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Loss of viability and induction of DNA damage in human leukemic myeloblasts and lymphocytes by *m*-AMSA*

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Summary. The effects of *m*-AMSA on in vitro viability and on the induction of DNA damage were examined in low-growth-fraction cell populations of human leukemic myeloblasts and normal lymphocytes. A significant individual variation in the drug-induced reduction of in vitro viability was observed in studies with five selected leukemic patients. The concentration of *m*-AMSA required to reduce viability by 50% within 48 h ranged from 0.25 μ*M* to in excess of 5.0 μ*M* for the leukemic myeloblasts as against about 2.0 μ*M* for the samples of normal lymphocytes. Alkaline elution studies showed that *m*-AMSA induced protein-associated DNA strand breaks (PADB) in both myeloblasts and lymphocytes. Depending upon the *m*-AMSA concentration, there was a 4- to 9-fold difference in the level of PADBs induced by a given drug concentration in the myeloblasts of eight patients studied. The level of PADBs was saturable with respect to both drug concentration (5–10 μ*M*) and exposure time (45–10 μ*M*). The PADBs were repaired rapidly in all the lymphocyte and myeloblast samples studied, with over 90% of this DNA damage being repaired within 45 min after resuspension of the cells in drug-free medium. These studies of *m*-AMSA in low-growth-fraction samples of human lymphocytes and myeloblasts show both similarities and differences in the action of this drug compared with previously published studies using the high-growth-fraction mouse L1210 system.

Introduction

4'-(9-Acridinylamino)-methanesulfon-*m*-anisidide, or *m*-AMSA as it is more commonly called, is a synthetic aminoacridine analogue synthesized by Cain et al. [4–6], which has shown significant activity in the treatment of refractory acute nonlymphocytic leukemia and is also being evaluated as a component of various multiple drug combinations for initial induction of untreated acute leukemia [2, 10].

In a comprehensive study using cultured mouse L1210 cells, Zwelling et al. have shown that *m*-AMSA causes protein-associated DNA single-strand breaks which are similar in nature to those caused by adriamycin and other DNA-intercalating agents [20]. However, the saturation dependence and the rapidity of repair of the *m*-AMSA-induced lesions was considerably different from that observed with adriamycin in various cell culture systems [3, 21].

In this study we have examined the loss of in vitro cell viability and DNA lesions induced by *m*-AMSA in human leukemic myeloblasts. Our objectives were to determine whether the action of this drug in the low-growth-fraction human leukemic cell population was comparable to that previously described in the high-growth-fraction mouse L1210 system, and whether significant individual variations in these parameters exist in leukemic cell populations from different patients.

Material and methods

Human leukemic myeloblasts were isolated from peripheral blood, which had been diluted 1 : 1 with RPMI-1640 medium, by centrifugation over a layer of 60% Percoll (Pharmacia Fine Chemicals, Inc.). The resulting cell preparations contained in excess of 90% leukemic blasts. Human lymphocytes were isolated from peripheral blood by the same procedure after removal of monocytes with carbonyl iron [1]. The incubation medium used in these studies was RPMI-1640 with 10% fetal calf serum (Grand Island Biological Co.).

The in vitro viability of myeloblasts and lymphocytes as a function of *m*-AMSA concentration and exposure time was determined after staining with fluorescein diacetate (5 μg/ml) for 15–30 min [12]. The proportion of viable fluorescent cells was determined, either by fluorescence microscopy or by flow cytometry using a Coulter Electronics Epics V system with an argon laser. The flow cytometric analysis was carried out with excitation at 488 nm, and the emission between 530 and 590 nm was measured using the standard three-log amplifiers on the Epics V system.

Freshly isolated cell preparations were used for the in vitro studies on the effect of *m*-AMSA on lymphocyte and myeloblast viability, while frozen cells were used in most cases for the studies of drug-induced DNA damage. The cells were frozen in RPMI-1640 medium containing 10% fetal calf serum and 10% dimethylsulfoxide using a programmable freezing apparatus; the cooling rate was 1° per

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min to −40°C and 3° per min to −70°C. Cell viability upon thawing was always in excess of 90%.

