Enhancement of X-ray Damage in HeLa Cells by Exposure to Lucanthone (Miracil D) following Radiation

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SUMMARY

The colony-forming ability of HeLa cells that survived an initial dose of X-irradiation proved abnormally sensitive to lucanthone. Surviving cells gradually regained normal resistance to lucanthone treatment, which implied that lucanthone was acting to inhibit a postradiation repair process. Enhancement of cell killing by lucanthone was comparable in degree to that induced by actinomycin D, with which it shares many properties. Lucanthone merits further study as a possible adjunct in clinical radiation therapy, since it is a relatively safe drug which has been widely used for many years in the treatment of human schistosomiasis.

INTRODUCTION

Lucanthone (Miracil D) is a thiaxanthene which has been widely used for many years in the treatment of human schistosomiasis (4). Its heterocyclic ring structure resembles that of actinomycin D; like actinomycin D, lucanthone binds to DNA and inhibits RNA synthesis in bacteria (15). Lucanthone inhibits both DNA and RNA synthesis to about the same extent in HeLa cells (3), in contrast to actinomycin D which is primarily an inhibitor of RNA synthesis (13). Another important difference between the two drugs resides in the fact that actinomycin D is 40 times as effective an inhibitor of RNA synthesis in HeLa cells but it is 5000 times as potent in its cell-killing effects (3). Moreover, the effects of actinomycin D on cell viability are not readily reversible (1), unlike those of lucanthone (3).

Previous studies suggested that actinomycin D enhances X-ray damage in animal cells by interfering with postradiation repair processes (2, 7, 9). Elkind et al. (8) reported that repair of sublethal damage in irradiated hamster cells was sensitive to actinomycin D and that the sensitivity of the repair progressively disappeared during 4 to 6 hr following radiation.

Although it is not known which molecular species are primarily involved in repair of ionizing radiation damage, similarities in the chemical structure of the two drugs and in their effects on macromolecular synthesis suggested that lucanthone might also enhance X-ray damage in animal cells. This is of special interest because actinomycin D has an established role as a useful adjunct in the radiation therapy of certain childhood tumors (5, 10). Unfortunately, its cytotoxic effects have restricted its general usefulness in clinical radiation therapy.

MATERIALS AND METHODS

Suspension cultures of HeLa S-3 were grown in Eagle's medium as modified for suspension cultures (6) with 5% fetal bovine serum. Under these conditions, the cells were virtually single and the cell populations doubled every 22 hr. About 50% of control cells plated directly from suspension cultures produced macroscopic colonies in 10 to 12 days of incubation in Eagle's minimum essential medium with 10% fetal bovine serum in a moist CO₂ atmosphere at 37°C.

X-rays (280 kV) were used to irradiate cells in plastic Petri plates or glass suspension culture bottles at dose rates of 430 rad/min or 310 rad/min, respectively. A Picker Vanguard constant potential X-ray machine was operated at 20 ma with 3.2 mm of beryllium filtration (equivalent to a half-value layer of 0.14 mm of copper) and doses were determined with a 250-R Victoreen chamber immersed in medium at the center of the culture vessels.

Lucanthone concentrations were verified by absorbance measurements at 330 nm (16). The lucanthone concentration used in studies with HeLa cells was 3.0 µg/ml. Under these conditions, ribosomal RNA synthesis was completely inhibited, synthesis of DNA-like RNA continued, DNA synthesis was depressed to about 30% of normal, while protein synthesis remained grossly undisturbed (3). However, the inhibitory effects of lucanthone were promptly and completely reversible if cells were removed from the drug within 2 hr. Longer exposures sterilized them, as judged by loss of ability of single cells to produce macroscopic colonies. Like lucanthone, low concentrations of actinomycin D selectively inhibit ribosomal RNA synthesis while allowing messenger RNA synthesis to continue (11–14).

