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β-LAPACHONE GREATLY ENHANCES MMS LETHALITY TO HUMAN FIBROBLASTS

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β-Lapachone is a naturally occurring tricyclic O-naphthoquinone. At μM concentrations it did not substantially affect viability, growth or DNA synthesis of cultured undamaged human fibroblasts. Cells exposed to minimally toxic concentrations of methyl methane sulfonate were strongly inhibited in these properties by β-lapachone. The effects were not reversed by further incubation in the absence of β-lapachone and were equal for initially quiescent or growing cells. Thus inhibitions were specific for damaged cells and did not involve replicative DNA synthesis. Inhibition of DNA strand break repair was demonstrated by alkaline elution, but unscheduled DNA synthesis was not inhibited. We propose that β-lapachone inhibits a ligation step of DNA repair, in a manner perhaps similar to that reported for carbamoylating nitrosoureas. Other repair inhibitors differ significantly from β-lapachone in their modes of action.

Much DNA damage is repaired; therefore repair inhibitors greatly enhance consequences of damage such as cell death. Pharmacological inhibition of DNA repair is of fundamental and medical interest, as shown by extensive literature on effects of substances such as methylxanthines (1,2) or 3-aminobenzamide (3,4,5) on cells damaged by a variety of agents. We report here that β-lapachone has similar effects when applied at μM concentrations. It has antiprotozoal (6) and antimalarial activities (7), but its synergism with DNA damaging drugs appears not to have been studied.

MATERIALS AND METHODS

Cell culture methods: The normal human foreskin fibroblast strain (GM 3652) was purchased from the Human Genetic Mutant Cell Repository, Camden, N.J. Methods for cell culture, colony and cell counting, etc. have been described (8). Other methods are described in Figure legends.

Drugs. MMS was purchased from Aldrich. β-lapachone was a gift from Ciba-Geigy of India. Other drugs and chemicals were purchased from Sigma or Aldrich.

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Abbreviations: methyl methane sulfonate, MMS; 3,4-dihydro-2,2-dimethyl-2H-naphtho 1,2-b pyran-5,6-dione, β-lapachone (see Figure 1).

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RESULTS AND DISCUSSION

Cell survival was much diminished when human fibroblasts treated with a 20-30% lethal dose of MMS for 1 hour were subsequently exposed for 4 hours to β-lapachone (Figure 1). Neither MMS nor β-lapachone alone caused nearly as great lethality. This synergistic lethality occurred with either quiescent (G_0) or S phase cells.

Increase in number of cells during 2 days was determined in experiments otherwise identical to those of Figure 1. The increases after MMS treatment were inhibited 30 and 100% by 2 and 4 μM β-lapachone, respectively, with either initially treated G_0 or S phase cells. Neither MMS or β-lapachone alone inhibited cell growth.

β-lapachone rapidly inhibited replicative DNA synthesis (^{3}H-thymidine incorporation), by MMS treated cells to a much greater extent than by

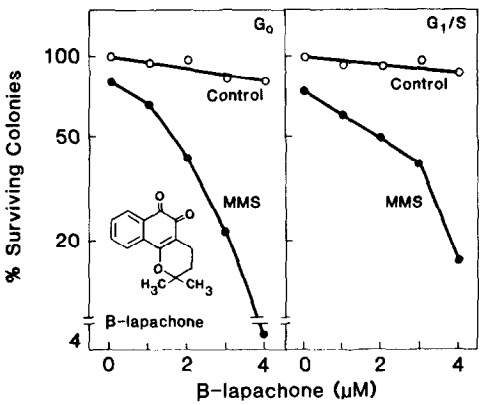

Figure 1: Effects of β-lapachone and MMS on survival of human fibroblasts.

Normal human fibroblasts GM3652 were plated at a density of 5 x 10^4 cells per 35 mm plastic dish. To obtain cells in G_0, they were shifted to DME medium + 0.5% fetal calf serum for 36 hours. To obtain cells at the G_1/S boundary, G_0 cells were shifted to DME + 10% fetal calf serum for 10 hours, and then to DME + 10% fetal calf serum + 0.25 mM hydroxyurea for an additional 10 hours. Cells were then washed and fresh DME + 10% fetal calf serum was added either in the presence (●) or absence (○) of 0.5 mM MMS. After 1 hour, the cells were washed, and indicated doses of β-lapachone were added for 4 hours in complete medium. Cells released from G_0 and treated with MMS did not enter S phase during the drug treatments, whereas cells treated with MMS at the G_1/S boundary traversed S. Cells were then trypsinized and replated at 500 cells per 60 mm dish in F12 medium + 15% fetal calf serum. This medium was replaced after 48 hours. After 12 additional days, colonies were fixed with Hank's balanced salt solution plus formalin and stained with crystal violet. Colonies of greater than 50 cells were counted. Plating efficiencies averaged 30-40% in control cultures. % surviving colonies is expressed relative to untreated control cultures.