The alkaline elution studies were carried out basically as described by Kohn's group [9, 16], although the DNA elution had to be quantitated with a fluorescent procedure since the DNA of normal lymphocytes and leukemic myeloblasts could not be prelabeled with radioactively labeled thymidine. The DNA from approximately 2 million lymphocytes or myeloblasts was eluted over 18 h from 2.5 cm polyvinyl chloride filters (2 μm pore size, Millipore Corp.) at a flow rate of 1.5 ml/h with 0.01 sodium EDTA buffer at pH 12.2. Samples were collected at 90 min intervals, and the single-stranded DNA was quantitated after staining with the Hoechst 33258 dye [17]. The DNA was eluted with sodium EDTA instead of tetrapropylammonium hydroxide, as described by Kohn et al., because of the high fluorescent background obtained when the latter buffer system was used. Protein-associated DNA single-strand breaks were defined using the proteinase-K procedure described by Ross et al. [16].

The *m*-AMSA used in this study was the intravenous formulation obtained from Parke-Davis. In this formulation the *m*-AMSA is dissolved in *N,N*-dimethylacetamine, and 0.035 *M* lactic acid is used as the diluent.

Results

The effects of various concentrations of *m*-AMSA on the in vitro viability of normal human lymphocytes and leukemic myeloblasts were first examined to select appropriate *m*-AMSA concentrations for the subsequent studies of drug-induced DNA strand breakage.

Dose – response curves were obtained using freshly prepared samples of lymphocytes from three normal donors and leukemic blasts from five patients with acute myelogenous leukemia. Leukemia blasts from these five patients were also frozen for subsequent studies of *m*-AMSA-induced DNA damage. The leukemic blasts were selected from patients with both new, untreated disease (A.H., R.P., A.L.) and with relapsed disease (S.A., S.K.). Patient A.L. was induced easily into remission, but patient A.H. died with a hyperplastic marrow while receiving our standard induction therapy consisting of cytosine arabinoside, adriamycin, and *m*-AMSA [2]. Two sequential cycles of induction therapy produced marrow hypocellularity in patient R.P. but did not result in the induction of a remission. However, a remission was subsequently obtained in this patient with high-dose cytosine arabinoside. One patient (S.A.) was undergoing a second relapse 3 years after the initial diagnosis. Another (S.K.) was in her first relapse 3 months after receiving a bone marrow transplant from an HLA-matched sibling, which was carried out 7 months after she entered her first remission. In this latter case the leukemic cells were proven by chromosomal analysis to be of patient origin.

The loss of in vitro viability as a function of time of continuous exposure to *m*-AMSA is shown in Fig. 1 for one sample of lymphocytes (Fig. 1 A) and for two samples of myeloblasts (Fig. 1 B, C). The results for the two leukemic patients indicate the range of in vitro susceptibility to *m*-AMSA that was observed in this study. Viabilities are average values from duplicate cultures and are expressed relative to untreated control cultures. The absolute viability of these control cultures was in excess of 85% over the 3-day culture period.

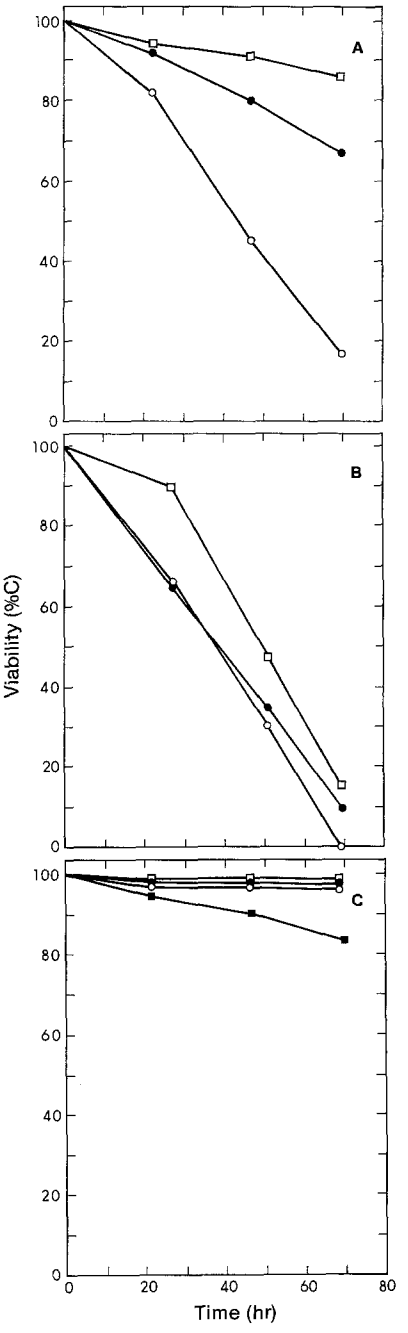

Fig. 1 A–C. Loss of lymphocyte and myeloblast viability induced by *m*-AMSA. Samples of normal lymphocytes *(A)* and leukemic myeloblasts from two patients, S. A. *(B)* and R. P. *(C)* were incubated with *m*-AMSA as described in the text, and the cell viabilities relative to those in untreated control cultures were determined using fluorescein diacetate at the times indicated. The concentrations of *m*-AMSA were 0.25 (□), 0.50 (●), 1.0 (○), and 5.0 (■) μM

