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MULTIPLE MECHANISMS OF ADRIAMYCIN RESISTANCE IN THE HUMAN LEUKEMIA CELL LINE CCRF-CEM*

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Abstract—CEM cells exhibiting a 25-fold (C25X) or 80-fold (C80X) increase in resistance to adriamycin were isolated and characterized. C25X cells were cross-resistant to daunomycin and etoposide (VP-16) but not to vincristine or colchicine. These cells were not defective in the cellular accumulation of drug and did not contain detectable levels of P-glycoprotein. Continued exposure of C25X cells to adriamycin resulted in increased levels of resistance and additional phenotypic changes. These cells (C80X) now contained high levels of P-glycoprotein and were cross-resistant to a variety of agents including vincristine and colchicine. A fluorometric assay for DNA unwinding was used to measure levels of drug-induced DNA breaks in sensitive and C25X resistant cells. Studies carried out with VP-16, 4′9-acridinylaminomethanesulfon-*m*-anisidide (*m*-AMSA), adriamycin, or daunomycin showed that the level of drug-induced DNA strand breakage in resistant cells was considerably less than that occurring in drug-treated sensitive cells. These studies, therefore, show that treatment of CEM cells with adriamycin resulted in a nuclear alteration that contributed to drug resistance. They also demonstrate that prolonged treatment of cells with adriamycin resulted in membrane alterations that affect cellular drug accumulation. Adriamycin resistance in CEM cells can thus occur as a result of at least two distinct mechanisms.

Previous studies have provided evidence that multidrug resistance in experimental cell lines can occur as a result of two distinct types of cellular changes. Thus, it has been observed that cells treated with agents such as adriamycin, vincristine or colchicine exhibit resistance as a result of enhanced levels of a drug efflux system [1–3]. Recent studies have provided considerable evidence that enhanced rates of drug efflux in many resistant isolates are related to overexpression of a surface membrane phosphoglycoprotein (P-glycoprotein) of 170–180 kilodaltons (P180) [4–7]. A number of other studies have shown that cells isolated for resistance to agents such as etoposide (VP-16‡), teniposide (VM-26), or 4′9-acridinylaminomethanesulfon-*m*-anisidide (*m*-AMSA) are not defective in cellular accumulation of drug and do not contain detectable levels of P-glycoprotein [8–10]. These isolates are, however, multidrug resistant exhibiting cross-resistance to a variety of agents including adriamycin and daunomycin [9–11]. The basis of resistance in these isolates appears to be related to an intranuclear change which reduces the ability of various drugs to induce DNA strand breaks [8, 11–13]. Evidence obtained in the last few years suggests that DNA strand breaks occur in drug-treated cells as a result of an interaction between drug and topoisomerase II [8, 11, 14, 15]. This interaction may result in a trapping of the topoisomerase II DNA cleavable complex [14, 15]. In resistant cells, topoisomerase II may be altered such that events leading to drug-induced breaks are greatly reduced [8, 14]. One major question which still needs to be resolved is whether agents such as adriamycin can induce nuclear changes that contribute to drug resistance. This question has been difficult to answer with *in vivo* studies since treatment of cells with adriamycin has resulted invariably in isolates that exhibit reduced cellular drug levels [1–3]. In the present study, we describe the characterization of adriamycin-resistant CEM cells that are not defective in the cellular accumulation of drug. Analysis of this isolate clearly indicates that resistance is related to an intranuclear alteration.

MATERIALS AND METHODS

Cells. CCRF-CEM cells (human leukemic lymphoblasts) were isolated for adriamycin resistance by growing cells in increasing levels of drug. Cells were maintained in RPMI medium containing 10% fetal calf serum. Cells exhibiting a 25-fold (C25X) or 80-fold (C80X) increase in resistance to adriamycin were isolated and characterized. The levels of resistance were determined by dividing the IC_{50} of the resistant cells by that of the sensitive parent cell line. The IC_{50} is defined as the drug concentration inhibiting cell growth by 50% in a 48-hr time period. Dose–response curves were determined by growing cells in various concentrations of drug and thereafter counting cells capable of excluding trypan blue in a hemacytometer.

