[CANCER RESEARCH 42, 107–111, January 1982]
0008-5472/82/0042-0000$02.00

Lethal Activity and Kinetic Response of Cultured Human Cells to 4′-(9-Acridinylamino)methanesulfon-*m*-anisidine[1]

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ABSTRACT

Cytotoxic and cytokinetic effects of 4′-(9-acridinylamino)methanesulfon-*m*-anisidine (m-AMSA) were studied on a cultured human colon carcinoma and on a human lymphoma (T_1) cell line. Proliferating cells were more susceptible (10-fold) to the cytotoxic effect of m-AMSA than were nonproliferating cells. The agent had minimal effects on DNA synthesis. At comparable exposure doses on m-AMSA, the degree of lethal cell damage exceeded inhibition of DNA synthesis by 4-fold. Cells synchronized in different stages of the cell cycle were equally sensitive to the cytotoxic effects of the drug, although the major cytokinetic effect was a block in G_2. A greater killing effect was obtained by fractionated delivery than by a comparable dose delivered at once. These results suggest that superior antitumor results may be achieved by adequately spaced low doses of m-AMSA in the treatment of sensitive human tumors.

INTRODUCTION

m-AMSA[3] (NSC 249992) is one of the series of acridines with antitumor activity synthesized by Dr. Bruce F. Cain and his collaborators in Auckland, New Zealand (2–6) that is presently undergoing evaluation in various clinical trials (11, 22, 24, 25, 28, 31). More than 120 analogs of acridines have been prepared and shown to have antiproliferating activity against L1210 cells, P388 lymphoid leukemia, and B16 melanoma (2–5, 19, 29, 32). Among these analogs, m-AMSA is one of the more potent drugs that can inhibit the growth of a spectrum of experimental tumors *in vivo* (2) and also possesses antiviral activity (1). m-AMSA is easily absorbed after p.o. administration (9), intercalates with DNA with no base specificity (8, 33), and inhibits DNA synthesis (20, 21) by inhibiting the enzymes that utilize nucleic acid templates (7, 21). m-AMSA can interact with thiols (6, 34), and although arylation of essential thiol groups could play a role in its antitumor properties, the capacity to react rapidly with thiols is neither necessary nor sufficient for the cytotoxic activity in the AMSA series (34).

The effect of an m-AMSA analog was examined on cycling and resting Chinese hamster ovary cells (10, 32), but no report has yet evaluated the effect of m-AMSA on human tumor cells *in vitro*. This paper describes the cytotoxic activity on m-AMSA on cultured human tumor cells and the associated effects on cell cycle progression.

[1] Supported by Grants CA 11520, CA 14528, and CA 23272 and by Contract CM 97277 from the National Cancer Institute, USPHS.

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[3] The abbreviations used are: m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidine; dThd, thymidine; PCA, perchloric acid.

Received June 19, 1981; accepted September 16, 1981.

MATERIALS AND METHODS

Cell Lines. A human carcinoembryonic antigen-producing colon carcinoma cell line (LoVo) (16) and a human lymphoma cell line (T_1) (14) were used in this study. Cells are maintained as monolayer cultures in Ham's Medium F-10 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal calf serum, glutamine, vitamins, and antibiotics. Under these conditions, the average length of cell cycle is 29 hr for LoVo cells and 31 hr for T_1 cells.

Drugs. m-AMSA was obtained from the Cancer Chemotherapy Evaluation Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and was dissolved by adding the sterile diluent provided by the manufacturer. The resulting solution contained 5 mg m-AMSA per ml in 10% (v/v) *N,N*-dimethylacetamide. Further dilutions were made by adding medium containing 10% dimethyl sulfoxide. The final concentrations of *N,N*-dimethylacetamide and dimethyl sulfoxide in solution ranged from 0.001% to 0.005% and 0.1% to 0.5%, respectively. These solvent concentrations failed to cause cytotoxic or cytokinetic effects.