A summary of the data on lymphocyte and myeloblast cell viability after 48 h of drug exposure is shown in Table 1. Considerable variation in the in vitro susceptibility to *m*-AMSA was observed with the concentration of *m*-AMSA required to reduce viability by 50% within 48 h, ranging from 0.25 μ*M* to in excess of 5.0 μ*M*. Studies with one patient (A. L.) indicated that *m*-AMSA exposures in

Table 1. Cell viability[a] at 48 h after addition of *m*-AMSA

Cell source	*m*-AMSA concentration (μ*M*)			
	0.25	0.50	1.0	5.0
Lymphocytes[b]	91±4	76±6	51±5	39±6
A.H. (m)	77	64	51	27
R.P. (m)	98	99	98	93
A.L. (m)	61	54	43	21
S.A. (f)	48	35	30	16
S.K. (f)	97	94	92	80

[a] Cell viabilities are expressed relative (percent) to untreated control cells

[b] Cell viability data for lymphocytes are average values for samples from three individuals together with the standard deviations

excess of 4 h resulted in loss of viability that was indistinguishable from that observed with continuous exposure. The loss of viability was progressively less when *m*-ASMA exposure times were reduced below 4 h. We did not study the long-term stability or degradation of *m*-AMSA in the culture medium during the continuous exposure experiments.

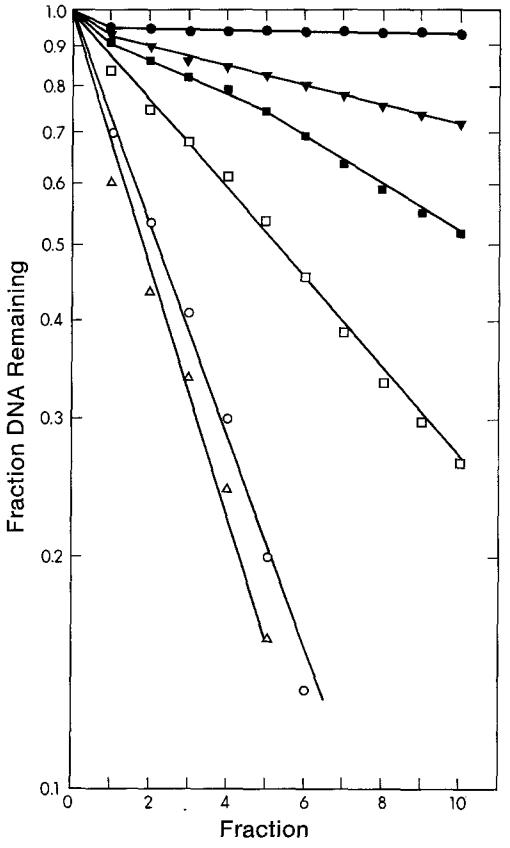

Fig. 2. Protein-associated DNA strand breaks induced by *m*-AMSA in human lymphocytes. Lymphocytes were incubated for 1 h with or without *m*-AMSA in culture medium, chilled, and analyzed by alkaline elution. Cell lysates were pretreated with proteinase K prior to the alkaline elution. Control cells were either irradiated with 400 R (○) or unirradiated (●). The *m*-AMSA concentrations were 0.25 (▲), 0.50 (■), 1.0 (□), and 5.0 (△) μM

The effect of *m*-AMSA on the induction of DNA damage in leukemic myeloblasts from eight patients was examined using the alkaline elution procedure over a concentration range of 0.25–5.0 μ*M*, since comparable levels of *m*-AMSA were required for the in vitro loss of viability. The patients studied included the five shown in Table 1 and three additional patients (J. W., H. S., S. E.) who had relapsed 27, 13, and 47 months after remission induction, respectively. Of the five relapsed patients studied, only S. E. had not been previously treated with *m*-AMSA.

