damage and cytotoxicity was studied. Using alkaline elution assays, ICRF-187 in a dose-dependent manner inhibited the formation of DNA single strand breaks (SSBs) as well as DNA–protein cross-links induced by drugs such as VP-16 (etoposide), m-AMSA [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide], daunorubicin and doxorubicin (Adriamycin®) which are known to stimulate DNA–topoisomerase II cleavable complex formation. Thus, 50% inhibition of DNA SSBs induced by 5 μM doxorubicin occurred already at equimolar ICRF-187. In contrast, ICRF-187 did not affect DNA SSBs induced by H_2O_2. In clonogenic assay, ICRF-187 in non-toxic doses antagonized both VP-16 and daunorubicin cytotoxicity in a dose-dependent manner. Our results indicate that the previously described acute *in vivo* protection by ICRF-187 against anthracycline toxicity may be due to inhibition of topoisomerase II activity. The antagonistic effect of ICRF-187 on daunorubicin cytotoxicity should be taken into consideration when planning clinical trials.

One of the dose-limiting side-effects of anthracycline treatment is cardiotoxicity and the bisdioxopiperazine ICRF-187 has been the subject of numerous preclinical *in vivo* studies as a cardioprotector in anthracycline therapy [see 1 and 2 for reviews]. Recently, a large-scale clinical trial in patients with carcinoma of the breast has demonstrated a beneficial effect of ICRF-187 as a cardioprotector in conjunction with doxorubicin treatment [3]. The way in which ICRF-187 acts as a cardioprotector is still unknown, although prevention of the toxicity of oxygen radicals by chelation of iron is at present considered to be the most likely explanation [1, 2]. Anthracyclines are thought to exert their antitumor effect by interaction with the nuclear enzyme topoisomerase II [4], as are several other clinically important drugs such as the epipodophyllotoxins etoposide (VP-16¶) and VM-26, and the aminoacridine 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA) [5]. A recent report has however implicated several bisdioxopiperazine derivatives including ICRF-159, the racemic form of ICRF-187, as inhibitors of topoisomerase II [6]. In addition, the derivative ICRF-193 inhibited VP-16- and m-AMSA-induced formation of enzyme-mediated DNA cleavage [7]. As ICRF-187 is the focus of great interest in clinical phase III trials, and indeed licensed in several European countries as a cardioprotector in conjunction with the topoisomerase II active anthracyclines, it would be of interest to examine any possible interactions between these two types of drug.

* Corresponding author: Dr M. Sehested, Dept of Pathology, Sundby Hospital, DK-2300 Copenhagen S, Denmark. Tel. (45) 32343735; FAX (45) 32343999.

¶ Abbreviations: m-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; DPC, DNA–protein cross-link; SSB, single strand break; VP-16, etoposide.

MATERIALS AND METHODS

Drugs. ICRF-187 [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane, ADR-529, dexrazoxan] was a gift from Farmitalia (Milan, Italy) and was received as the formulation used in clinical trials. The drug was stored according to the manufacturer's instructions and dissolved in water immediately before use. Daunorubicin and doxorubicin (Adriamycin®) were purchased from Farmitalia, VP-16 and mAMSA from Bristol-Myers Squibb (NJ, U.S.A.). All other chemicals were of analytical grade.

Cell lines and clonogenic assay. The human small cell lung cancer cell line OC-NYH [8] as well as L1210 cells were grown in RPMI 1640 medium with 10% fetal calf serum, and cytotoxicity was measured by a 3 week clonogenic assay following 1 hr drug exposure as described [9].

