Abrogation by Novobiocin of Cytotoxicity Due to the Topoisomerase II Inhibitor Amsacrine in Chinese Hamster Cells


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ABSTRACT

Using cultured V79 Chinese hamster cells, we found that novobiocin (or 2,4-dinitrophenol) can abrogate, almost completely, the cytotoxicity due to the topoisomerase II inhibitor amsacrine (mAMSA). V79 cells were sensitive to mAMSA killing at all stages in the cell cycle but mainly in S phase followed by late G phase; however, novo rescued cells of all ages.

The properties of two kinds of radiation-sensitive Chinese hamster cells were also examined, i.e., the line of V79 cells that can be rescued by caffeine, designated S-10 (H. Utsumi and M. M. Elkind, Radiat. Res., 96: 348-358, 1983); and Chinese hamster ovary cells (P. A. Jeggo and L. M. Kemp, Mutat. Res., 112: 313-327, 1983) which are also sensitive to other DNA-damaging agents. As is the case for exposure to radiation, after mAMSA treatment caffeine rescued V79/S-10 cells. Although Jeggo's Chinese hamster ovary cells were more responsive to novo, novo still abrogated mAMSA toxicity in the mutant cells as well as in the parental Chinese hamster ovary cells. 2,4-Dinitrophenol acted similarly to novo with respect to mAMSA killing, but neither compound reduced the ATP content of V79 cells.

We propose that one reason for the rescue from mAMSA killing of at least S-phase cells by novo or 2,4-dinitrophenol is their ability transiently to inhibit replicative DNA synthesis.

INTRODUCTION

The intercalative inhibitor of topoisomerase II, mAMSA, is thought to arrest "cleavable complexes" in DNA which result in DNA breaks as well as DNA-protein cross-links, lesions which could be responsible for the cytotoxicity which is effected by mAMSA (1-4). Biochemical and cellular studies support the idea that the DNA-protein cross-links involve covalent linkages between DNA and a monomer of topoisomerase II (3-5) which, in turn, can give rise to single- and double-strand breaks in the DNA. Although a correlation between DNA cleavage and cell killing is not always observed (6), the cause and effect connection between DNA damage and cytotoxicity has remained an attractive association.

Radiation is a strong inhibitor of replicative DNA synthesis. Because an association had been demonstrated between the DNA-sequestering property of the DNA intercalator actinomycin D and the enhanced killing of Chinese hamster cells by radiation (7, 8), possible interactive effects between topoisomerase inhibitors and radiation have been under study. These led to some novel findings concerning the combined action of mAMSA and the nonintercalative inhibitor of topoisomerase II, novo, which we report here. In brief, we have found that novo can abrogate mAMSA killing; DNP has a similar effect as was reported earlier for mouse leukemia cells (3). However, additional findings have led to an interpretation of our results that does not hinge upon a suppression of ATP synthesis. In a paper to follow, extensive molecular data will be presented of the effects of novo and DNP on the DNA-damaging properties of mAMSA.

MATERIALS AND METHODS

Cell Culture. The Chinese hamster cells that were used are: a repair-proficient line of V79 cells, V79-B310H; a radiation-sensitive repair-deficient line, V79-A1162/S-10 (referred to hereafter as S-10 cells (9)); repair-competent CHO cells, K-1C; and repair-deficient cells designated by Jeggo and Kemp as line xrs-5 and xrs-6e cells (10). S-10 cells are sensitive to ionizing radiation primarily because of their reduced capacity for sublethal damage (9), whereas the sensitivity of xrs-5 and xrs-6e cells is indicated by a steep survival curve and essentially no capacity for sublethal damage (10). Methods of cell culture and the procedures that were used for measuring survival by colony formation have been described (e.g., Ref. 9). In brief, cells were incubated overnight (2% CO2 at 37°C) in the medium of Eagle's minimum essential medium (Sigma Chemical Co., St. Louis, MO) that contained 10% fetal calf or fetal serum, or a mixture of 2.5% fetal calf plus 7.5% fetal serum (from various sources). The following morning they were treated and then returned to the incubator for an additional 7-10 days for colony formation. Colony counts were made after cells had been stained with methylene blue, rinsed, and dried. In some experiments, cells were synchronized using the method of shaking asynchronously, actively growing cultures to dislodge mitotic cells.