An increase in the rate of DNA elution from *m*-AMSA-treated cells, against that from control cells, is indicative of either drug-induced single-strand breaks or alkaline-sensitive sites. The alkaline elution profiles for *m*-AMSA-treated normal human lymphocytes and for two samples of leukemic myeloblasts are shown in Figs. 2 and 3, respectively. In all cases there was an increase in the rate of DNA elution from the *m*-AMSA-treated cells, but this increased rate of elution was only evident when the filter-bound cell lysates were treated with proteinase K prior to the actual alkaline elution (Fig. 3 A). In the absence of the proteinase K treatment there was no increase in the elution rate of DNA after drug exposures to 10 μ*M*, which was the highest concentration examined in this study. This indicates that in human leukemic myeloblasts the major type of DNA damage induced by *m*-AMSA is protein-associated DNA single-strand breaks.

Table 2 shows the level of DNA single-strand breaks, expressed as rad equivalents, that were induced by various concentrations of *m*-AMSA-treated cells and control cells that had been exposed to known levels of X-irradiation.

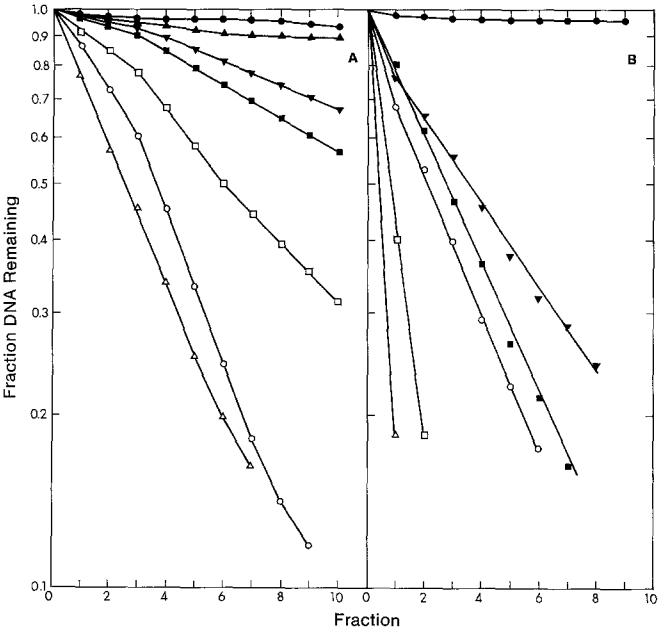

Fig. 3 A, B. Protein-associated DNA strand breaks induced by *m*-AMSA in myeloblasts from patient S. A. *(A)* and A. H. *(B)*. Myeloblasts were incubated for 1 h with or without *m*-AMSA in culture medium, chilled, and analyzed by alkaline elution. Cell lysates were pretreated with proteinase K prior to the alkaline elution. Control cells were either irradiated with 400 R (○) or unirradiated (●). The *m*-AMSA concentrations were 0.25 (▲), 0.50 (■), 1.0 (□), and 5.0 (△ μ*M*. The elution profile of myeloblasts from S. A. which had been treated with 5.0 μ*M* *m*-AMSA without proteinase K treatment is also shown in *A* (▲)

Table 2. DNA strand breaks induced by 1 h exposure to *m*-AMSA

Patient	DNA breaks (rad equivalents)			
	m-AMSA concentration (μ*M*)			
	0.25	0.50	1.0	5.0
Lymphocytes	80	135	210	480
A.H. (m)	275	480	1200	2400
R.P. (m)	50	115	280	570
A.L. (m)	135	280	500	760
S.A. (f)	130	175	270	550
S.K. (f)	50	110	350	600
J.W. (f)	70	110	220	540
H.S. (m)	50	175	280	650
S.E. (f)	30	115	300	1100

Studies with cell preparations of lymphocytes from three individuals and with myeloblasts from two patients demonstrated that there were no significant differences in the dose – response curves of the DNA single-strand breaks whether freshly isolated cells or frozen cells were used. Consequently, for reasons of convenience frozen cell preparations were used to obtain the data shown in Table 2.

Depending upon the *m*-AMSA concentration, it is seen that there is a 4- to 9-fold difference in the level of DNA single-strand breaks induced by a given drug concentration in the eight patients studied. The level of DNA single-strand breaks observed in a given sample of myeloblasts was essentially linear with *m*-AMSA concentrations to about 1 μ*M*. Further increases in the *m*-AMSA concentrations indicated that the level of DNA damage was saturable. In studies with normal lymphocytes and myeloblasts from four patients (A. H., R. P., S. A., S. K.), was little additional damage induced, as the *m*-AMSA concentration was increased in the 5 to 10-μ*M* range.

