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Circumvention of resistance by doxorubicin, but not by idarubicin, in a human leukemia cell line containing an intercalator-resistant form of topoisomerase II: evidence for a non-topoisomerase II-mediated mechanism of doxorubicin cytotoxicity

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Abstract—The novel, topoisomerase II-reactive anthracycline intercalator idarubicin (IDA) was demonstrated to produce protein-associated DNA cleavage in HL-60 human leukemia cells. Like a host of other antineoplastic intercalating agents, IDA produced this effect to a much lesser extent in HL-60/AMSA cells, a line that is primarily resistant to the intercalator amsacrine, but is cross-resistant to a variety of topoisomerase II-reactive DNA intercalating agents including IDA. This resistance is thought to be secondary to the resistance of the topoisomerase II within HL-60/AMSA cells. Surprisingly, HL-60/AMSA cells were minimally resistant to the cytotoxic and DNA cleaving actions of another anthracycline, doxorubicin (ADR). Comparing other effects of the two anthracyclines revealed that IDA, but not ADR, produced endonucleolytic cleavage, a marker of apoptosis. These results suggest that DNA intercalating anthracyclines can have different effects in human leukemia cells. In the case of IDA, drug actions were similar to those produced by the majority of intercalating agents examined in this cellular system. In the case of ADR, the ability to circumvent the resistance of HL-60/AMSA suggests additional, non-topoisomerase II-mediated mechanisms of cytolysis that may also explain the broad spectrum of clinical activity of ADR.

Idarubicin (IDA)* is a novel anthracycline that is being used to treat a variety of human malignancies [1–3]. The drug, like other anthracyclines, produces protein-associated DNA cleavage in mammalian cells [4, 5] and inhibits the catalytic action of topoisomerase II [5], the putative target of this class of drugs [6]. We have been investigating the actions of a variety of topoisomerase I- and II-directed agents in an amsacrine-sensitive/resistant human leukemia cell pair developed by Beran and Anderson [see Refs. 7 and 8]. The resistant cell line, HL-60/AMSA, is 50–100 times less sensitive to the cytotoxic actions of amsacrine than is its parent line, HL-60. The topoisomerase II from HL-60/AMSA is commensurately resistant to amsacrine inhibition [9], and the topoisomerase II gene in that line contains a single base change that may underlie the cellular and enzyme drug resistance [10, 11]. However, HL-60/AMSA is not resistant to etoposide, a nonintercalating topoisomerase II-directed drug. Additionally, it has been reported that HL-60/AMSA is not resistant to doxorubicin (ADR) [7]. This latter report is surprising given that ADR is an intercalator, like a host of other topoisomerase II-reactive drugs to which HL-60/AMSA cells are resistant [9, 12]. The current study was undertaken to ascertain whether IDA could overcome the resistance of HL-60/AMSA, as ADR had been reported to do. The initial results led us to investigate some unique aspects of the actions of IDA in these cells and to distinguish clearly the actions of IDA from those of ADR on both the cellular and biochemical levels.

Materials and Methods

HL-60 and HL-60/AMSA were initially provided by Drs. M. Beran and B. Andersson of the Department of Hematology, the University of Texas, M.D. Anderson Cancer Center. Descriptions of the conditions for the cultivation of these cells have been extensively published previously [7–9, 12]. Soft-agar colony formation assays [13] and the DNA alkaline elution technique of Kohn *et al.* [14] were performed as previously described [9, 12, 15]. Endonucleolytic cleavage was performed as previously described [16], as were biochemical assays of drug effects on purified topoisomerase II [9, 12, 15].

IDA was a gift from Adria Laboratories, Columbus, OH. We used the clinical formulation of this compound dissolved in normal saline at 1 mM and stored at −20°.

Results and Discussion

The ability of IDA to reduce the survival of HL-60 or HL-60/AMSA cells is shown in Fig. 1 (left panel). As previously demonstrated with a number of other topoisomerase II-reactive DNA intercalating agents [9, 12, 15], HL-60/AMSA cells resist the cytotoxic actions of these drugs. HL-60/AMSA cells were 17-fold more resistant to IDA than were HL-60 cells. By contrast, HL-60/AMSA cells were only 2-fold more resistant to the cytotoxic actions of ADR than were HL-60 cells (Fig. 1, right panel). These findings are consistent with the results of Odaimi *et al.* [7]. Thus, ADR is the only topoisomerase II-reactive intercalator yet examined that can overcome the resistance of HL-60/AMSA [9, 12, 15].