Cell Synchronization. LoVo cells were synchronized with a single treatment of 7 mM dThd for 24 hr as described before (13, 16). Postsynchronization cell cycle transit was monitored with the labeling index, the mitotic index, and by flow cytometry as described below. At the end of the dThd block, about 85% of the cells were accumulated in early S phase; synchrony was maintained in G_2, with 80% of the cells accumulated in that stage, but rapidly decreased when cells reached G_1. Twenty-four hr after the release from the dThd block, the compartment distribution was similar to that observed for asynchronous cells.

Dose-Response Survival of Asynchronous Exponentially Growing Populations. Stock cultures were harvested by standard procedures reported previously (16, 18) and counted with the aid of a Coulter Counter Model ZBI electronic particle counter. Cell suspension aliquots were seeded into 60-mm Petri dishes (5×10^5 cells/dish). The cells were incubated at 37° in a 5% CO_2 atmosphere in air for 48 to 72 hr to achieve exponential growth and for 8 days to obtain cells in stationary phase of growth. The medium was discarded, and the cells were exposed to increasing drug concentrations for exactly 1 hr at 37°. The drug was decanted, and the cells were washed twice in Hanks' balanced salt solution, harvested as a monodispersed suspension, and counted. Because m-AMSA did not induce cell detachment, the decanted supernatant medium and the washings were not combined with the monolayer harvest. Known aliquots of the cell suspension were dispensed into 60-mm Petri dishes so that 50 to 100 colonies would appear after 21 days of incubation in a 5% CO_2 humidified atmosphere at 37°. The colonies were stained with 2% crystal violet in 95% ethanol. Viability was defined as the ability of single cells to give rise to a colony of ≥50 cells. In each experiment, the plating efficiency of at least 6 control cultures was assessed simultaneously. Control cultures consisted of cells treated in exactly the same manner as the test cells but without receiving drug. The survival fractions for the different drug concentrations were normalized with respect to the individual controls for each experiment. All experiments were repeated at least twice with triplicate samples for each drug concentration.

Time-dependent Survival Response. Cell survival as a function of

duration of treatment was investigated by exposing replicate cultures simultaneously to a single dose of drug. Petri dishes containing 5×10^5 cells in asynchronous exponential growth were incubated at Time 0 with the same drug concentration. Replicate cultures were harvested at regular intervals and processed for colony formation assays as described above. Plating efficiency controls were obtained at regular intervals. To demonstrate the stability of the lethal activity of m-AMSA, a separate control system was instituted. This consisted of dishes also containing 5×10^5 cells that were kept growing without adding drug throughout the experiment. Every time dishes were harvested, the supernatant media were transferred to the control dishes after discarding their drug-free medium. The latter were then incubated for 1 hr at 37°, and survival was assessed as usual.

Recovery from Potentially Lethal Damage. To investigate recovery from potentially lethal damage, we used the method described by Little (26). Both exponentially growing and stationary phase cells (5×10^5 cells/dish) were treated with m-AMSA for 1 hr at 37°, washed thoroughly with Hanks' balanced salt solution, and reincubated for 24 hr with spent median before harvesting for colony formation. Spent medium was the cell-free supernatant obtained from 2-week-old stock cultures. The survival of these cells was compared to that of cells harvested immediately after drug exposure was terminated.

Recovery from Sublethal Damage. To investigate recovery from drug-induced sublethal damage, cells were exposed to fractionated doses of the integral dose. Integral dose was defined as the product of the concentration times the time of incubation ($C \times t$). Both stationary phase and exponentially growing cells received the total integral dose in 2 equal separate exposures spaced in time, and their survival was compared to that resulting from cells receiving the total dose at one time. Controls obtained at regular intervals consisted of cells receiving (*a*) half the integral dose, (*b*) the total integral dose, and (*c*) the total integral dose in which only the time parameter was changed.

Cell Cycle Stage-dependent Cell Survival. Age-dependent survival was investigated by incubating synchronized cells with increasing concentrations of drug at regular intervals throughout the cell cycle. Plating efficiency controls were obtained at each time point.