Studies with normal lymphocytes and two samples of myeloblasts (A. H., S. A.) showed that the degree of DNA damage induced with a given concentration of *m*-AMSA increased with time for 45–60 min, but longer exposure times of up to 4 h did not result in any significant increase in the level of DNA damage.

The DNA single-strand breaks induced by *m*-AMSA are repaired relatively rapidly when the drug is removed from cultures of either normal lymphocytes or of leukemic myeloblasts (Fig. 4). Greater than 90% of the DNA single-strand breaks induced by *m*-AMSA were repaired within 45 min after resuspending the cells in drug-free medium at 37° C. This rapid and essentially complete repair occurred at all the drug concentrations tested (maximum 5 μ*M*) and with exposure times of up to 4 h. The rapid DNA repair occurred in all the myeloblast samples, including those that were relatively sensitive to the cytotoxic effects of *m*-AMSA as evidenced by the viability studies using flourescein diacetate. After 24 h of continous drug exposure the DNA damage was still evident, although the extent of the damage was always less than the maximum level observed after a 1 h exposure (Fig. 4). We have not monitored *m*-AMSA levels in the culture medium to determine whether this reduced level of DNA damage is the result of drug inactivation, as the product literature states that *m*-AMSA is stable in solution for 24 h. This observation will require further investigation.

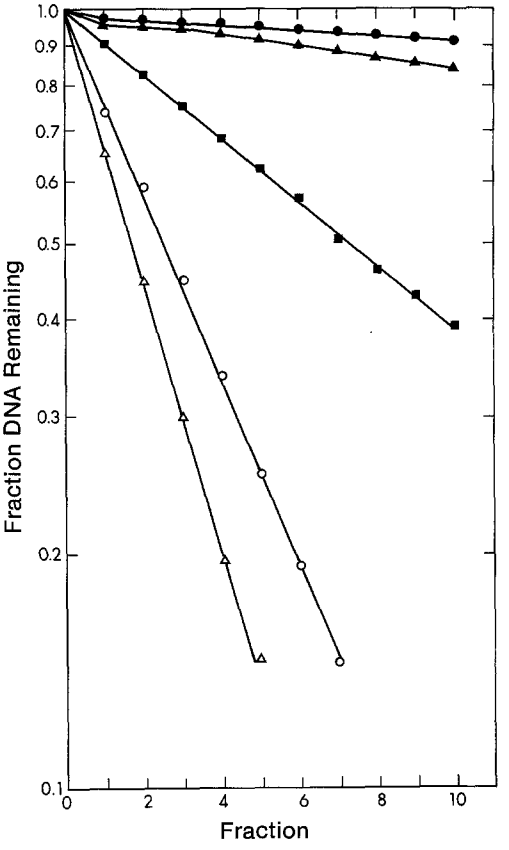

Fig. 4. Protein-associated DNA strand breaks induced by *m*-AMSA in myeloblasts from patient R. P. Myeloblasts were analyzed by alkaline elution after incubation with 5 μ*M m*-AMSA for either 1 h (△) or for 24 h (■). Control cells were either irradiated with 400 R (○) or unirradiated (●). The elution profile is also shown for cells that were incubated in drug-free medium for 1 h after a 1 h exposure to 5 μ*M m*-AMSA (▲)

Discussion

A major goal of chemotherapy research is the understanding of the development of the biochemical differences in drug action, at either host or tumor level, that lead to the clinical failures. It is generally believed that such information will allow either for more effective usage of the drugs in question or for the design of newer and better agents. The data presented in this study show that *m*-AMSA causes loss of viability and induces DNA single-strand breaks in human lymphocytes and in leukemic myeloblasts in vitro. Significant individual variations were observed in both the *m*-AMSA-induced loss of cell viability and in the level of DNA damage. Although the extent of the individual variations in the loss of viability and in the level of DNA damage induced were less than one order of magnitude, we believe that variations of this extent may be significant at the clinical level, since the degree of resistance to DNA intercalators, such as *m*-AMSA, that has been observed in model systems is also of this magnitude [19]. The samples of myeloblasts studied with respect to *m*-AMSA-induced loss of in vitro viability could be divided into sensitive and insensitive categories. Samples of myeloblasts from two patients, one who was untreated (R. P.) and one who was in relapse (S. K.), were quite insensitive to *m*-

AMSA in vitro, and it is interesting to note that neither patient could be induced into remission with our conventional therapy, which includes *m*-AMSA. The remaining samples of myeloblasts were considerably more sensitive to *m*-AMSA in vitro and our conventional therapy produced either a remission or drug-induced marrow hypocellularity in these patients. Normal lymphocytes had a sensitivity that was intermediate to the sensitive and insensitive samples of myeloblasts. Peak blood levels obtained with conventional bolus doses of *m*-AMSA are in excess of 5 μM, whereas blood levels obtained with continuous infusion are in the range of 0.15–0.25 μM [13, 18].