Both anthracyclines produced characteristic DNA cleavage and protein cross-linking as quantified by the alkaline elution method of Kohn [see Refs. 17 and 18] (Fig. 2). In the case of IDA, DNA–protein cross-linking was measured. DNA cleavage measurements were not used as excessive amounts of cellular DNA eluted from the filters during the lysis step precluding accurate quantification of IDA-induced DNA cleavage. However, because the amount of this excessive lysis paralleled the amount of cleavage detected and because neither this cleavage nor the excessive lysis was detectable without the use of proteinase in the lysis solution (data not shown), both the lysis and the cleavage were probably topoisomerase II-mediated [17, 18]. Thus, the use of DNA–protein cross-link measurements as an indicator of the effects of IDA on cellular topoisomerase II was justified.

The DNA effects of IDA were biphasic, a phenomenon reported by others [4, 5] and reported by us for mitoxantrone and benzisoquinolinedione [12]. We have attributed this decrease in DNA effects at high drug concentrations to drug-induced alterations in the relationship between topoisomerase II and its DNA binding sites [12];

* Abbreviations; IDA, idarubicin or 4-demethoxydaunorubicin hydrochloride; ADR, doxorubicin or Adriamycin®; and SV40, simian virus 40.

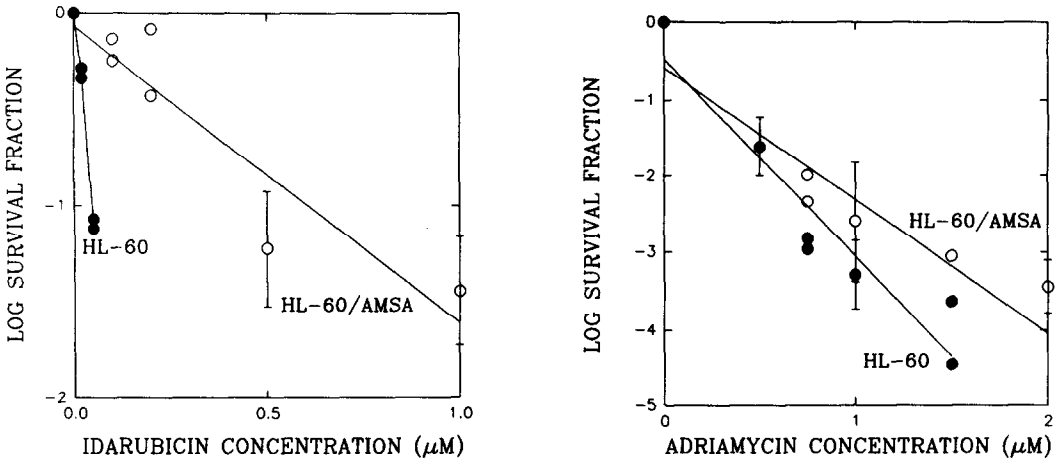

Fig. 1. Cytotoxicity of IDA (left panel) or ADR (right panel) in HL-60 and HL-60/AMSA cells. Exponentially growing HL-60 (●) or HL-60/AMSA (○) cells were exposed to various concentrations of IDA or ADR for 1 hr at 37° followed by soft-agar colony formation as previously described [9, 12, 13, 15]. The lines are linear regressions through the individual points shown. Error bars are ±1 SD for at least three independent experiments. If fewer than three experiments were performed, individual points are shown. The colony formation efficiencies for these experiments were: IDA: for HL-60, 0.57 and 0.55; for HL-60/AMSA, 0.52 ± 0.09. ADR: for HL-60, 0.46 ± 0.13; for HL-60/AMSA, 1.01 ± 0.14.