Flow Cytometry. For flow cytometry studies, cells were washed twice in Hanks' balanced salt solution and harvested with pepsin (0.5%) in HCl acid (pH 1.7) for 5 min at 37°, obtaining a monodispersion of greater than 97%. Cells were washed with 0.9% NaCl solution and fixed in 70% ethanol. DNA fluorochromation was performed with 5 ml of ethidium bromide (25 μg/ml in Tris buffer with 0.6% NaCl solution, pH 7.4; Serva, Heidelberg, Germany) for 10 min followed by 5 ml of mithramycin (50 μg/ml containing 7.5 mM $MgCl_2$ and 12.5% ethanol; Pfizer, New York, N. Y.) to yield a final concentration of 12.5 μg/ml for ethidium bromide and 25 μg/ml for mithramycin. RNase (0.1%; Serva) in 0.3 M NaCl was added for 5 min at room temperature. The samples were measured in a Phywe ICP-11 pulse cytophotometer (Phywe Co., Göttingen, Germany). Routinely, 30,000 to 50,000 cells were measured, and a 128-channel histogram was generated. Histograms were evaluated as described previously (23). The coefficient of variation for the $G_{1/0}$ compartment was routinely less than 5%. All experiments were performed at least twice with duplicate samples per each concentration and time point. Differences between controls and treated samples were analyzed by utilizing a one-way analyses of variance with the Student-Newman-Keuls multiple range test.

For labeling index determinations, cells were pulse labeled (30 min) with [^{3}H]dThd (1 μCi/ml; specific activity, 3.0 Ci/mmol), washed twice with Hanks' balanced salt solution, and harvested with hyaluronidase and trypsin. Cytocentrifuge slide preparations were made and processed for autoradiography with Kodak NTB-2 emulsion. For a determination of the percentage of labeled nuclei, 500 cells were counted. Cells with 5 or more grains over the nucleus were considered labeled. For mitotic index determinations, cytocentrifuge preparations were made and stained with Wright-Giemsa, and the index was calculated for 1000 cells/duplicate dish.

Incorporation of [^{3}H]dThd into DNA. Cells in exponential growth were exposed to 1 and 2 μg of m-AMSA per ml for 2 hr at 37°, followed by [^{3}H]dThd (1 μCi/ml, 6 Ci/mM) added directly to each dish for 1 hr. Cells were then harvested and washed 3 times with 0.4 M cold PCA. The acid-insoluble fraction was hydrolyzed and extracted with 0.4 M PCA by heating in a boiling water bath for 15 min. The incorporation of [^{3}H]dThd was measured by counting an aliquot of the PCA extract with a Packard Tri-Carb Model 3255 liquid scintillation spectrometer (Packard Instrument Co., Downers, Ill.). The percentage of inhibition of DNA synthesis was calculated as the ratio of cpm of drug-treated cells to the cpm obtained in untreated cells.

RESULTS

Dose-dependent Survival of Asynchronous Cells. The survival curve of exponentially growing LoVo cells treated with increasing concentrations of m-AMSA for 1 hr was characterized by a biphasic exponential type B curve (Ref. 17; Chart 1). The numerical values of the pertinent parameters (17) are summarized in Table 1. Treatment in stationary phase of growth resulted in a similar type B pattern but with an almost 10-fold decrease in sensitivity. The survival curves of T_1 cells in exponential and stationary phase of growth were virtually identical to those defined for LoVo cells (Table 1).

Kinetics of Cell Kill. Cell survival as a function of duration of treatment was investigated by exposing cells to a single concentration of m-AMSA for increasing periods of time. The biological stability of the drug was monitored by exposing control cells for 1 hr to the drug-containing supernatants obtained at regular intervals from the treated cells and comparing their survival to that obtained from the cells exposed for 1 hr to freshly prepared drug. In addition, the biological stability of m-AMSA in a cell-free medium solution or in a solution containing 35 mM lactic acid was determined in identical fashion.

When LoVo cells were continuously treated with a single dose (1 μg/ml) of m-AMSA, there was a rapid increase in killing activity, which reached 99% by 8 hr (Chart 2). The rate of cell kill declined subsequently achieving another log by 24 hr. At lower concentrations (0.25 μg/ml), the rate of cell kill after 8 hr was decreased even further.