RESULTS AND DISCUSSION

Chart 1 shows that unirradiated HeLa cells in suspension cultures did not lose viability unless exposed to lucanthone for more than 2 hr, unlike survivors of 300 rad or 500 rad, which lost viability promptly and more rapidly during exposure to the drug. Only 3% of the survivors of 500 rad remained viable after 10.5 hr of exposure to the drug; in contrast, 18% of the unirradiated cells were still viable at that time.
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irradiated and unirradiated S or G2 cells was only one-half as great as that observed for G1 cells. The effects of lucanthone on radiation dose-response curves of HeLa cells were examined in order to judge its effects on the D0 and on the shoulder of these curves. Chart 3 shows radiation dose-response curves for single cells irradiated soon after attachment in plastic Petri dishes. One group was exposed to lucanthone for 4 hr beginning immediately after irradiation. Lucanthone was then removed and normal medium replaced; the other group was treated identically except that no lucanthone was used. The plating efficiency of unirradiated cells of both groups was virtually identical (i.e., complete reversibility of drug effects). Irradiated cells exposed to lucanthone for 4 hr suffered relatively greater loss of viability and this increased with radiation dose. The D0's (radiation dose leaving 37% survivors on the straight portion of the curve) were estimated at 170 rad and 150 rad in the two groups. Some reduction of the shoulder also is evident in the dose-response curve of lucanthone-treated irradiated cells. If

Chart 1. Increased sensitivity of irradiated HeLa cells to lucanthone.
A suspension culture containing $1 \times 10^6$ HeLa cells/ml in modified Eagle's medium was divided into three identical subcultures. Two received 300 rad or 500 rad; the third served as an unirradiated control. Lucanthone was immediately added to all 3 cultures in a final concentration of 3.0 $\mu$g/ml. At the indicated times, cell samples were removed, diluted, and plated for survivors in Eagle's minimal essential medium containing 10% fetal bovine serum. Residual lucanthone carried into assay plates underwent dilution to a concentration 50-fold less than the minimum detectable inhibitory one. Control cells had 40% plating efficiency in this experiment; 3.0 $\mu$g/ml of lucanthone caused no decrease in plating efficiency in unirradiated cells exposed to it for up to 4 hr before rescue. •, relative survival of unirradiated cells; △, relative survival of colony-forming cells present after 300 rad (0.46 compared with unirradiated controls); ◇, relative survival of colony forming cells present after 500 rad (0.16 compared with unirradiated controls). Assays were performed in duplicate or triplicate. Determinations at 10.5 hr of exposure were made in quadruplicate; the standard errors of means were calculated and are shown if larger than the symbols.

Results comparable to those of Chart 1 also were obtained with synchronized cell populations tested in the G1, S, or G2 phases of their mitotic cycle. Cultures were synchronized by exposure to 0.002 M thymidine (17); following return to normal growth medium cells were selected at appropriate times as determined by monitoring thymidine-$^3$H incorporation in aliquots of the cultures and by enumerating the cells. Chart 2 shows results of an experiment with G1 cells. In Chart 2, the lucanthone sensitivity of unirradiated G1 cells is slightly greater than with random cells (Chart 1). This may be related to the thymidine treatment itself but is not inconsistent with variations encountered with random cells in other experiments.

Cell age does not greatly influence the sensitivity of irradiated cells to lucanthone. However, unirradiated cells in S and G2 were more sensitive to lucanthone than those in G1. Because of this, the difference between the drug sensitivity of