Drug accumulation. Daunorubicin accumulation in L1210 cells was assessed as described for Ehrlich ascites cells [10]. Briefly, after washing the tumor cells four times with ice-cold Ringer's solution, these were incubated at 2×10^6/mL in phosphate-buffered saline (57.0 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 9 mM NaH_2PO_4, 51 mM Na_2HPO_4,

pH 7.45) containing 5% (v/v) calf serum and 10 mM glucose. Increasing concentrations of ICRF-187 from 5 to 100 μM were added 5 min prior to 1 hr incubation with 5 μM daunorubicin at 37°. Following incubation, 2 mL cell suspension was withdrawn and rapidly injected into 8 mL ice-cold Ringer solution followed by centrifugation at 2400 *g* for 1 min. After two washes with ice-cold Ringer solution to remove extracellular drug, the pellet was extracted with equal volumes of 0.3 N HCl:ethanol, and the daunorubicin content determined by spectrofluorometry.

Alkaline elution assay. This was performed as described by Kohn *et al.* [11] for protein-associated single strand breaks (SSBs) and for DNA–protein cross-links (DPCs) with minor modifications as in Ref. 12. Thus, for determination of SSBs lysis of cells on polycarbonate filters (2 μM pore size, Nucleopore, Pleasanton, CA, U.S.A.) with 5 mL SDS–EDTA lysis solution (2% sodium dodecyl sulfate–0.1 M glycine–0.025 M disodium EDTA, pH 10) was followed by treatment with 0.5 mg/mL proteinase K (Sigma Chemical Co., St Louis, MO, U.S.A.) in 5 mL SDS–EDTA lysis solution, and the filters were then eluted with 35 mL tetrapropylammonium hydroxide-EDTA, pH 12.1 containing 0.1% SDS at a rate of 0.130 mL/hr. Fractions were collected at 20 min intervals for 3 hr. For determination of DPCs, 0.8 μm pore-size Metricel DM-800 filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) were used. Lysis was performed with 5 mL sarcosyl-EDTA lysis solution (2 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. Exposure on ice to either 100 μM or 5 mM hydrogen peroxide for 60 min corresponding to an irradiation of 300 or 3000 rad, respectively, as described in Ref. 13 was performed.

RESULTS

Drug accumulation

ICRF-187 at concentrations from 5 to 100 μM had no effect on daunorubicin accumulation in L1210 cells (not shown).

Cytotoxicity

Figure 1 demonstrates that ICRF-187 in a dose-dependent manner antagonized both daunorubicin (Fig. 1A) and VP-16 (Fig. 1B) cytotoxicity to OC-NYH cells in clonogenic assay involving simultaneous 1 hr incubations with the two drugs.