The decline in cytotoxic activity is attributed to the rapid inactivation of m-AMSA in cell culture supernatants (Chart 3).

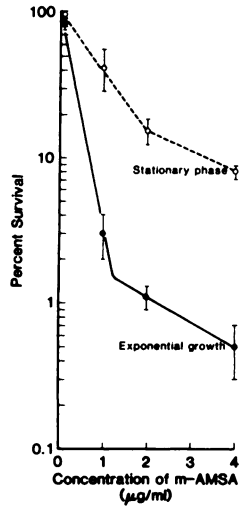

Chart 1. Dose-dependent survival of LoVo cells in exponential and stationary phase of growth. *Bars*, S.D.

Table 1
Survival parameters of cells treated with m-AMSA

Cell line	Phase of growth	D_{o_1}[a] (μg/ml)	D_{o_2} (μg/ml)	IP (μg/ml)
LoVo	Exponential	0.3	2.5	1.3
	Stationary	1	3.1	2
T_1	Exponential	0.6	3.4	3.0
	Stationary	1.3	3.9	4.1

[a] D_o, mean lethal dose equal to the concentration required to reduce survival by 63% on exponential part of survival curve; IP, inflexion point.

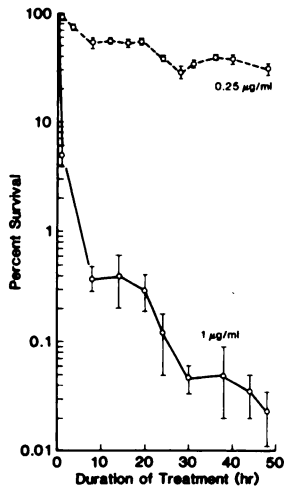

Chart 2. Time-dependent survival of LoVo cells exposed continuously to m-AMSA. *Bars*, S.D.

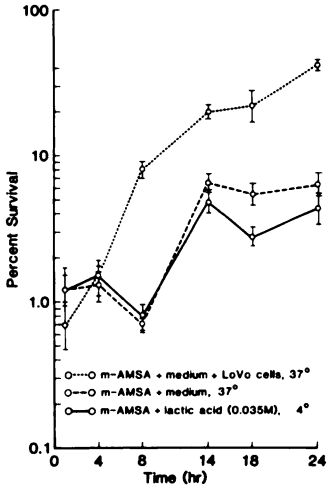

Chart 3. Lethal activity of m-AMSA following different incubation conditions for various periods of time. *Bars*, S.D.

When m-AMSA (5 μg/ml) was in solution without LoVo cells, there was only a moderate loss in potency (about 5-fold) after 14 hr; after continuous exposure to LoVo cells in medium, the lethal activity of the drug was reduced by 10-fold of control in 8 hr and by 15-fold in 24 hr. All of these experiments were conducted in identical fashion under a darkened hood and in a light-tight incubator receiving only minimal indirect fluorescent lighting during the transfer from hood to incubator. Thus, although the mechanism for the loss of potency after exposure to the cells is unclear, it seems related to the presence of cells (*i.e.*, binding of m-AMSA to cell membrane or interaction with secretory products of the cells) rather than to the serum proteins (27) contained in the medium.

Recovery from Potentially Lethal and Sublethal Damage. LoVo cells in exponential and stationary growth treated for 1 hr with graded concentrations of m-AMSA, washed, and reincubated with spent medium for 24 hr before processing for colony formation showed no difference in survival with respect to those processed immediately after treatment.

Cells receiving m-AMSA in split-dose treatment were unable to recover from sublethal damage (Chart 4). In fact, split treatment (1 μg/ml for 15 min plus 1 μg/ml for 15 min) resulted in a small, although statistically insignificant, increase in cell kill with respect to that resulting when the total dose (2 μg/ml for 15 min) was delivered at once. However, if the integral dose ($C \times t = 30$) was considered as a function of time rather than concentration (1 μg/ml for 30 min), a statistically significant increase in cell kill was observed for the split-dose delivery.