Analysis of VP-16 uptake. Sensitive and resistant cells were centrifuged and suspended in fresh RPMI at a concentration of 7×10^6 cells/ml. Portions (1 ml)

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‡ Abbreviations: VP-16, etoposide; VM-26, teniposide; m-AMSA, 4′,9-acridinylaminomethanesulfon-*m*-anisidide; and PBS, 0.01 M phosphate (pH 7.6)–0.15 M NaCl.

of the cells were placed in corex tubes, and [^{3}H]VP-16 (90 cpm/ng, final concentration 5μM) was added. The cells were maintained at 37°, and at various time intervals 7.0 ml of cold 0.85% NaCl was added and the cells were centrifuged. The cell pellet was washed once with 5.0 ml of 0.85% NaCl. THe final pellet was solubilized in 0.1 N NaOH, and a portion was taken for radioactivity and protein determination.

Analysis of daunomycin uptake. Sensitive and resistant cells were suspended in 2.0 ml of RPMI at a final concentration of 3×10^5 cells/ml. [^{3}H]Daunomycin (600 cpm/ng) was added and the cells were incubated at 37°. After various time periods the cells were centrifuged, the cell pellet was solubilized in NaOH, and an aliquot was taken for radioactivity and protein determination.

Analysis of intracellular degradation of drug. Sensitive and resistant cells were incubated with [^{14}C]adriamycin for 5 hr at 37°. The cells were centrifuged and thereafter extracted with 50% ethanol. The extracts were evaporated, and the residue was dissolved in 0.01 M Tris–HCl (pH 7.6). Portions were thereafter applied to silica gel thin-layer sheets, and chromatography was carried out in a solution of 65% chloroform, 30% methanol and 5% water. After chromatography the plates were dried, and labeled drug was detected after autoradiography.

DNA breakage. The CEM cells used for these studies were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ. Subsequent to the selection of the adriamycin-resistant isolates, we found that both sensitive and resistant cells were defective in the incorporation of [^{3}H]thymidine into DNA. The nature of this defect is not known. In view of this, drug-induced DNA strand breaks were assessed with a fluorometric assay [16] which does not require the use of radioactively labeled DNA. This assay is based on the partial, time-dependent alkaline unwinding of DNA and quantitation of residual duplex structures with the fluorometric probe bisbenzamide (Hoechst 33258). In these experiments, CEM-sensitive or -resistant cells growing in log phase were incubated in the absence or presence of drug for various time periods and then suspended in PBS at a concentration of 10^6 cells/ml. The DNA unwinding assay and calculations to determine residual double-stranded DNA (F) were carried out under conditions identical to those described by Kanter and Schwartz [16].

Immunoblot analysis for P-glycoprotein. Analysis of sensitive and resistant cells for the presence of P-glycoprotein by immunoblot analysis was carried out as described previously [17]. The monoclonal antibody (C219) [18] which is highly specific for P-glycoprotein was provided by Dr. Victor Ling.

RESULTS

Cross-resistance pattern of the CEM/ADr isolates. Analysis of resistance patterns of the CEM/Adr cells revealed distinct differences in isolates selected for low and high levels of resistance. Both C25X and C80X cells exhibited cross-resistance to daunomycin, VP-16, *m*-AMSA and mitoxantrone (Table 1). However, C25X showed no increase in resistance to vincristine or colchicine, whereas C80X cells exhibited substantial levels of resistance to these agents (Table 1).

Table 1. Cross-resistance pattern of the CEM/Adr isolates

Drug	Relative resistance*	
	CEM25X	CEM80X
Adriamycin	24	83
Daunomycin	18	90
Vincristine	1	100
Colchicine	1	20
VP-16	15	70
Mitoxantrone	20	60
m-AMSA	20	40

* Levels of drug resistance were determined as described in Materials and Methods.