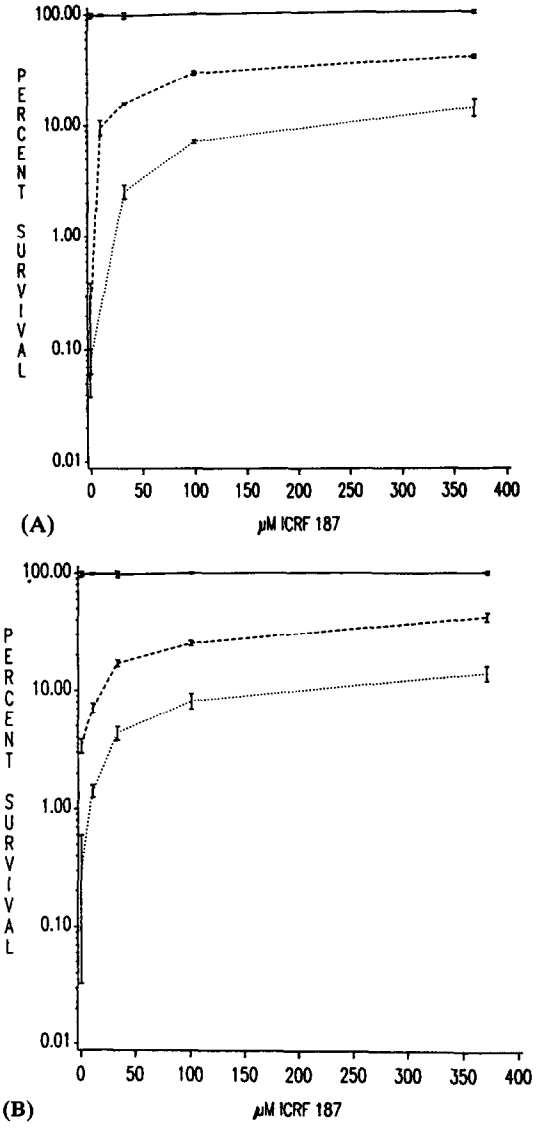

Fig. 1. Clonogenic assay demonstrating inhibition of daunorubicin (A) and VP-16 (B)-induced cytotoxicity in OC-NYH cells by increasing concentrations of ICRF-187 as shown on the abscissa. Drug exposure was for 1 hr at 37°. Colonies were counted after 3 weeks; 100% corresponds to approximately 3000 colonies. Bars are SEM of determinations on three separate dishes. (A) (—) ICRF-187 alone; (– – –) ICRF-187 + 0.15 μM daunorubicin; (· · · · · ·) ICRF-187 + 0.3 μM daunorubicin. (B) (—) ICRF-187 alone; (– – –) ICRF-187 + 10 μM VP-16, (· · · · · ·) ICRF-187 + 20 μM VP-16.

Alkaline elution experiments

As shown in Table 1, ICRF-187 inhibited the formation of protein-associated DNA SSBs induced by the topoisomerase II-directed drugs m-AMSA, VP-16, daunorubicin and doxorubicin. In Table 2, similar inhibition of topoisomerase II-directed drug induction of DPCs is shown. ICRF-187 itself up to 1 mM did not result in DNA fragmentation. As doxorubicin is the clinically most used anthracycline, this combination was studied in detail. As shown in Fig. 2 and Table 1, ICRF-187 inhibited doxorubicin-induced DNA SSBs at a concentration as low as the equimolar level of 5 μM, and a clear dose–response effect was seen in the 5–100 μM range. In Table 2, a similar result is observed for doxorubicin-induced DPCs. In contrast, no effect of ICRF-187 was observed on DNA SSBs induced by H_2O_2 (Table 1).

DISCUSSION

The bisdioxopiperazine ICRF-187 has been the

Table 1. DNA SSBs in L1210 cells incubated with different drugs for the time periods indicated

Drug	Dose (μM)	Time (hr)	SSBs in rad-equivalents: Co-incubation with ICRF-187 (μM) 0	5	10	20	100	1000
VP-16	3	1	203		103		25	12
	3	1	222				30	
	3	3	477			200		
m-AMSA	0.5	1	293	240		164	164	
	1	1	345				239	199
DNR	1	1	168				0	0
	1	1	142		44		44	44
	3	1	334					0
DOX	5	1	90				71	40
	10	1	132				40	
	1	3	73			35	35	
	5	3	392			74	74	
	5	3	282	144	89	37	0	
H_2O_2	50	1	59				59	
	100	0.5	241				240	238
	100	1	238				237	242
ICRF-187	10	1	12					
	100	1	12					
	1000	1	29					

DNR, daunorubicin; DOX, doxorubicin.

Table 2. DPCs in L1210 cells incubated with different drugs for the time periods indicated

Drug	Dose (μM)	Time (hr)	DPC in rad-equivalents: Co-incubation with ICRF-187 (μM) 0	5	10	20	100	1000
VP- 16	3	1	316				0	18
	3	1	238		144		0	
	3	3	900			302		
	3	3	775			207		
	10	1	1267		1052		328	
m-AMSA	0.5	1	409		303		0	
	1	1	549		597		359	
	10	1	1819		1856		1115	
DOX	5	2	168	78	42		0	
	5	3	445	82	0		0	0
	5	3	677				42	0
ICRF-187	100	1	53					
	100	3	0					
	100	3	0					
	1000	3	0					

DOX, doxorubicin.

subject of considerable interest as a cardioprotector in conjunction with anthracycline therapy [2]. The mechanism whereby ICRF-187 protects against the clinically important phenomenon of anthracycline-induced chronic cardiac cardiotoxicity is considered to be due to iron chelation in the cardiac myocyte leading to reduced free oxygen radical toxicity [1]. The present study does not test this hypothesis. However, we do show that the protective effect of ICRF-187 against formation of DNA SSBs is not due to inhibition of H_2O_2-induced free oxygen radical damage (Table 1), as is the case for other iron chelators such as 1,10-phenanthroline [14].