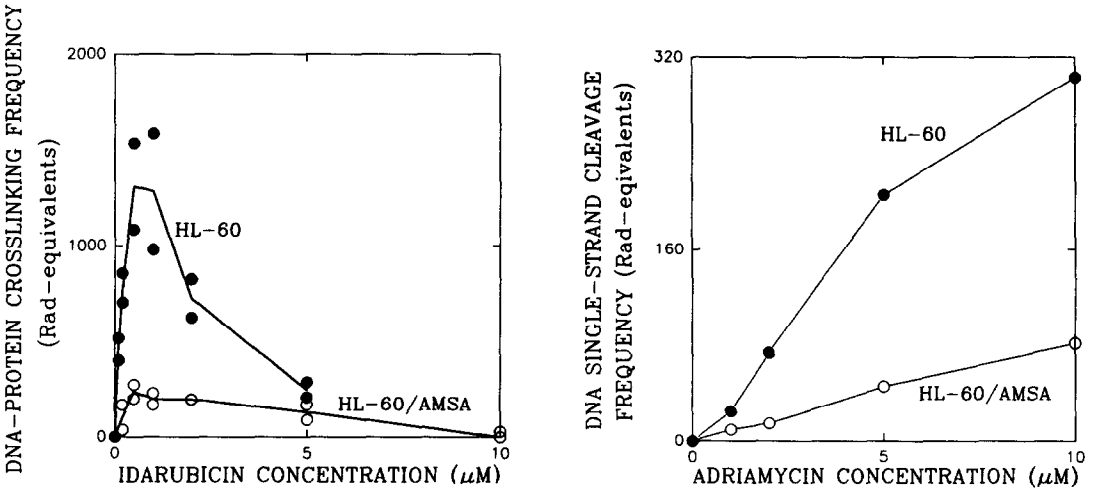

Fig. 2. DNA effects of IDA (left panel) and ADR (right panel) in HL-60 (●) and HL-60/AMSA (○) cells. Cells were treated with IDA or ADR for 1 hr at 37° after which the DNA effects of those treatments were analyzed using the alkaline elution method of Kohn as described previously [9, 12, 14, 15]. DNA–protein cross-linking frequency was quantified when IDA was the drug used; single-strand cleavage was quantified when ADR was the drug used (see Results and Discussion). All DNA effects are expressed in rad-equivalents and were calculated as described elsewhere [17, 18].

these alterations probably disrupt the normal DNA–topoisomerase II complex. In contrast, the topoisomerase II-directed effects of ADR could be readily quantified using measurements of DNA cleavage, since in this case excessive lysis was not produced (Fig. 2, right panel). Although cleavage produced by ADR in HL-60/AMSA was less than that produced in HL-60, the difference in effects between the two cell lines was smaller than that seen using IDA or a number of other DNA intercalators [9, 12, 15].

Capranico *et al.* [4] noted that the DNA effects of IDA persisted following removal of that drug and subsequent incubation of the cells in drug-free medium. This effect, also previously reported for ADR [18], has been attributed to the intracellular retention of these agents [4, 18]. We examined this possibility in HL-60 cells. DNA–protein