Cellular accumulation of drug. Analysis of drug uptake patterns showed that the cellular accumulations of [^{3}H]daunomycin in sensitive cells and the C25X isolate were essentially the same (Fig. 1). In contrast, cells exhibiting an 80-fold increase in resistance to adriamycin were highly defective in the cellular accumulation of this drug (Fig. 1). Similar studies with [^{3}H]VP-16 showed that there was essentially no difference in the levels of drug accumulation in sensitive and C25X cells (Fig. 2). The higher level resistant isolate (C80X) showed, however, a major defect in the accumulation of [^{3}H]VP-16 (Fig. 2).

Intracellular degradation of drug. Sensitive and resistant cells were incubated for prolonged periods with [^{14}C]adriamycin, and the drug extracted from cells was analyzed after thin-layer chromatography as described in Materials and Methods. The results of these studies showed that adriamycin did not undergo detectable structural changes in either sensitive or resistant cells (not shown).

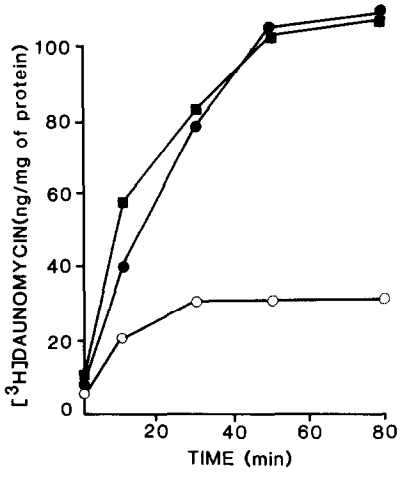

Fig. 1. Accumulation of daunomycin in sensitive and resistant CEM cells. Sensitive and resistant CEM cells were incubated with [^{3}H]daunomycin, and cellular accumulation of drug was determined as described in Materials and Methods. Key: CEM-sensitive (■); C25X (●); and C80X (○).

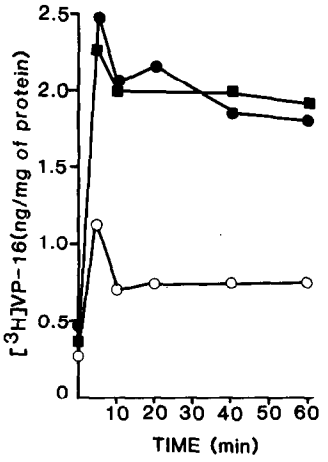

Fig. 2. Accumulation of VP-16 in sensitive and resistant CEM cells. Sensitive and resistant CEM cells were incubated with [^{3}H]VP-16, and cellular accumulation of drug was determined as described in Materials and Methods. Key: CEM-sensitive (■); C25X (●); and C80X (○).

Analysis of drug-induced DNA breaks in sensitive and resistant cells. Sensitive and C25X resistant cells were incubated in the absence or presence of adriamycin, daunomycin, *m*-AMSA or VP-16, and drug-induced DNA breaks were analyzed with the fluorometric assay described in Materials and Methods. In this assay the fraction of DNA (F) remaining in duplex form after a 30-min exposure to alkaline conditions decreases with increasing levels of DNA breaks. The experiments show that treatment of sensitive cells with adriamycin or daunomycin resulted in a dose-dependent increase in the levels of DNA breaks (Fig. 3). In parallel experiments with resistant cells, there was also DNA strand breakage but the extent of degradation was considerably less than that occurring in sensitive cells (Fig. 3). Additional studies showed that the levels of DNA strand breaks induced in resistant cells by either VP-16 (Fig. 4A) or *m*-AMSA (Fig. 4B) were also considerably less than those occurring in sensitive cells treated with these agents.

Analysis of DNA repair in sensitive and resistant cells. Sensitive and C25X cells were incubated with *m*-AMSA for a 1-hr time period such that F values determined from the fluorometric assay [16] were reduced to 40–50% of that in untreated cells. The cells were centrifuged and suspended in fresh medium, and the repair of DNA, as measured by an increase in F values, was determined with the fluorometric assay. The results of these studies demonstrated that the rate of repair of induced breaks was essentially the same in sensitive and resistant cells. Thus, during a 1-hr incubation in the absence of drug, 80% of the DNA breaks were repaired (not shown).