The results in Tables 1 and 2 clearly indicate that ICRF-187 inhibits the formation of DNA breaks induced by drugs which stabilize cleavable complexes. Recent experiments using yeast mutants have further established topoisomerase II as the cellular target of VP-16 [15], strongly indicating that ICRF-187 acts on this enzyme. This agrees with the finding that the two analogs ICRF-193 and ICRF-154 inhibited VP-16-induced DNA breaks analysed by alkaline

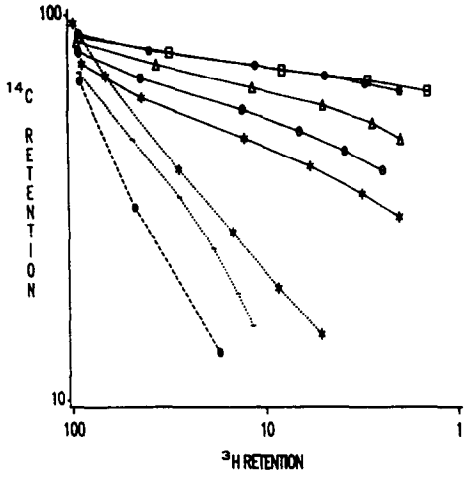

Fig. 2. Alkaline elution assay demonstrating inhibition of doxorubicin-induced DNA SSBs in L1210 cells by increasing concentrations of ICRF-187. Drug exposure for was 3 hr at 37°. Ordinate is ^{14}C retention in %, abscissa is ^{3}H retention in %. (●—●) no drug; (●- -●) 3 μM VP-16; (★·····★) 3 μM VP-16 + 20 μM ICRF-187; (|·····|) 5 μM doxorubicin; (★—★) 5 μM doxorubicin + 5 μM ICRF-187; (○—○) 5 μM doxorubicin + 10 μM ICRF-187; (△—△(; 5 μM doxorubicin + 20 μM ICRF-187; (□—□) 5 μM doxorubicin + 100 μM ICRF-187.

sedimentation analysis [7]. With respect to VP-16 and daunorubicin, the results of the alkaline elution assay translate into inhibition of cytotoxicity in Fig. 1, which also agrees with a reported antagonistic effect on VP-16-induced growth inhibition in RPMI 8402 cells by ICRF-193 [7]. To our knowledge, our study shows for the first time that ICRF-187 can also inhibit an anthracycline's toxicity *in vitro*. Wadler *et al.* [16] stated that ICRF-187 had a synergistic effect on doxorubicin toxicity in clonogenic assay at 24 hr incubation [16]. However, when the tabulated data in Ref. 16 is shown graphically in the following paper [17], the effect of ICRF-187 appears to be additive only. The difference in the ICRF-187 effect on the cytotoxicity of daunorubicin as presented in the present study and of doxorubicin as shown in Refs 16 and 17 can be explained by the well-known differences in cellular pharmacokinetics, especially in the slow membrane transport of doxorubicin, between these two anthracyclines. When we co-incubate ICRF-187 with doxorubicin in clonogenic assay, we also only achieve an additive toxicity similar to that described in Refs 16 and 17 (data not shown). The present study, together with the experiments using other ICRF bisdioxopiperazines described in Refs 6 and 7 on VP-16, indicate that inhibition of daunorubicin-induced topoisomerase II-mediated DNA damage by ICRF-187 could explain the reduction by ICRF-187 of daunorubicin-induced acute *in vivo* toxicity described in Refs 18–20. Accordingly, preliminary results from our laboratory show a significant increase in mean survival time in mice treated with a high dose of VP-16 administered together with ICRF-187 compared to VP-16 alone.