Cell Cycle Stage-dependent Survival. No major changes in cell kill as a function of position in the cycle were noted for treatment with 2 different concentrations of m-AMSA (Chart 5).

Effect on DNA Synthesis. Treatment with 1 and 2 μg of m-AMSA per ml for 3 hr decreased incorporation of [^{3}H]dThd into the acid-insoluble fraction by 18% and 31%, respectively.

Cytokinetic Effects. Treatment with 0.01 and 0.1 μg of m-

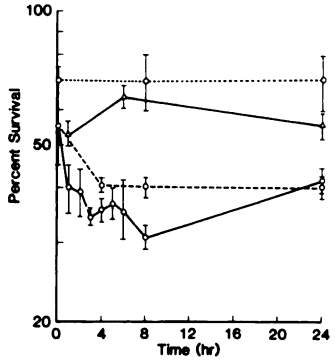

Chart 4. Survival of LoVo cells following protracted treatment with m-AMSA. O—O, survival of cells exposed to 1 μg/ml for 15 min at times indicated; Δ—Δ, survival of cells treated with 1 μg/ml for 30 min at times indicated; O- - -O, survival of cells treated with 2 μg/ml for 15 min at times indicated; O—O, survival of cells treated with 1 μg/ml for 15 min at Time 0, and then washed, reincubated with fresh medium, and finally treated with a second dose of 1 μg/ml for 15 min at times indicated. *Bars*, S.D.

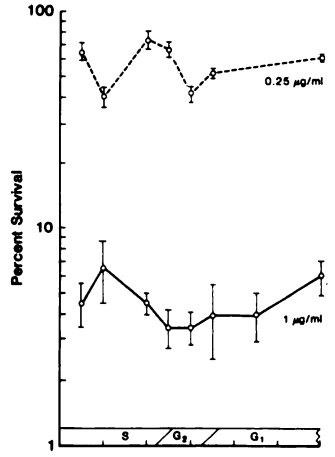

Chart 5. Cell survival as a function of position in the cell cycle at the time of drug administration. *Bars*, S.D.

AMSA per ml for 1 hr did not induce DNA compartment changes significantly different from those of control cells. Treatment with 1 μg/ml for 1 hr induced a steady decrease of the $G_{1/0}$ and S compartments mirrored by an ever-increasing accumulation of cells in G_2 + M, peaking 20 hr after drug removal (Chart 6). The mitotic index never exceeded 4%, indicating that the cells were indeed accumulated in G_2. After 20 hr, the magnitude of the G_2 compartment decreased, while that of the $G_{1/0}$ compartment increased. Treatment for 1 hr with higher concentrations of m-AMSA (10 and 100 μg/ml) showed a somewhat similar pattern. The accumulation in G_2 also increased steadily but reached peak values with delay accounted for by up to 12-hr delays in the exit from S phase. After 24 hr, the compartment distribution remained stable, and no subsequent decreases in G_2 or increases in G_1 were noted.

Treatment with m-AMSA for 1 hr followed by replenishment with fresh medium containing 0.05 μg Colcemid per ml (12) revealed finer details of the perturbation effects of the agent on cell cycle traverse (Chart 7). Even at a concentration of 0.01 μg/ml, onset of evacuation of the $G_{1/0}$ compartment was delayed by 4 hr with respect to control. Evacuation of S phase was delayed by 8 hr, the same time required for beginning accumulation in G_2 + M. With increasing concentration of m-AMSA, delays in $G_{1/0}$ evacuation lasted longer. Onset of evacuation from S phase remained the same, but the rates of G_2 + M accumulation decreased. At a concentration of 100 μg m-AMSA per ml (data not shown), delays were more severe, and the evacuation from $G_{1/0}$ and S was less pronounced.

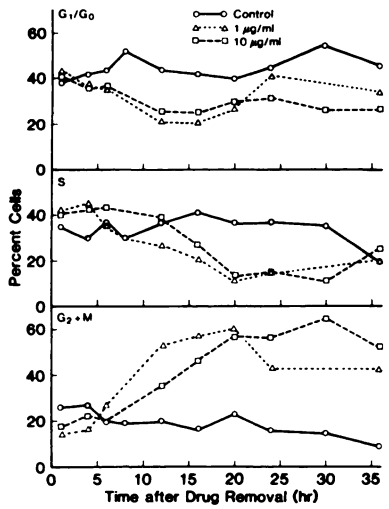

Chart 6. DNA compartment distribution of LoVo cells following treatment with m-AMSA for 1 hr.