X-Irradiation. Cells growing in 90-mm plastic dishes were exposed to 50-kV X-rays, at a dose rate of 1500 cGy/min, after the growth medium was removed from the dishes. Following exposure, medium was returned to the dishes and they were then incubated for 7-10 days for colony formation depending upon the survival level that was expected. Further details about our irradiation techniques may be found in earlier reports (e.g., Ref. 9).

Treatment with Inhibitors. After overnight growth, cells were incubated with medium containing topoisomerase II inhibitors, and/or DNP, at 37°C for the time periods to be indicated. Treatments were terminated by aspirating the medium containing the compounds, rinsing the dishes with fresh medium, and then adding medium for colony formation. mAMSA (NSC 249992) was obtained from the Drug Synthesis Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, and DMSO, novo, and DNP were obtained from Sigma. A stock of mAMSA at 10 mM was prepared in DMSO and stored frozen; novo was prepared as a 100 mM stock solution in distilled water and was stored frozen; DNP was prepared fresh, for each use, as a 100 mM stock solution in high performance liquid chromatography grade acetone (Fisher Scientific Co., Fair Lawn, NJ).

ATP/Protein Measurements. Ninety-mm dishes inoculated with 6 x 106 cells were grown overnight and then incubated in fresh medium, in medium containing an inhibitor, or in PBS (Gibco Laboratories, Grand Island, NY) that contained DNP. To determine total cellular protein and ATP contents, cells were rinsed twice with ice-cold PBS and then suspended in DMSO by scraping with a rubber policeman. An aliquot of the suspension was hydrolyzed with an equal volume of 0.2 N NaOH (Mallinckrodt, Inc., Paris, KY) for a protein determination. A second aliquot was diluted 5-fold in 0.025 M Tris Ultrl buffer,
Samples for protein measurements were precipitated at room temperature by trichloroacetic acid, pelleted, and suspended in 25% glycerol/water, v/v, in order to eliminate the DMSO and NaOH. A protein assay kit (Bio-Rad Laboratories, Richmond, CA) was used to measure total protein via measurements of absorbance at 595 nm (Gilford Spectrometer Model 260; Gilford Instrument Labs., Oberlin, OH). ATP content was determined in triplicate using an ATP Bioluminescent Assay Kit (Sigma) and a Beckman LS 7800 scintillation counter equipped with a single-photon monitor (Beckman Instruments, Inc., Palo Alto, CA) for measurements of the light generated.

RESULTS

Repair-competent Cells. Fig. 1 shows the killing effectiveness of exposures to mAMSA alone or to mAMSA plus graded concentrations of novo, using 30-min treatments. The convex upward shape of the survival curve for large concentrations of mAMSA suggests that either a small moiety of the cells were resistant to this inhibitor or that cells were induced to become resistant with increasing concentration (11). Progressively increasing concentrations of novo applied with the mAMSA progressively abrogated the killing due to mAMSA without changing the shape of the survival curve.

Fig. 1. Survival of V79-B310H Chinese hamster cells exposed to graded concentrations of mAMSA or mAMSA plus novo. P.E., plating efficiency; N, cell multiplicity at the time of the treatments. Uncertainties, standard errors (bars), are shown where they are larger than the size of the points.