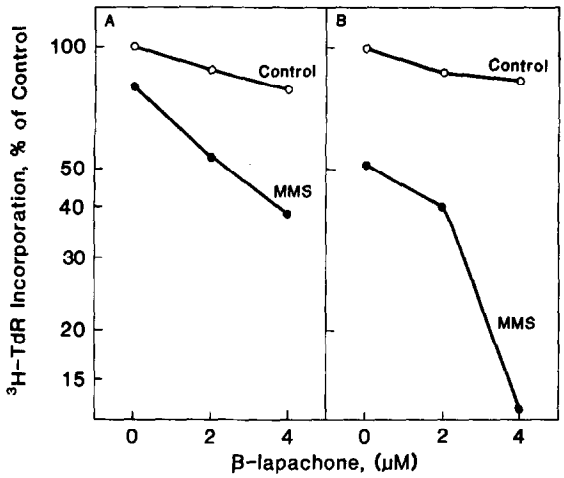

Figure 2: Effect of β-lapachone on ^{3}H-thymidine incorporation.

Cells were synchronized at the $G_{1/S}$ boundary and treated with MMS and β-lapachone as described in Figure 1. Cells were incubated continuously with 2 μCi/ml ^{3}H-thymidine during the 4 hour interval with β-lapachone (Panel A) or during the following 20 hour interval (Panel B). The cells were washed three times with PBS, incubated for 20 min at 4°C with 5% TCA, and dissolved in 0.2 N NaOH. Percent of incorporation relative to control cultures is shown. Approximately 3 times as much incorporation was measured in control cultures in the 4-24 hour interval as in the 0-4 hour interval.

untreated cells (Figure 2). Inhibition was already evident by 4 hours, and was greater during the next 20 hours. Inhibition was minimal with undamaged cells. Similar treatment of initially G_0 cells with MMS followed by 4 μM β-lapachone inhibited ^{3}H-thymidine incorporation by 99% over the next 24 hours.

Flow microfluorimetry (9) was used to locate the phase in the cell cycle of growth arrest. Cells synchronized with hydroxyurea at the $G_{1/S}$ boundary, treated with MMS and then with β-lapachone. They were delayed in their passage through S and stopped in mid to late S. Cells treated only with MMS or β-lapachone progressed through the cycle with normal kinetics. Quiescent cells treated with MMS and β-lapachone and then stimulated with serum were unable to enter S at all. These results are consistent with inhibition of DNA replication.

These effects of β-lapachone on viability, increase in cell number and DNA synthesis were very similar. They required prior MMS treatment, and were rapidly suppressed and not reversible. MMS is generally thought to be

lethal through DNA methylations which are repairable. β-Lapachone might inhibit this repair, at the step of DNA incision, or excision of nucleotides, or insertion of new nucleotides, or ligation. MMS creates single strand incisions as demonstrated by the alkaline elution technique (Figure 3). After 4 hours incubation some lesions were removed, but most remained if β-lapachone was present. Thus, β-lapachone inhibited repair of single stranded DNA lesions.

Unscheduled DNA synthesis (11) by MMS treated cells was not blocked by β-lapachone; rather, this process was stimulated (Figure 4). Several possible explanations for this increase will need investigation; the point here is that β-lapachone did not inhibit nucleotide insertion. A reasonable hypothesis at this point is that β-lapachone inhibits the ligation step of

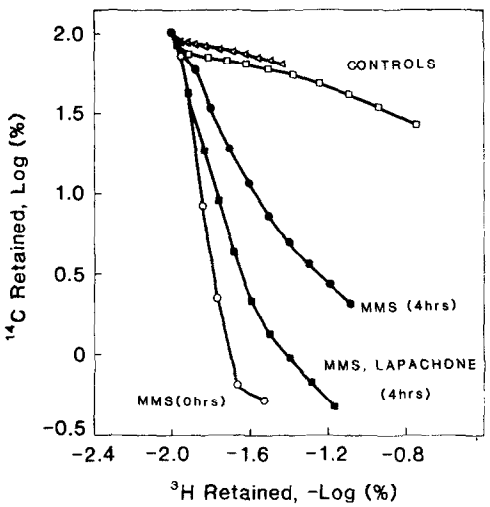

Figure 3: Effects of β-lapachone on the alkaline elution profiles of MMS treated human fibroblasts.