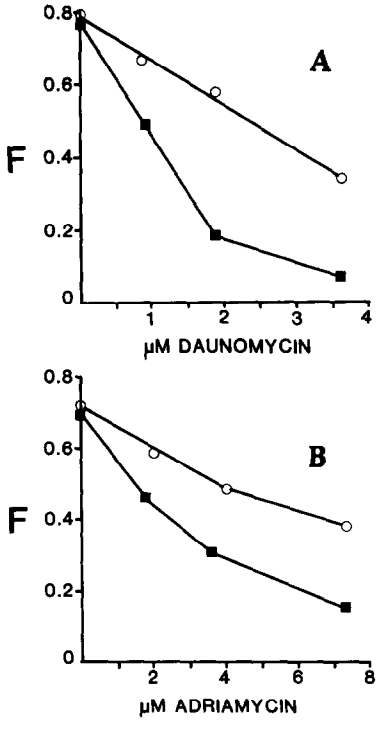

Fig. 3. Adriamycin- and daunomycin-induced DNA breaks in sensitive and resistant cells. Sensitive and resistant cells were incubated in the absence or presence of various concentrations of daunomycin (A) or adriamycin (B), and drug-induced breaks were measured with the fluorometric assay as described in Materials and Methods. Incubation with adriamycin was for 4 hr, while incubation with daunomycin was for 1.5 hr. Key: CEM-sensitive (■); and C25X (○).

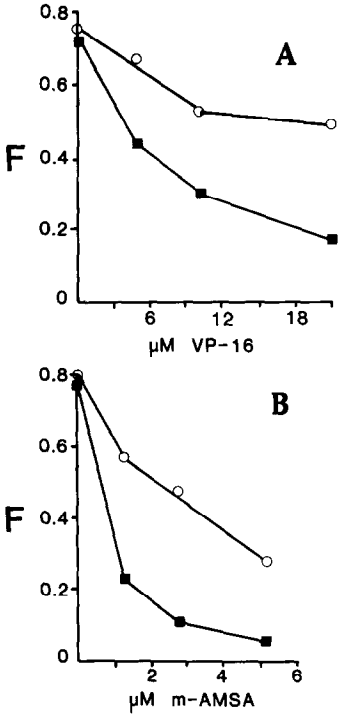

Fig. 4. VP-16- and *m*-AMSA-induced DNA breaks in sensitive and resistant cells. Sensitive and resistant CEM cells were incubated in the absence or presence of various concentrations of VP-16 or *m*-AMSA, and drug-induced breaks were measured with the fluorometric assay as described in Materials and Methods. Incubation with VP-16 (A) or *m*-AMSA (B) was for 1 hr. Key: CEM-sensitive (■); and C25X (○).

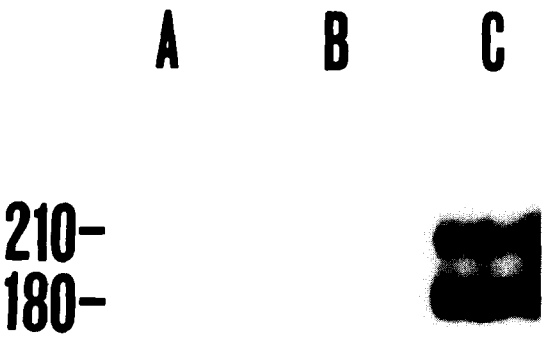

Fig. 5. Analysis of CEM cells for P-glycoprotein. Immunoblot analysis of membrane proteins using the C219 monoclonal antibody [18] was carried out as described in Materials and Methods. Lane A, CEM sensitive; Lane B, C25X; and Lane C C80X.

Analysis of CEM cells for P-glycoprotein. Previous studies have provided evidence that multidrug resistance in a number of experimental cell lines is related to the presence of a 150–180 kilodalton surface membrane phosphoglycoprotein (P-glycoprotein) [4–7]. Sensitive and resistant CEM cells were analyzed for the presence of this protein using the highly specific P-glycoprotein monoclonal antibody C219 [18]. The results of these studies demonstrated that, with immunoblot analysis, P-glycoprotein was not detected in membranes prepared from CEM-sensitive cells or the C25X isolate (Fig. 5, lanes A and B). In contrast, membranes from C80X cells contained P-glycoprotein which was highly reactive with the C219 monoclonal antibody (Fig. 5, lane C). In this system we found that the antibody reacted with two proteins of 180 and 210 kilodaltons.