Treatments with novo after exposure to mAMSA was found to be without effect but, as shown in Fig. 2, pretreatment with novo resulted in some survival sparing. The shoulder on the time dependence toxicity curve of mAMSA suggests that its concentration must build up before the fraction of cells surviving decreases. The two curves for pretreatment with novo before mAMSA suggest that the survival sparing resulted from increases in the time required for the concentration of mAMSA to reach effective levels. However, the time dependence of killing when cotreatment was used clearly indicates that novo was most effective when present along with mAMSA.

Fig. 2. Dependence of the length of exposure of the survival of V79-B310H Chinese hamster cells exposed to: mAMSA only; novo added first for 30 or 60 min, followed by mAMSA; or mAMSA plus novo. Other details as for Fig. 1.

With the use of the technique of harvesting synchronous cells, by shaking dishes in which asynchronous cultures were growing to suspend mitotic cells, repair-competent V79 cells were treated as a function of time thereafter as cells progressed through their growth cycle. The work of Sinclair and Morton (12) showed that V79 cells are the most resistant to a brief exposure to X-rays when they are in the second half of S phase. These authors also showed that a period of sensitivity exists at the G1-S phase border.

Fig. 3. Dependence of the survival of V79-B310H Chinese hamster cells on age in their growth cycle for fixed exposures to X-rays, mAMSA alone (20 min) or mAMSA plus novo (20 min). Zero on the abscissa corresponds to the age of mitotic cells and 12 h to about the next mitosis. Other details as for Fig. 1.

In Fig. 3, we have used the X-ray age response pattern as a reference in determining the dependence on cell cycle age of the response of V79 cells to topoisomerase II inhibitors. The features of the age response pattern for X-rays, as worked out by Sinclair and Morton (12), are evident. Also shown is the dependence of survival on exposure to mAMSA; 20-min exposures were used in order to increase the resolution of the mAMSA age response pattern. In addition to S-phase cells being the most sensitive, as was also reported for CHO Chinese hamster cells (13), V79 cells were lethally affected at all ages with late G1-phase to early S-phase cells having an intermediate response. Cotreatment with mAMSA and novo rescued cells at all ages but somewhat less effectively in late G1 phase than at other ages. It is apparent that, roughly speaking, the age response pattern of mAMSA survival is the mirror image of that for X-ray survival.

Repair-deficient Cells. Because of the likelihood that topoisomerase II is closely associated with the DNA-replisomal complex in mammalian cells (e.g., Ref. 14) and the suggestions

pH 7.75 (Calbiochem-Behring Corp., La Jolla, CA) for an ATP determination.

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of various kinds that this complex may be important in the inhibition of DNA synthesis and cell killing by radiation (e.g., Ref. 15), the survival responses of two kinds of repair-deficient, radiation-sensitive Chinese hamster cells to topoisomerase inhibitors were examined.

The first of these are S-10 cells which have a reduced capacity for sublethal radiation damage and an anomalous response to postirradiation treatment with caffeine (9). Whereas repair-competent cells are sensitized to radiation by nontoxic concentrations of caffeine, 1–2 mM, S-10 cells are rescued by caffeine [and other methyl xanthines, but not by dibutyryl cyclic AMP (9)] mainly because the capacity of the cells for sublethal damage is increased. Fig. 4 shows that although repair-competent cells, V79-B310H, were not sensitized by caffeine, caffeine still rescued S-10 cells. The results in Fig. 5 show: (a) that the survival of S-10 cells to mAMSA alone was similar to that for V79-B310H cells (Fig. 1); (b) that caffeine shifted the entire survival curve upward; (c) that novo abrogated mAMSA killing as effectively in S-10 cells as did in V79-B310H cells; and (d) that caffeine appeared to act independently of novo because the effect of caffeine appeared to be additive to that of novo.

