Response of 9L Tumor Cells In Vitro to Spirohydantoin Mustard

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ABSTRACT

The effects of spirohydantoin mustard (SHM), a potential antitumor agent for central nervous system tumors, in the in vitro 9L rat brain tumor model were studied. In cell culture medium at 37°C, the drug was totally detoxified within 30 min. Dose-response curves for exponentially growing and plateau-phase cells were similar and indicated that a small fraction of cells were resistant to SHM. When exponentially growing cells were treated with SHM (5 μg/ml for 1 hr), recovery from potentially lethal damage occurred within 28 hr.

When cells were perturbed by SHM, the S, G2, and M phases were prolonged, there was a G2 block, some cells entered mitosis, but few divided, and cells tended to accumulate in mid-S, then moved synchronously to G2-M. The rate at which cells moved was concentration dependent and was much slower at high concentrations. The ability of SHM to both synchronize cells and block DNA synthesis may be useful in multitagent therapy regimens.

INTRODUCTION

In 1975, Peng et al. (6) reported the synthesis of a new family of drugs that incorporated a known antitumor functional group into 5,5-diphenylhydantoin, an anticonvulsant drug with no antitumor activity that penetrates the blood-brain barrier in significant concentrations (2) and localizes in brain tumors relative to surrounding normal brain tissue (8). Five compounds with nitrogen mustard linked to the hydantoin ring showed significant activity against several murine tumor systems, including an ependymoblastoma (6).

In SHM, nitrogen mustard is attached via a 2-N-ethyl group to N-3, and the 2 phenyl groups of 5,5-diphenylhydantoin are replaced by a spiro-cyclohexyl group (Chart 1). Plowman et al. (7) showed that SHM in dog blood has a distribution phase (half-life, 1.8 min) followed by an elimination phase (half-life, 11 min), and crosses the blood-brain barrier of dogs.

We have reported the effects of SHM in combination with radiation on 9L brain tumor cells in vitro (1). A schedule-dependent enhanced radiation response resulted when cells were exposed to a low concentration of SHM either 4 or 24 hr before or after irradiation, with the greatest enhancement found when the drug was given 4 hr after irradiation. This report summarizes our findings on the effects of the drug on this cell line.

MATERIALS AND METHODS

Cell Culture Conditions

Monolayer cultures of 9L cells were grown in CM consisting of Eagle’s minimum essential medium supplemented with fetal calf serum (10%, v/v), streptomycin (87 μg/ml), and penicillin (87 units/ml). Cells had a doubling time of 18 to 20 hr and a plating efficiency of 60 to 80% when they were maintained in this medium at 37°C in a humidified 95% air:5% CO2 environment.

Colony Formation Assay

This assay has been described (1, 10). In these studies, (saline A containing 0.05% trypsin and 0.02% versene) was used to remove cells from the flasks, and the optimal feeder number (5 × 10⁵) and incubation time (12 to 14 days) were determined in preliminary experiments. PE’s for untreated and treated cells were calculated as the ratio of the number of colonies observed to the number of cells plated. SF’s were calculated as the ratio of the PE of treated cells to the PE of untreated cells.

Stability of SHM Solutions

The drug was dissolved in either ethanol or DMSO at 23°C and allowed to stand for up to 2 hr. These solutions were diluted with CM, and exponentially growing cells were exposed to 2-μg/ml doses of the drug for 1 hr at 37°C and then assayed as above. Temperature effects on the stability of SHM in solution were determined in CM with or without fetal calf serum at 4, 23, or 37°C for various times before exposing the cells to SHM (2 μg/ml) for 1 hr at 37°C.

Dose-Response Studies

For monolayer cultures, SHM was dissolved in either ethanol or DMSO and diluted with CM to a final 1:10 dilution (15 ml total) in treatment flasks that contained either exponentially growing or plateau-phase 9L cells. Unless the temperature or pH was deliberately altered, the cells were maintained at 35–37°C and pH 7.2 to 7.4. Cells were trypsinized and assayed for colony formation immediately after drug treatment.

Recovery from PLD

Exponentially growing monolayer cells were exposed to SHM (5 μg/ml) for 1 hr, rinsed (2 times; 10 ml), overlaid with 37°C buffered EBSS, and incubated at 37°C. At various times, cells were assayed for colony formation as described above.

In a separate experiment, exponentially growing cells were exposed for 1 hr to various concentrations of SHM and then either trypsinized and plated immediately, or rinsed, overlaid with EBSS, and reincubated at 37°C for 24 hr before trypsinizing and plating.
In Vitro Effects of Spirohydantoin Mustard

Growth Rate Studies

Cultures containing $5 \times 10^6$ cells initially were exposed 48 hr later for 1 hr to SHM (0, 5, 10, and 20 $\mu$g/ml). On each of 5 successive days after SHM treatment, 3 cultures from each experimental group were trypsinized, and the number of cells was counted.