DISCUSSION

Cell lines isolated for resistance to adriamycin have invariably shown a major defect in the intracellular accumulation of drug [1–3]. This phenotype clearly poses major problems for *in vivo* studies which seek to identify intracellular changes that contribute to drug resistance. In the present study we describe the isolation and characterization of adriamycin-resistant CEM cells (C25X) which were not defective in cellular drug accumulation. This isolate showed no defect in transport of drug to the nucleus and did not convert adriamycin to non-reactive species. Detailed analysis of these cells suggests that resistance was related to a nuclear alteration which reduced the capability of certain drugs to induce DNA strand breaks. Thus, the C25X isolate exhibited resistance to a variety of agents capable of inducing DNA degradation. In contrast, the cells were not resistant to agents such as vincristine or colchicine. Additional studies showed that VP-16, *m*-AMSA, adriamycin and daunomycin induced DNA breaks in sensitive cells and that the level of DNA degradation was reduced markedly in cells isolated for drug resistance. The assay used for analyzing DNA strand breaks measures a time-dependent alkaline unwinding of DNA prepared from cells incubated in the absence or presence of drug [16]. This assay which measures DNA unwinding under alkaline detergent conditions does not determine if the strand breaks are protein-linked as occurs during the action of topoisomerase II on DNA [12]. Previously, murine leukemia cells isolated for adriamycin resistance were analyzed for patterns of drug-induced DNA strand breaks [19, 20]. Although these isolates were defective in cellular drug accumulation, some evidence was obtained that resistance was related, in part, to a nuclear change which reduces the ability of adriamycin to induce strand breakage of DNA [19, 20]. Similar results were obtained with isolated nuclei which should not present a barrier to drug accumulation [20].

The results of the present study clearly indicate that the C25X isolate has properties similar to those for cell lines selected for resistance to VP-16, VM-26 or *m*-AMSA [8–11]. Thus, there was a close similarity with regard to cross-resistance patterns, drug accumulation and absence of P-glycoprotein [8–11]. These isolates have also been found to have a nuclear alteration which reduces the levels of drug-induced breaks [8, 11, 13]. Recent studies have suggested that the antitumor activity of drugs such as the epipodophyllotoxins and intercalating agents occur as a result of an interaction of drug with topoisomerase II [21–23]. This interaction may result in the inhibition of the covalent closure of a topoisomerase II catalyzed DNA break [22]. Alternatively, these drugs may stimulate the DNA breakage activity of topoisomerase II [8]. In the resistant cell, topoisomerase II may be altered such that drug–enzyme interactions are reduced, thus resulting in a decrease in DNA breaks [8, 12]. The present results thus raise the possibility that adriamycin, like VP-16, is capable of inducing a stable genetic change that results in a reduced interaction between drugs and topoisomerase II. The results of the present study also suggest that multiple mechanisms can contribute to adriamycin resistance. Thus, resistance may occur as a result of at least two components which include nuclear changes possibly involving topoisomerase II

and membrane changes which reduce cellular accumulation of drug. It will be of considerable importance to determine if either of these mechanisms actually accounts for clinical drug resistance.