Several cytostatic drugs including ICRF-193 [6, 7], aclarubicin [12, 21], fostriecin [22], merbarone [23] and suramin [24] also inhibit DNA breaks due to topoisomerase II-directed drugs such as m-AMSA and VP-16. Common to all these drugs is their inhibition of topoisomerase II catalytic activity without formation of a cleavable complex. Though we now know that aclarubicin is able to prevent the topoisomerase II enzyme from performing its initial non-covalent DNA binding reaction and thus prevent cleavage activity of typical topoisomerase II-directed drugs [21], the mechanism of ICRF-187 inhibition is presently completely speculative and includes such possibilities as chelation of the Mg^{2+} necessary for topoisomerase II catalytic activity.

The obvious question is whether the inhibition by ICRF-187 of DNA damage induced by anthracyclines and other typical topoisomerase II inhibitors as shown in the present study is of clinical relevance. The clinically used 20 : 1 ratio of ICRF-187 : doxorubicin is well above that shown in Table 1 to exert inhibition of doxorubicin-induced DNA SSBs. A large-scale prospective trial on patients with breast cancer has demonstrated significant protection against doxorubicin-induced cardiotoxicity by ICRF-187 [3]. However, no improvement of clinical response was observed despite an increase in the total doxorubicin dose given to ICRF-187-treated patients, a finding attributed by the authors to the development of drug resistance [3]. The present study indicates that the inhibitory effect of ICRF-187 on doxorubicin-induced DNA breaks may have played a role in this lack of clinical response. On the other hand, ICRF bisdioxopiperazines are cytostatic agents in their own right [2]. Further, their alternative interaction with topoisomerase II may lead to a possible lack of cross-resistance to drugs which stimulate DNA–topoisomerase II cleavable complex formation, and we have preliminary data showing lack of cross-resistance to ICRF-187 in the altered topoisomerase II multidrug-resistant OC-NYH/VM subline. Thus, certain sequential anthracycline/ICRF-187 schedules may be advantageous. The *in vivo* situation would therefore appear to be very complex and needs further elucidation. However, we feel that the antagonism of ICRF-187 towards daunorubicin cytotoxicity demonstrated in the present study should be taken into account when planning clinical trials with these two drugs in, for example, childhood leukemia.

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REFERENCES

1. Green MD, Alderton P, Gross J, Muggia FM and Speyer JL, Evidence of the selective alteration of anthracycline activity due to modulation by ICRF-187 (ADR-529). *Pharmacol Ther* **48**: 61–69, 1990.
2. Herman EH, Witiak DT, Hellmann K and Waravdekar VS, Biological properties of ICRF-159 and related