Cell Progression Analysis

**Pulse Chase Method.** Exponentially growing cells in 75-sq cm flasks were exposed to SHM (0, 10, and 20 $\mu$g/ml) for 1 hr. Thirty min after initiation of the drug treatment, each flask received 1 $\mu$Ci of $[^3]$H]thymidine (specific activity, 0.36 Ci/mmole; Schwarz/Mann, Orangeburg, N. Y.). After the 1-hr drug treatment, cells were rinsed twice with cold medium containing 0.5 mM thymidine (Sigma Chemical Co., St. Louis, Mo.). The remainder of this experiment, which resulted in construction of PLM curves, was carried out as described previously (5).

**FCM Analysis.** A random sample from each of 3 groups of exponentially growing cultures [treated for 1 hr with SHM (5, 10, and 20 $\mu$g/ml)] was harvested every 3 hr by trypsinization and fixed in 10% buffered formalin. The cells were stained with acriflavin after hydrolysis with HCl (4) and analyzed using the flow cytometer at the Lawrence Livermore Laboratories of the University of California (3, 9).

Radiosensitizer Study

Cells ($6 \times 10^5$) were seeded into 60-mm glass Petri dishes 24 hr before the experiment; $20 \mu$l of SHM in ethanol were added to walled-off compartments within some dishes. Selected dishes were placed in gas-tight aluminum chambers, the 95% air:5% CO$_2$ environment was evacuated using a vacuum pump, and the cultures were flushed with a 95% N$_2$:5% CO$_2$ mixture. The evacuation and flushing process was repeated 4 times during the first hr. At the end of the first hr, the chambers were tilted to mix the SHM solution and growth medium (final concentration, 2 $\mu$g/ml). During the second hr, the cultures were irradiated (230 kvp; 15 ma; 1.23 GY/min) in the aluminum chambers. Cultures were at ambient temperature ($\approx$23°) for the entire 2 hr. After the 1-hr drug treatment, the cells were assayed as above.

RESULTS

Stability of SHM Solutions

The same survival level resulted when SHM was dissolved in either ethanol or DMSO and allowed to remain at $\approx$23° for up to 2 hr (Chart 2A). However, in aqueous cell culture medium, cytotoxicity was lost at rates that increased with temperature (Chart 2, B to D). At 37°, cytotoxicity was completely eliminated by a 30-min preexposure to cell culture medium.

Dose-Response Studies

The 1-hr dose-response curve using exponentially growing cells showed a slight shoulder followed by an exponential region of cell kill (Chart 3). A plateau region, where the SF was $\approx1 \times 10^{-4}$, was observed for concentrations $>9$ $\mu$g/ml. One-hr dose-response curves using either plateau-phase or exponentially growing cells were similar (Chart 4), and the efficacy of the drug for killing exponentially growing cells was the same when cells were exposed for 2 hr at either ambient temperature or 37° (data not shown). However, when cells were exposed to graded concentrations of SHM at a higher pH (1-hr exposure; 37°; pH 8.2 versus 7.4), less cell kill resulted (data not shown).

The survival of 9L single cells in suspension exposed to SHM (5 $\mu$g/ml) at 37° decreased very rapidly with time. Cell kill was complete after 30 min of exposure, similar to that seen for the 2-$\mu$g/ml concentration used in the drug stability experiments (Chart 2D).

Growth Rate. After 1-hr exposures to increasing concentrations of SHM, control cells continued to grow exponentially for 2 days, after which they plateaued at about $6 \times 10^6$ for the next 3 days (Chart 5). Exposure to SHM (5 $\mu$g/ml) moderately inhibited both the growth rate after treatment and the level at which cells plateaued. Exposure to SHM (10 or 20 $\mu$g/ml) prevented cell growth.

Recovery from PLD. When exponentially growing cells were allowed to recover in EBSS after a 1-hr treatment with SHM (5 $\mu$g/ml), the fraction of cells surviving increased by a factor of 3 during the first 5 hr and by a factor of 50 to 80 during the first 28 hr (Chart 6). A constant number of cells per flask was recovered, and the PE of control cells decreased only slightly at 24 to 28 hr. At several concentrations of SHM and a 24-hr recovery period, similar recovery resulted (Chart 7). Survival after a 1-hr exposure to 5-$\mu$g/ml doses of the drug increased about 2.5 orders of magnitude after 24 hr of recovery.
**Cell Progression Analysis**

**Pulse Chase Method.** The PLM curve for cells treated with 10 μg/ml relative to the PLM curve for control cells showed: (a) a delayed initial appearance of labeled mitoses; (b) a slower increase in PLM from 0% at 5 hr to 100% at 15 hr after SHM exposure; (c) a plateau at 100% from 15 hr to about 30 hr after SHM exposure; and (d) a gradual decrease in PLM for times >30 hr (Chart 8, A and B). The frequency of mitoses was <1% during the 3- to 20-hr period after exposure to SHM, but increased from about 1% at 20 hr to >6% at 45 hr after exposure (Chart 8C). No mitoses were observed after a 1-hr exposure to SHM (20 μg/ml).