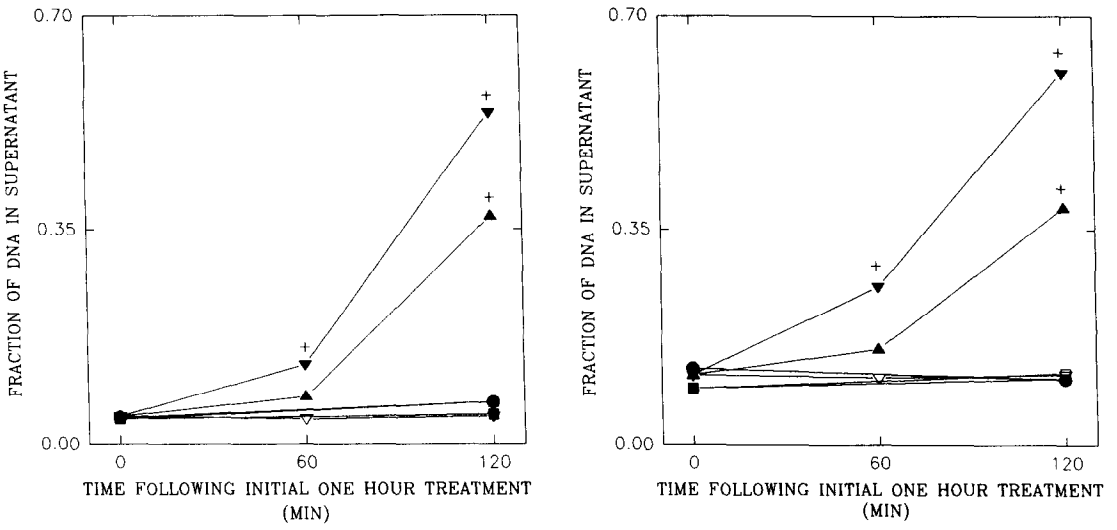

Fig. 3. Fragmentation of HL-60 cellular DNA following treatment with IDA (▲, ▼, ▽) or ADR (■, □). Cells were exposed to 1 μM IDA or 5 μM ADR at 37°. Drug was then either removed by centrifugation and the cells incubated in drug-free medium at 37° (▼, □), removed by centrifugation and the cells incubated in drug-free medium at 0° (▽), or not removed (▲, ■). The closed circles (●) are untreated cells. The graphed values are the fraction of cellular DNA released following cell lysis (i.e. in the supernatant fraction) in the quantitative endonucleolytic cleavage assay (see Materials and Methods and Refs. 16 and 19). Those points where the quantitative endonucleolytic cleavage was associated with endonucleolytic cleavage as visualized on an agarose gel (see Materials and Methods) have a + next to them. The results of two independent experiments are shown.

cross-linking did persist following IDA removal. In some experiments, its frequency actually increased above that detectable at the conclusion of the 1-hr incubation. However, along with this increased cross-linking, excessive amounts of cellular DNA were detected in the lysis fraction, even in the *absence* of proteinase digestion. This observation, which was not noted by Capranico *et al.* [4], was true whether we removed the drug 1 hr following its addition and quantified DNA effects over the next 2 hr or prolonged the incubation of IDA with the cells for up to 3 hr (data not shown). The excessive lysis and increased cross-linking *did not* occur if the cells were incubated at ice temperature following the removal of the IDA. These results indicated that the excessive lysis was not protein-associated and, thus, was not a direct indicator of the effect of IDA on cellular topoisomerase II (e.g. double-strand cleavage). Rather, this was frank DNA cleavage that might be mechanistically related to, yet biochemically distinct from, the actions of IDA on topoisomerase II.

We therefore investigated the nature of this excessive lysis by quantifying endonucleolytic cleavage, a feature of apoptosis [19, 20], as we have described previously [16]. Figure 3 displays the results of two of these experiments. Indeed, the conditions under which excessive lysis was seen in elution experiments without proteinase were the same as those producing endonucleolytic cleavage. Excessive lysis was not produced by ADR and was not detectable if cells were incubated at ice temperature following drug treatment. This suggests that this rapid effect was not a uniform response to lethal drug action and that some enzymatic component was responsible for the DNA breakdown. That component is unlikely to be topoisomerase II itself, but it might be part of a cascade initiated by the drug-induced production of topoisomerase II–DNA complexes. Alternatively, this cascade may be unrelated to topoisomerase II, but be initiated by the retention of IDA. We demonstrated this retention by treating either HL-60 cells (1 μM) or HL-60/AMSA cells (5 μM) with IDA, thoroughly washing the cells free of extracellular drug, and then irradiating the cells with 3000 rad of X-radiation. The cells were then placed in soft-agar tubes for measurement of colony formation, into which 100 unirradiated and untreated but otherwise identical cells were also added. Any retained drug would be released from the lethally irradiated cells, would raise the IDA concentration in the agar, and would kill the added untreated cells. That is precisely what occurred, thus substantiating the retention of IDA in both cell lines.