bis(dioxopiperazine) compounds. *Adv Pharmacol Chemother* **19**: 249–290, 1982.
3. Speyer JL, Green MD, Zeleniuch-Jacquotte A, Wernz JC, Rey M, Sanger J, Kramer K, Ferrans V, Hochster H, Meyers M, Blum RH, Feit F, Attubato M, Burrows W and Muggia FM, ICRF-187 permits longer treatment with doxorubicin in woman with breast cancer. *J Clin Oncol* **10**: 117–127, 1992.
4. Tewey KM, Rowe TC, Yang L, Halligan BD and Liu LF, Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226**: 466–468, 1984.
5. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58**: 351–375, 1989.
6. Tanabe K, Ikegami Y, Ishida R and Andoh T, Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives. *Cancer Res* **51**: 4903–4908, 1991.
7. Ishida R, Miki T, Narita T, Yui R, Sato M, Utsumi KR, Tanabe K and Andoh T, Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. *Cancer Res* **51**: 4909–4916, 1991.
8. Leij L de, Postmus PE, Buys CHCM, Elema JD, Ramaekers F, Poppema S, Brouwer M, Veen AY van der, Mesander G and The TH, Characterization of three new variant cell types derived from small cell carcinoma of the lung. *Cancer Res* **45**: 6024–6033, 1985.
9. Jensen PB, Roed H, Sehested M, Demand EJF, Vindeløv L, Christensen IJ and Hansen HH, Doxorubicin sensitivity pattern in a panel of small cell lung cancer cell lines: correlation to etoposide and vincristine, and inverse correlation to carmustine sensitivity. *Cancer Chemother Pharmacol* **31**: 46–52, 1992.
10. Skovsgaard T, Carrier-mediated transport of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells. *Biochem Pharmacol* **27**: 1221–1227, 1978.
11. Kohn KW, Ewig RAG, Erickson LC and Zwelling LA, Measurement of strand breaks and cross-links by alkaline elution. In: *DNA Repair: a Laboratory Manual of Research Techniques* (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
12. Jensen PB, Jensen PS, Demant EJF, Friche E, Sørensen BS, Sehested M, Wassermann K, Vindeløv L, Westergaard O and Hansen HH, Antagonistic effect of aclarubicin on daunorubicin-induced cytotoxicity in human small cell lung cancer cells: relationship to DNA integrity and topoisomerase II. *Cancer Res* **51**: 5093–5099, 1991.
13. Szmigiero L and Studzian K, H_2O_2 as a DNA fragmenting agent in the alkaline elution interstrand crosslinking and DNA–protein crosslinking assays. *Anal Biochem* **168**: 88–93, 1988.
14. Mello-Filho AC and Meneghini R, Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals. *Mutat Res* **251**: 109–113, 1991.
15. Nitiss JL, Liu Y-X, Harbury P, Jannatipour M, Wasserman R and Wang J, Amsacrine and etoposide hypersensitivity of yeast cells overexpressing DNA topoisomerase II. *Cancer Res* **52**: 4467–4472, 1992.
16. Wadler S, Green MD and Muggia FM, Synergistic activity of doxorubicin and the bisdioxopiperazine (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187) against murine sarcoma S180 cell line. *Cancer Res* **46**: 1176–1181, 1986.
17. Wadler S, Green MD, Basch R and Muggia FM, Lethal and sublethal effects of the combination of doxorubicin and the bisdioxopiperazine, (+)-1,2-bis(3-5-dioxopiperazinyl-1-yl)propane (ICRF-187), on murine sarcoma S180 *in vitro*. *Biochem Pharmacol* **36**: 1495–1501, 1987.
18. Woodman RJ, Cysyk RL, Kline I, Gang M and Venditti JM, Enhancement of the effectiveness of daunorubicin (NSC-82151) or adriamycin (NSC-123127) against early mouse L1210 leukemia with ICRF-159 (NSC-129943). *Cancer Chemother Rep* **59**: 689–695, 1975.
19. Giuliani F, Casazza AM, Di Marco A and Savi G, Studies in mice treated with ICRF-159 combined with daunorubicin or doxorubicin. *Cancer Treat Rep* **65**: 267–276, 1991.
20. Wang GM, Finch M, Trevan D and Hellmann K, Reduction of daunomycin toxicity by razoxane. *Br J Cancer* **43**: 871–877, 1981.
21. Sørensen BS, Sinding J, Andersen AH, Alsner J, Jensen PB and Westergaard O, Mode of action of topoisomerase II-targeting agents at a specific DNA sequence: uncoupling the DNA binding, cleavage and religation events. *J Mol Biol* **228**: 778–786, 1992.
22. Boritzki TJ, Wolfard TS, Besserer JA, Jackson RC and Fry DW, Inhibition of type II topoisomerase by fostriecin. *Biochem Pharmacol* **37**: 4063–4068, 1988.
23. Drake FH, Hofmann GA, Mong S-M, Bartus JL, Hertzberg RP, Johnson RK, Mattern MR and Mirabelli CK, *In vitro* and intracellular inhibition of topoisomerase II by the antitumor agent merbarone. *Cancer Res* **49**: 2578–2583, 1989.
24. Bojanowski K, Lelievre S, Markovits J, Couprie J, Jacquemin-Sablon A and Larsen AK, Suramin is an inhibitor of DNA topoisomerase II *in vitro* and in Chinese hamster fibrosarcoma cells. *Proc Natl Acad Sci USA* **89**: 3025–3029, 1992.