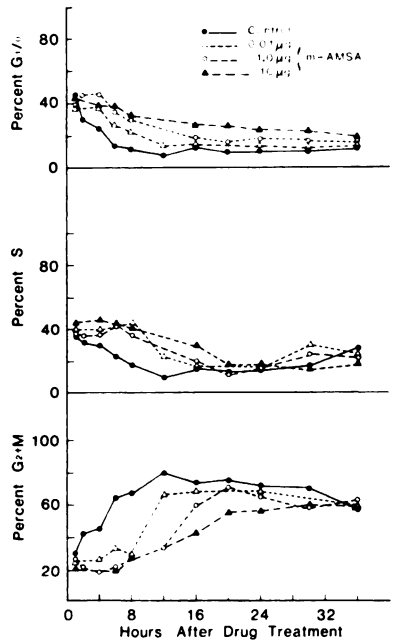

Chart 7. DNA compartment distribution of LoVo cells in the continuous presence of 0.05 μg Colcemid per ml following treatment with m-AMSA for 1 hr.

DISCUSSION

m-AMSA is a new anticancer drug that has demonstrated activity against a variety of experimental tumors, although it was not particularly efficient against human tumors xenografted into nude mice (30). Our *in vitro* studies have shown its potent cytotoxic effect on 2 established human malignant cell lines that presented survival parameters similar to those described for the response of Chinese hamster ovary cells (10, 32). In a fashion similar to that described for many other antitumor agents (15), the activity of m-AMSA decreased markedly when applied to nonproliferating cells.

The mechanism by which m-AMSA achieves lethality is unclear. The biochemical effect of m-AMSA, measured by the inhibition of [^{3}H]dThd incorporation, is much lower in comparison to that shown by experiments involving colony formation technique. When LoVo cells were exposed to 1 μg m-AMSA per ml for 3 hr, incorporation of [^{3}H]dThd into the acid-insoluble fraction was inhibited by only 18%, and yet the inhibition of cell survival reached 75%. These results suggest that inhibition of DNA synthesis by m-AMSA may not be related to its antitumor activity, in agreement with the conclusions reported by Gormley *et al.* (21).

LoVo cells failed to recover from potentially lethal and sublethal damage induced by m-AMSA and exhibited an increased cell killing effect if delivered in a fractionated fashion. Apparently, the initial interaction with the drug sensitizes the critical cellular target to the subsequent exposure so that a greater cytotoxic effect is elicited.

No apparent cell cycle stage-dependent increase in cell lethality was observed for m-AMSA, precluding its utilization in cell kinetics-oriented clinical regimens. This absence of cell cycle stage-dependent cytotoxicity could be due to the long-lasting binding of the drug to its intracellular target. Thus, although exerting its lethal effect in a particular stage of the cycle, the agent can bind to the target at any stage and kill cells when they enter the sensitive stage.

In a fashion similar to that described for other DNA intercalating agents (13), the greatest cytokinetic perturbation effect of m-AMSA consisted of a marked delay in G_2 transit, which developed into a complete irreversible block at higher concentrations (>10 μg/ml), suggesting cell death in that stage. The agent also caused delays in both G_1 and S transit, which accounted for prolongation of the interval required for the G_2 accumulation. At higher concentrations, m-AMSA caused partial blocks in G_1 and S, preventing the magnitude of the evacuation of these compartments into G_2 + M while in the presence of Colcemid.

In conclusion, m-AMSA appears as a very powerful cell cycle stage nonsensitive cytotoxic agent on 2 human malignant cell lines. The agent has the greatest activity on proliferating cells and when delivered in a protracted manner. After interaction with the drug, cells experience delays in their traverse through the cycle and eventually accumulate and die in G_2.

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