The second kind of repair-deficient, radiation-sensitive Chinese hamster cells that we have used are those the sensitivity of which is made evident mainly by a significantly increased steepness of the radiation survival curve. Fig. 6 shows the responses of two of the mutants isolated by Jeggo and Kemp (10) and designated by them xrs-5 and xrs-6e ("e" referring to a clonal reisolation from xrs-6). Also indicated in Fig. 6 by the dashed lines are the responses of the parental CHO cells, clone K-1C, to treatment with mAMSA alone, or mAMSA plus novo. (The data points for the latter two curves have been omitted for clarity because they trace survival responses essentially the same as those for V79-B310H cells.) It is evident that although both radiation-sensitive mutants were more responsive to mAMSA than their parental cells, nonetheless, the sector through which their survival curves were shifted was significant and about the same as for the parental CHO cells.

ATP and Novo/DNP Rescue. It has been inferred that, in bacteria (16) and some mammalian cells (3), ATP is a requirement for cytotoxicity effected by inhibitors of topoisomerase II. Hence, the possibility was examined that novo abrogates mAMSA killing because it inhibits the production of ATP. Accordingly, using the conditions of incubation which were applied in the measurements of cell killing and rescue, we measured ATP/protein ratios in repair-competent cells over time periods up to 3 h. The results in Fig. 7 show that when cells were incubated in growth medium, neither mAMSA alone, novo alone, nor the two inhibitors together progressively affected ATP/protein ratios to a degree greater than did a change of medium alone. Similar results were obtained when cells were incubated in medium containing 2 μM mAMSA plus 5 mM DNP or 5 mM DNP only (data not presented). In contrast, when cells were incubated in PBS+ plus 5 mM DNP, in 30 min the level of ATP/protein was 25–30% of that of untreated cells.

The foregoing result prompted us to examine the effect on cell killing when the treatment with inhibitors was in PBS+ instead of in medium. In the experiment shown in Fig. 8, open, half-filled, and closed circles refer to treatment with mAMSA alone, mAMSA plus DNP, or mAMSA plus novo, respectively. These results show that in medium 5 mM DNP abrogated mAMSA killing as effectively as did 0.8 mM novo. When cells were treated with mAMSA in PBS+, the open squares show that they were killed somewhat more effectively than they were.
in medium. Novobiocin, 0.8 mM, in PBS by itself (for 30 min) reduced the survival to 30–35% of the controls. However, mAMSA plus novo in PBS resulted in increased survival as did novo when cells were cotreated with mAMSA in medium.

The essential points contained in Fig. 8 are: (a) although treating with mAMSA alone in PBS resulted in lower survivals than did mAMSA alone in medium (open squares compared to open circles), in both situations, novo was able to abrogate mAMSA killing; (b) although in medium, neither novo nor DNP significantly affected the ATP/protein content of the cells (Fig. 7) but either compound was still able significantly to abrogate the killing effectiveness of mAMSA; (c) mAMSA plus DNP in PBS produced an even larger sector of rescue than mAMSA plus DNP in medium. Because ATP/protein was significantly reduced when cells were incubated in PBS containing DNP, this last result suggests the possibility that part of the enhanced survival sparing due to DNP may have resulted from the inhibition of ATP content (3).

**DISCUSSION**

As already noted, the DNA intercalator mAMSA and ionizing radiation produce dissimilar effects in mammalian cells; i.e., the shapes of the survival curves are characteristically different because the radiation survival curve does not have upward curvature. Further, even though the lethal effects due to radiation and mAMSA may originate from lesions registered in or near the same anatomical location in the nuclei of cells, it is clear from Fig. 3 that these two agents effect lethality in different ways. Not only is the age response dependence of mAMSA killing qualitatively different from that for ionizing radiation but also the mAMSA cycle dependence is qualitatively different from that of another DNA intercalator, actinomycin D (8, 17). The age response dependence of killing by far- and near-UV light also shows that S-phase cells are the most sensitive (18, 19) and, in this respect, mAMSA is more similar to these radiations than it is to X-rays. However, relative to the induction of sister chromatid exchanges by mAMSA, S-phase cells have been reported to be the most responsive and novo to reduce the frequency of exchanges in S-phase cells (20), suggesting a connection between the exchanges and cell killing.