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REFERENCES

1. Inaba M, Fujikura R and Sakurai Y, Active efflux common to vincristine and daunorubicin in vincristine-resistant P388 leukemia. *Biochem Pharmacol* **30**: 1863–1865, 1981.
2. Skovsgaard TM, Mechanisms of resistance to daunorubicin in Ehrlich ascites tumor cells. *Cancer Res* **38**: 1785–1791, 1978.
3. Inaba M and Johnson RK, Uptake and retention of adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. *Biochem Pharmacol* **27**: 2123–2130, 1978.
4. Juliano RL and Ling V, A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455**: 152–162, 1976.
5. Peterson RHF and Biedler JL, Plasma membrane proteins and glycoproteins from Chinese hamster cells sensitive and resistant to actinomycin D. *J Supramol Struct* **9**: 289–298, 1978.
6. Beck WT, Mueller TJ and Tanzer LR, Altered surface membrane glycoproteins in vinca alkaloid resistant human leukemic lymphoblasts. *Cancer Res* **39**: 2070–2076, 1979.
7. Garman D and Center MS, Alterations in cell surface membranes in Chinese hamster lung cells resistant to adriamycin. *Biochem Biophys Res Commun* **105**: 157–163, 1982.
8. Glisson B, Gupta R, Smallwood-Kentro S and Ross W, Characterization of acquired epipodophyllotoxin resistance in a Chinese hamster ovary cell line: Loss of drug-stimulated DNA cleavage activity. *Cancer Res* **46**: 1934–1938, 1986.
9. Beran M and Andersson BS, Development and characterization of a human myelogenous leukemia cell line resistant to 4′-(9-acridinylamino)-3-methanesulfon-*m*-anisidide. *Cancer Res* **47**: 1897–1904, 1987.
10. Beck WT, Cirtain MC, Danks MK, Felsted RL, Safa AR, Wolverton JS, Suttle DP and Trent JM, Pharmacological, molecular and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. *Cancer Res* **47**: 5455–5460, 1987.
11. Glisson B, Gupta R, Hodges P and Ross W, Cross-resistance to intercalating agents in an epipodophyllotoxin-resistant Chinese hamster ovary cell line: Evidence for a common intracellular target. *Cancer Res* **46**: 1939–1942, 1986.
12. Pommier Y, Schwartz RE, Zwelling L, Kerrigan D, Mattern MR, Charcosset JY, Jacquemin-Sablon A and Kohn KW, Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res* **46**: 611–616, 1986.
13. Bakic M, Chan D, Andersson BS, Beran M, Silberman L, Estey E, Ricketts L and Zwelling LA, Effect of 1-β-D-arabinofuranosylcytosine(ara-C) on nuclear topoisomerase II activity and on the DNA cleavage and cytotoxicity produced by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA) and etoposide in *m*-AMSA-sensitive and -resistant human leukemia cells. *Biochem Pharmacol* **36**: 4067–4077, 1987.
14. Pommier Y, Kerrigan D, Schwartz RE, Swack JA and McCurdy A, Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res* **46**: 3075–3081, 1986.
15. Yang L, Rowe TC, Nelson EM and Liu LF, *In vivo* mapping of DNA topoisomerase II-specific cleavage sites in SV40 chromatin. *Cell* **41**: 127–132, 1985.
16. Kanter PM and Schwartz HS, A fluorescence enhancement assay for cellular DNA damage. *Mol Pharmacol* **22**: 145–151, 1982.
17. Marsh W and Center MS, Adriamycin resistance in HL60 cells and accompanying modification of a surface membrane protein contained in drug-sensitive cells. *Cancer Res* **47**: 5080–5086, 1987.
18. Kartner N, Evernden-Porelle D, Bradley G and Ling V, Detection of P-glycoprotein in multidrug resistant cell lines by monoclonal antibodies. *Nature* **316**: 820–823, 1985.
19. Goldenberg GJ, Wang H and Blair GW, Resistance to adriamycin: Relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res* **46**: 2978–2983, 1986.
20. Capranico G, Riva A, Tinelli S, Dadia T and Zunino F, Markedly reduced levels of anthracycline-induced DNA strand breaks in resistant P388 leukemia cells and isolated nuclei. *Cancer Res* **47**: 3752–3756, 1987.
21. Ross W, Rowe T, Glisson B, Yalowich J and Liu L, Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. *Cancer Res* **44**: 5857–5860, 1984.
22. Tewey KM, Chen GL, Nelson EM and Liu LF, Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem* **259**: 9182–9187, 1984.
23. Nelson EM, Tewey KM and Liu LF, Mechanism of antitumor drugs: Poisoning of mammalian DNA topoisomerase II on DNA by an antitumor drug *m*-AMSA. *Proc Natl Acad Sci USA* **81**: 1361–1365, 1984.