**FCM Analysis.** After exposure to SHM (10 μg/ml) for 1 hr, a cohort of cells left the G₀ peak and moved to middle S, where the entire population was amassed by 15 hr (Chart 9). This cohort moved in synchrony to the G₂-M position by about 30 hr and remained there for the duration of the 45-hr posttreatment observation period. Cells treated with SHM (5 μg/ml) showed a similar accumulation in S phase, followed by a faster synchronous move towards G₂-M. Cells treated with SHM (20 μg/ml) also showed similar but slower changes to those seen for the lower concentrations, and the amassed cohort in middle S phase never reached G₂ phase during the 72-hr posttreatment observation period.

**Radiosensitizer Study**

A 1-hr exposure with SHM (2 μg/ml) at 23° did not sensitize either oxic or hypoxic cells to concurrent X-irradiation (Chart 10). Oxygen enhancement ratios of about 3 were obtained in this experiment.

**DISCUSSION**

Results for the dose-response studies indicate that exponentially growing and plateau-phase cells were equally sensitive to SHM (Chart 4). Because the experimental conditions such as temperature (Chart 2, B to D) and pH that could affect the cytotoxicity via changes in pharmacokinetics (11) were carefully controlled, SHM appears to be a cell cycle-nonspecific drug. Survivors of high concentrations (>9 μg/ml) of SHM may represent a small resistant subpopulation within the 9L cell line (Chart 3). Verification of resistance should be confirmed by isolating these survivors and performing a subsequent dose-response curve.

Although this cell line could recover from PLD (Charts 6 and 7), recovery might be an artifact of the in vitro system and may...
The delayed initial appearance of labeled mitoses after cells were treated with SHM (10 μg/ml) indicated that cellular progression through G2 was prolonged by 2 to 2.5 hr (Chart 8). The slow increase in PLM of treated cells relative to control or may not occur in vivo. If cells in a tumor actually can recover to this extent from SHM-induced damage, single doses of SHM may be of limited usefulness in chemotherapy. This phenomenon is being investigated.
cells implies either a prolonged mitotic phase or a transient G2-M block, which is supported by the low frequency of mitoses observed during this period. The plateau at 100% in PLM and the small number of mitotic cells at 15- to 30-hr posttreatment indicated a prolonged S phase. The gradual decrease in PLM for times >30 hr indicated that some cells initially in G1 phase reach mitosis; however, the marked increase in the number of mitoses during this period showed that most of the cells that enter mitosis could be blocked in metaphase. The growth curve confirmed that few cells exposed to SHM (10 μg/ml) retain the capacity to divide (Chart 5). The FCM profiles showed that the cohort of cells accumulated in G2-M, with no cells entering G1 (Chart 9).

Extrapolation of the observed SHM-induced perturbations in high growth fraction in vitro cells to relatively low growth fraction in vivo cells is complicated. In preliminary studies, rats bearing the intracerebral 9L tumor treated with single doses of SHM (1, 2, 4, 8, 10, and 16 mg/kg) failed to show an increase in life span,4 which may reflect the apparent ability of tumor cells to recover from SHM-induced PLD in vitro. We do not know if the in vitro cells that accumulate at the G2-M boundary after treatment with the lower SHM concentrations are doomed to die or if similar events occur in vivo. In addition, the recovery potential of in vivo cells is unknown. However, our in vitro results clearly show that SHM might be utilized in 2 ways: (a) as a strong inhibitor of DNA synthesis; and (b) as a drug that synchronizes cells at either S or G2. Preliminary data from our laboratory indicate that low concentrations of SHM (0.5 to 1.5 μg/ml) also synchronize the cells as above. Therefore, it is possible that the synchronizing capability of SHM would exist also for in situ brain tumor cells, and SHM could be used to advantage in multiagent anticancer regimens.

Finally, although SHM enhanced the effects of X-rays on 9L cells (1), it is not a classical radiosensitizer of either oxic or hypoxic cells (Chart 10), and the observed enhancement must be attributed to some other cause. The time and schedule dependencies of radiation enhancement were not explained by the perturbations shown by the present experiments.

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