The cyclic variation in sensitivity to mAMSA that we have observed with V79 Chinese hamster cells is qualitatively similar to those reported by Wilson and Whitmore (13) for CHO cells and by Estey et al. (6) for HeLa cells, when an accounting is made of the somewhat different treatment conditions. However, for HeLa cells G1 phase was the least responsive in contrast to what we observed (Fig. 3). The latter difference could reflect the human epithelial (HeLa) versus rodent fibroblast (V79) properties of the cells and/or the considerably longer G1 period of HeLa cells compared to V79 cells.

In spite of the differences noted in mAMSA killing compared to X-ray killing, the repair-deficient cells showed some systematic similarities in their responses to these two agents. The S-10 variant of V79 cells has a response to mAMSA killing quantitatively similar to that of repair-competent V79 cells. Except for the reduced shoulder width, the radiation survival curve of S-10 cells is also similar to that of repair-competent V79 cells. Further, S-10 cells are rescued by caffeine posttreatment in both cases. However, high concentrations of caffeine (i.e., >1 mM) neither decreased the extent of the survival rescue of S-10 nor enhanced the killing of repair-competent V79 cells following mAMSA treatment in contrast to what was found with radiation (9).

The CHO Chinese hamster cell mutants are significantly more sensitive to mAMSA than the parental cells, suggesting that similarities exist in their responses to both agents. These mutants have been reported to be deficient in the repair of DNA radiation-induced double-strand breaks (21) which leads to the suggestion that double-strand breaks may be involved in the killing by mAMSA. However, novo rescues essentially equally the mutants as it does the parental cells in the instances of mAMSA treatment, whereas novo has been found not to influence the X-ray survival of repair-competent V79 cells. Therefore, it would appear that mAMSA and radiation killing share a common pathway only in part.

Relative to the mechanism of the abrogation of mAMSA killing by novo, or by DNP, our results differ from those obtained with L1210 mouse leukemia cells (3). In contrast to L1210 cells, V79 cells apparently have an adequate metabolic reserve to maintain ATP/protein concentrations for several h at levels equal to those of untreated cells even when the incubation medium contained a concentration of DNP that would
have been effective in L1210 cells. In mouse L-cells, 50 µM DNP was shown to lower appreciably ATP levels (22) but, in this study, the incubation was in Hanks’ saline solution. We too observed a significant reduction in ATP content when V79 cells were incubated with DNP in a saline solution (Fig. 7).

In the present study, the inability of novo or DNP to affect ATP levels in V79 cells when medium was used, plus the lack of a qualitative change in the rescuing properties of DNP when cells were cotreated with mAMSA contained in PBS, do not support a general involvement of an ATP-requiring process in cell killing by the topoisomerase II inhibitor mAMSA subsequent to the formation of cleavable complexes. In a further study of the production and repair of cleavable complexes due to mAMSA, evidence in support of a large enough reduction in the frequency of such complexes to explain the increases in survival by cotreatment with novo or DNP was not found. However, in contrast to the significant inhibition of replicative DNA synthesis by mAMSA, an inhibition that persists in time as does the inhibition produced by radiation, novo and DNP produce only transient, although significant, depressions in DNA synthesis consistent with the action of a metabolic as opposed to a template-damaging effect (23). Other metabolic inhibitors of replicative DNA synthesis were also found to be able to abrogate mAMSA toxicity. The metabolic inhibition of DNA synthesis, together with the lack of any survival sparing if the novo treatment followed that with mAMSA, leads us to propose that the slowing of replicative DNA synthesis plays a role in the abrogation of toxicity. Hence, at least in S-phase cells, our results suggest that mAMSA is cytotoxic because of the disruption of the orderly, isomeric changes which DNA must undergo as it leaves the replication complex and is organized into compact, superhelical chromatin.

REFERENCES


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