Alkaline elution was performed with syringe-type filter holders and poly-carbonate filters at pH 12.1, according to Kohn, et al., (10) with minor modifications (8). Cells used as internal standards were labeled for 24 hours with 0.05 μCi/ml ^{3}H-thymidine and then irradiated with 150 rads from a ^{137}Cs source. Experimental cells were prelabeled with 0.01 μCi/ml ^{14}C-thymidine for 24 hours and then arrested at G_0 as described in Figure 1. Immediately after release with complete medium, cells were treated with MMS for 1 hour and then with 4 uM β-lapachone for 4 hours. (△) control, 0 hours incubation, (□) control, 4 hours incubation with 4.0 uM β-lapachone. (○) MMS, 0 hours incubation. (●) MMS, 4 hours incubation. (■) MMS, 4 hours incubation with 4 uM β-lapachone. The elution of experimental cells' ^{14}C-labeled DNA is expressed as a function of eluted ^{3}H-labeled DNA from cells used as an internal standard. Data are expressed as the log (%) of DNA retained on the filters (10). Steeper slopes of the lines as plotted represent increasing amounts of single strand breaks. A slope of -1 indicated 150 rad-equivalents of DNA damage.

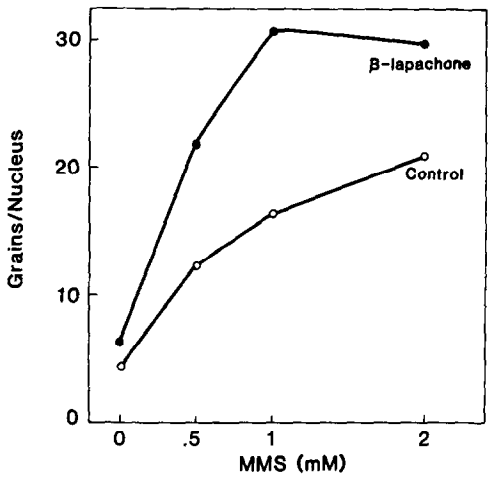

Figure 4: Effect of β-lapachone on unscheduled DNA synthesis.

Quiescent (G_0) normal human fibroblasts were shifted to DME medium + 10% fetal calf serum and treated with MMS for 1 hour. The MMS was removed, and 4 μM β-lapachone were added to half of the cultures. After 2 hours' incubation with 10 μCi/ml ^{3}H-thymidine, the cells were washed and incubated with 10^{-4} M thymidine and 10^{-5} M uridine for 1 hour. After autoradiography (11), grains in at least 40 cells per sample were counted. Labeled S phase nuclei represented less than 2% of cells and were not counted.

repair. Irreversibility of the lethal effects suggests that this repair inhibition is irreversible.

A variety of DNA repair inhibitors have been reported. The ones with properties most similar to those of β-lapachone are carbamoylating nitrosoureas (12). 3-Aminobenzamide inhibits repair and enhances lethality of MMS (3,4,5,8); it has been reported to inhibit ligation (3). It is required at a 1000-fold higher concentration than is β-lapachone. Its action differs from that of β-lapachone in several respects. Enhanced lethality is dependent on replicative DNA synthesis; physiologically or pharmacologically inhibiting DNA synthesis blocks 3-aminobenzamide enhancement (8,23). In contrast, β-lapachone is effective with either replicating or quiescent cells. Also, 3-aminobenzamide arrests MMS treated cells in G_2 phase (8), whereas β-lapachone blocks them in S phase. Enhanced lethality due to caffeine also differs from that by β-lapachone; caffeine has only minor effects on human cells damaged by alkylating agents (8,14).

β-lapachone acts differently from chloroquine, another antimalarial, whose effects on DNA repair (15) are probably nonspecific since they inhibit

replicative DNA synthesis and repair synthesis to the same extent (16). Chloroquine (10-500 μM) inhibited DNA synthesis equally in control or MMS treated cells (data not shown). β-lapachone is a tricyclic derivative of a 1,2-naphthoquinone (Figure 1), structurally different from the 4-aminoquinolines including chloroquine, and from lapachol, a bicyclic 1,4-naphthoquinone. It contains several reactive groups which might permit it to bind covalently to damaged DNA or to enzymes involved in DNA repair, perhaps similarly to nitrosoureas (12).

Detailed chemical and enzymological studies will be needed to understand the molecular basis for or action of this interesting compound. Its low toxicity to undamaged cells, together to its striking synergistic effects with DNA damaging agents at low concentrations of both suggest uses in cancer chemotherapy, and investigations of DNA damage and repair.

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