Chart 2. Increased sensitivity of irradiated G1 cells to lucanthone.
Cells were synchronized by 14 hr of exposure to 0.002 M thymidine, after which the cells were resuspended in normal medium. The cell population remained constant for 11 hr following return to normal medium and then it abruptly doubled during a 2-hr interval. An experiment similar to that of Chart 1 was then performed. •, relative survival of unirradiated cells; ◇, relative survival of cells after 300 rad (0.46 compared with unirradiated controls); ◇, relative survival of colony forming cells present after 500 rad (0.16 compared with unirradiated controls). Assays were performed in duplicate or triplicate. Determinations at 10.5 hr of exposure were made in quadruplicate; the standard errors of means were calculated and are shown if larger than the symbols.
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and 180 rad to 135 and 140 rad. The shoulders of the curves also were shortened. Reduction in $D_0$ by radiation may not alone be sufficient to account for this, since it can also be appreciated from Charts 3, 4, and 5 that dose-response curves of cells treated with lucanthone extrapolate back to lower numbers on the ordinates than do curves for control cells. Survival of lucanthone-treated HeLa cells was reduced 2- to 6-fold at the radiation doses tested, comparing well with results previously obtained with actinomycin D (1).

Results with synchronous cells suggested that the enhancement of radiation damage by lucanthone could not be accounted for simply as a selection of subpopulations sensitive to one agent by pretreatment with the other. Instead, it appears more likely that lucanthone acts to inhibit postradiation repair.

Normal sensitivity to lucanthone returned soon after irradiation. Chart 6 shows progressive recovery of normal sensitivity to a 4-hr treatment with 3µg/ml of lucanthone; recovery was still incomplete 5 hr after exposure to 500 rad. However, normal sensitivity of HeLa cells to a 2-hr treatment with 3 µg/ml returned by 5 hr after 300 rad (Chart 7). In both of these experiments, the resistance of irradiated cells to lucanthone increased most notably during the 1st hr following radiation, too early to be accounted for by cell synchronization effects of the experimental operations used. These results also speak against selection of lucanthone-sensitive cells by radiation.

Charts 4 and 5 show experiments in which longer lucanthone treatments after radiation reduced the $D_0$ from 170 rad to 160 rad, 140 rad, and 135 rad. The shoulders of the curves also were shortened. Reduction in $D_0$ by radiation may not alone be sufficient to account for this, since it can also be appreciated from Charts 3, 4, and 5 that dose-response curves of cells treated with lucanthone extrapolate back to lower numbers on the ordinates than do curves for control cells. Survival of lucanthone-treated HeLa cells was reduced 2- to 6-fold at the radiation doses tested, comparing well with results previously obtained with actinomycin D (1).

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Chart 3. X-ray dose-response curves for HeLa cells attached to plastic Petri plates. ●, no lucanthone treatment; ○, exposure to 3.0 µg/ml of lucanthone for 4.0 hr following irradiation. All determinations were made in replicates of 3 or 6, and the standard errors of the means are indicated where larger than the symbol size. The plating efficiency of control cells was 48%, and for lucanthone-treated cells it was 45%.

Chart 4. X-ray dose-response curves for cells attached to plastic Petri plates. Symbols are as described in the legend for Chart 3. Lucanthone treatment lasted 7.5 hr. Control plating efficiency was 44% and the $D_0$ was 170 rad; plating efficiency for lucanthone-treated cells was 28% and their $D_0$ was 135 rad.

Chart 5. X-ray dose-response curves for cells attached to plastic Petri plates. Symbols are as described in the legend for Chart 3. Lucanthone treatment lasted 8.0 hr. Control plating efficiency was 50% and the $D_0$ was 180 rad; plating efficiency for lucanthone-treated cells was 23% and their $D_0$ was 140 rad.
Finally, blood concentrations of 3.0 μg/ml of lucanthone are routinely achieved in man and are recommended in the treatment of human schistosomiasis (4). At that time of Blair's review, no deaths or lasting side effects could be attributed to lucanthone despite the fact that hundreds of thousands of patients had received it.

**ADDENDUM**

Recent experiments have shown that sublethal doses of lucanthone significantly enhance mortality in mice receiving sublethal doses of total-body radiation. In one such experiment, the cumulative 28-day mortality for mice receiving 400 rad was 0/23, for mice receiving 180 mg/kg of lucanthone methanesulfonate subcutaneously it was 0/22, while for mice receiving the lucanthone immediately after the radiation it was 13/24.

**REFERENCES**


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