While the cause-and-effect relationship between the *m*-AMSA-induced DNA damage and the loss of cell viability has not been absolutely established, it is generally believed that the DNA damage induced is an important factor in the cytotoxic action of *m*-AMSA [8, 20]. A comparison of the effects of *m*-AMSA on the DNA of human lymphocytes and leukemic myeloblasts, which have low growth fractions, with those described by Kohn et al. in the mouse L1210 system, which has a high growth fraction, shows both similarities and significant differences [20].

In both systems, *m*-AMSA causes cell kill as well as DNA single-strand breaks. These DNA strand breaks are associated in some manner with a cellular protein(s), as shown by the fact that they are not observed unless filter-bound cell lysates are pretreated with proteinase K prior to the actual alkaline elution. There is evidence that these *m*-AMSA-induced DNA strand breaks may be the result of inhibition of topoisomerase II [14, 15].

The level of DNA damage observed in both systems is dose-dependent at lower concentrations of *m*-AMSA, but saturates as the drug dose is escalated. The saturating drug concentrations was about 2 μM in the L1210 system [20], but between 5 and 10 μM in our study of lymphocytes and myeloblasts. The maximum level of DNA single-strand breaks produced by a given concentration of drug occurred within 30–60 min in both systems, after which there was no significant increase in the extent of the DNA damage even though the drug was still present. This saturation in the level of DNA strand breaks with respect to time is not observed with adriamycin, which is another DNA intercalator that produces protein-associated DNA single-strand breaks in mouse and human cell culture systems [3, 21]. The maximum level of single-strand breaks observed in the L1210 system was approximately 3000 rad equivalents. However, in this study the level of DNA strand breaks ranged from about 600 to 2500 rad equivalents, depending on the myeloblast sample in question, and was about 500 rad equivalents for the normal lymphocytes. It is currently not known whether this difference in the level of induced damage in the different cell systems reflects differences in the actual induction of the breaks or in the rate of repair.

Upon removal of the *m*-AMSA from the culture medium, human lymphocytes, leukemic myeloblasts, and mouse L1210 cells rapidly repair essentially all of the DNA single-strand breaks that have been produced. This repair occurs even after exposure to drug concentrations that will result in essentially complete cell kill. This repair is effected within 30–60 min at 37° C. The mechanism and the fidelity of the repair process is currently not understood, although topoisomerase activity has been implicated [8, 11]. The rapid repair of *m*-AMSA-induced DNA single-strand breaks is similar to the rate of repair observed after cytotoxic exposures to X-irradiation, but is much faster than the repair rate observed after treatment with adriamycin [21, 22]. There is evidence that the large difference in the rates of repair of *m*-AMSA – and adriamycin-induced DNA damage may be related to the rapidity of efflux of the intracellular drug once external drug is removed [7].

A major difference in the action of *m*-AMSA on leukemic myeloblasts or normal lymphocytes, as compared with the cultured mouse L1210 cells, is in the concentration of drug required to cause comparable levels of DNA single-strand breaks. For instance, data obtained by Kohn et al. with L1210 show that a 1 h exposure to 1 μM *m*-AMSA causes approximately 2300 rad equivalents of DNA single-strand breaks, whereas in our study a comparable drug exposure resulted in 200–1200 rad equivalents of strand breaks, depending upon the myeloblast sample in question [20]. Unfortunately, it is not possible to compare our *m*-AMSA cell kill data directly with those presented for the L1210 system, since our data are presented as the initial rate of loss of viability and the L1210 data, which were obtained in cloning experiments, are presented as total loss of viability. However, it appears that the L1210 cells may be several times more sensitive to *m*-AMSA than human lymphocytes or leukemic myeloblasts.

It is not possible from this initial data to establish any relationships between the loss of in vitro viability, the level of DNA damage, or the outcome of therapy. However, of the five samples of myeloblasts studied with respect to both loss of viability and level of DNA damage, the two samples of myeloblasts whose viability was relatively unaffected by *m*-AMSA in vitro also had the lowest level of DNA damage, particularly at the lower concentrations of drug.

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