Finally, we demonstrated the ability of IDA to stabilize a complex between HL-60 topoisomerase II and DNA (Fig. 4). HL-60/AMSA topoisomerase II was not sensitive to IDA in this assay. However, the catalytic (strand-passing) ability of *both* enzymes was inhibited by IDA, a discrepancy between assays of topoisomerase II noted previously [15]. The effect on strand passage may result from the steric effects of the drug on DNA that result from intercalation and that alter the DNA–topoisomerase II relationship. This, in turn, may be related to the biphasic curve observed when quantifying the effects of increasing drug concentrations on DNA–enzyme complex formation in cells (Fig. 2).

IDA produced protein-associated DNA cleavage in HL-60 cells but, unlike ADR, could not overcome the resistance of HL-60/AMSA to topoisomerase II-reactive, DNA intercalating agents. Like ADR, IDA is retained in cells following its removal from the extracellular milieu [4]. Unlike ADR treatment, IDA treatment is associated with the rapid production of endonucleolytic cleavage, a feature of apoptosis. IDA does target topoisomerase II, but the rapid production of endonucleolytic cleavage may or may not be a direct result of topoisomerase II-directed actions of this agent. The clear differences between the effects of IDA and those of ADR in these two cell lines, the most important of which is the ability of ADR to overcome most

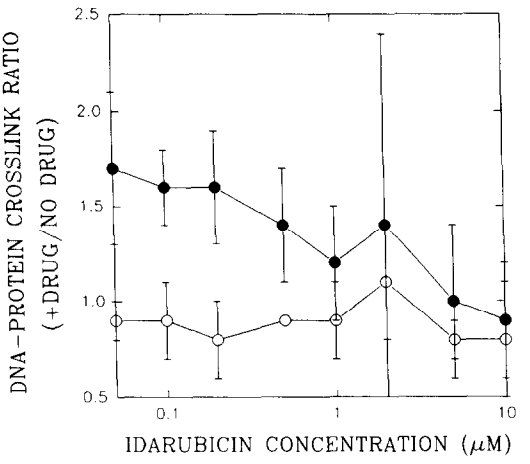

Fig. 4. Ability of IDA to stabilize a covalent complex between purified HL-60 (●) or HL-60/AMSA (○) topoisomerase II and SV40 DNA. The sodium dodecyl sulfate-KCl method [9, 15] was used to quantify these covalent complexes. Incubations were for 30 min at 37°. The amount of topoisomerase II used was twice the amount of each purified preparation needed to decatenate 50% of the kinetoplast DNA used in assays to quantify topoisomerase II activity (see Ref. 21). The data are expressed as the amount of radiolabeled DNA precipitated in the presence of drug divided by that amount precipitated in the absence of drug. All points are the means ±1 SD (error bars) of at least three independent experiments.

of HL-60/AMSA's resistance, suggest that the cellular actions of IDA are similar to those of amsacrine, mitoxantrone, 5-iminodaunorubicin, benzisoquinolinedione, and other topoisomerase II-directed intercalators. However, although ADR is both an intercalator and a topoisomerase II-directed agent [6], it may have additional tumor cell-killing mechanisms, unavailable to IDA, that allow it to circumvent topoisomerase II-mediated drug resistance. This may well explain the particular effectiveness of ADR in the clinic.

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REFERENCES

1. Berman E, Raymond V, Daghestani A, Arlin ZA, Gee TS, Kempin S, Hancock C, Williams L, Stevens Y-W, Clarkson BD and Young C, 4-Demethoxydaunorubicin (idarubicin) in combination with 1-β-D-arabinofuranosylcytosine in the treatment of relapsed or refractory acute leukemia. *Cancer Res* **49**: 477–481, 1989.
2. Case DC Jr, Hayes DM, Gerber M, Gams R, Ervin TJ and Dorsk BM, Phase II study of oral idarubicin in favorable histology non-Hodgkin's lymphoma. *Cancer Res* **50**: 6833–6835, 1990.
3. Hochster HS, Green MD, Blum RH, Wernz JC, Speyer JL and Muggia FM, Oral 4-demethoxydaunorubicin (idarubicin) in bronchogenic lung cancer; Phase II trial. *Invest New Drugs* **4**: 275–278, 1986.
4. Capranico G, Tinelli S and Zunino F, Formation, resealing and persistence of DNA breaks produced by 4-demethoxydaunorubicin in P388 leukemia cells. *Chem Biol Interact* **72**: 113–123, 1989.
5. Ferrazzi E, Woynarowski JM, Arakali A, Brenner DE and Beerman TA, DNA damage and cytotoxicity induced by metabolites of anthracycline antibiotics, doxorubicin and idarubicin. *Cancer Commun* **3**: 173–180, 1991.
6. Tewey KM, Rowe TC, Yang L, Halligan BD and Liu LF, Adriamycin-induced DNA damage mediated by mammalian topoisomerase II. *Science* **226**: 466–468, 1984.
7. Odaimi M, Andersson BS, McCredie KB and Beran M, Drug sensitivity and cross-resistance of the 4′-(9-acridinylamino)methanesulfon-*m*-anisidide-resistant subline of HL-60 human leukemia. *Cancer Res* **46**: 3330–3333, 1986.
8. Beran M and Andersson BS, Development and characterization of a human myelogenous leukemia cell line resistant to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *Cancer Res* **47**: 1897–1904, 1987.
9. Zwelling LA, Hinds M, Chan D, Mayes J, Sie KL, Parker E, Silberman L, Radcliffe A, Beran M and Blick M, Characterization of an amsacrine-resistant line of human leukemia cells. Evidence for a drug-resistant form of topoisomerase II. *J Biol Chem* **264**: 16411–16420, 1989.
10. Hinds M, Deisseroth K, Mayes J, Altschuler E, Jansen R, Ledley FD and Zwelling LA, Identification of a point mutation in the topoisomerase II gene from a human leukemia cell line containing an amsacrine-resistant form of topoisomerase II. *Cancer Res* **51**: 4729–4731, 1991.
11. Lee M-S, Wang JC and Beran M, Two independent amsacrine-resistant human myeloid leukemia cell lines share an identical point mutation in the 170 kDa form of human topoisomerase II. *J Mol Biol* **223**: 837–843, 1992.
12. Zwelling LA, Mayes J, Hinds M, Chan D, Altschuler E, Carroll B, Parker E, Deisseroth K, Radcliffe A, Seligman M, Li L and Farquhar D, Cross-resistance of an amsacrine-resistant human leukemia line to topoisomerase II reactive DNA intercalating agents. Evidence for two topoisomerase II directed drug actions. *Biochemistry* **30**: 4048–4055, 1991.
13. Chu M-Y and Fischer GA, The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemia cells (L5178Y). *Biochem Pharmacol* **17**: 753–767, 1968.
14. Kohn KW, Ewig RAG, Erickson LC and Zwelling LA, Measurement of strand breaks and cross-links by alkaline elution. *DNA Repair: A Laboratory Manual of Research Procedures* (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
15. Zwelling LA, Mitchell MJ, Satitpunwaycha P, Mayes J, Altschuler E, Hinds M and Baguley BC, Relative activity of structural analogues of amsacrine against human leukemia cell lines containing amsacrine-sensitive or -resistant forms of topoisomerase II: Use of computer simulations in new drug development. *Cancer Res* **52**: 209–217, 1992.
16. Zwelling LA, Altschuler E, Cherif A and Farquhar D,

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N-(5,5-Diacetoxypentyl)doxorubicin: A novel anthracycline producing DNA interstrand cross-linking and rapid endonucleolytic cleavage in human leukemia cells. *Cancer Res* **51**: 6704–6707, 1991.

17. Ross WE, Glaubiger D and Kohn KW, Qualitative and quantitative aspects of intercalator-induced DNA strand breaks. *Biochim Biophys Acta* **562**: 41–50, 1979.
18. Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M and Kohn KW, Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and adriamycin. *Biochemistry* **20**: 6553–6563, 1981.
19. Kaufmann S, Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: A cautionary note. *Cancer Res* **49**: 5870–5878, 1989.
20. Cotter TG, Lennon SV, Glynn JG and Martin SJ, Cell death via apoptosis and its relationship to growth, development and differentiation of both tumor and normal cells. *Anticancer Res* **10**: 1153–1160, 1990.
21. Bakic M, Chan D, Andersson BS, Beran M, Silberman L, Estey E, Ricketts L and Zwelling LA, Effect of 1-β-D-arabinofuranocytosine (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.