

Enhancement of Mammalian Cell Killing by 5-Fluorouracil in Combination with X-Rays¹

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ABSTRACT

The enhanced cytotoxic effect of a combination of X-rays and 5-fluorouracil was investigated by means of colony-forming ability in mouse L-cells. Cells were treated with 5-fluorouracil immediately preceding (preirradiation treatment) or following (postirradiation treatment) irradiation. In either pre- or postirradiation treatment with various concentrations of the drug for a fixed time, the enhanced effect was augmented with increasing concentrations of 5-fluorouracil up to 20 $\mu\text{g}/\text{ml}$. When cells were subjected to postirradiation treatment with a fixed concentration of drug for varying times, the cytotoxic effect was further enhanced with increasing duration of drug treatment. In preirradiation treatment, however, drug treatment for longer than 3 hr did not exhibit any further enhancement.

Postirradiation treatment with 5-fluorouracil to synchronous cells at various ages demonstrated enhancement at all ages during the cell cycle. The greatest enhancement was observed in the DNA-synthetic phase.

Postirradiation treatment with 5-fluorouracil (2.5 $\mu\text{g}/\text{ml}$) for 24 hr markedly reduced the width of the shoulder of the X-ray survival curve without significantly altering the slope of the exponential portion of the curve. Recovery from sublethal radiation damage was not suppressed by 5-fluorouracil when cells were treated with drug between fractionated X-ray doses. These results indicate that damages caused by 5-fluorouracil and X-rays interact additively to induce cell killing.

INTRODUCTION

There is increasing evidence that human neoplasms are more responsive to combined therapy than to treatment with drug or X-ray alone. A combined effect of FUra³ and X-rays was first observed by Heidelberger *et al.* (13). Because of the wide clinical use of this drug, there have been a number of clinical reports in which the combined effect of FUra and radiation was studied. Some investigators thought that FUra significantly enhanced the effect of radiation and prolonged the survival of patients (1, 6, 10, 14-16). Others, however, observed no benefit from the combination therapy (5, 11). These conflicting results may be attributed to the difference in the combination schedules, the types of cancer, the clinical stages at the time of treatment, and the difficulty in evaluating the response objectively.

Experimental studies on the combined effect of radiation and chemotherapy provide important information on the proper

sequence of one or more treatment modalities. Bagshaw (2) has reported that HeLa cells grown in a low concentration of FUra exhibited a potentiated response to radiation. On the other hand, Vietti *et al.* (19) reported a synergistic cytotoxic effect of the combination of FUra and X-rays mainly when FUra was administered after irradiation.

In this study, we investigated the interaction between FUra and X-ray damages with respect to the enhanced cytotoxic effect in cultured mouse L-cells. We also examined the effect of FUra on radiation damage repair which was detected by means of dose fractionation.

MATERIALS AND METHODS

Cell Line. Experiments were performed with a subline of mouse L-cells (B929-L2J) designated L5, which has been passaged approximately 500 times in our laboratory. The cells were maintained in monolayer culture in F10 medium (12) containing 5% calf serum, 0.05% heart infusion broth, and antibiotics. Cultures were incubated in humidified air containing 5% CO₂ at 37°. The average generation time of cells under these conditions was 18 hr: G₁, 5 hr; S, 9 hr; G₂, 3 hr; and M, 1 hr.

Asynchronous Cultures. Exponentially growing cells were dispersed with 0.1% trypsin and diluted with growth medium, and cell counts were performed with a Coulter counter. An appropriate number of cells which may result in 50 to 150 colonies was seeded into each plastic dish (60 x 15 mm). The cells were attached firmly to the bottom of the dish within 4 hr of incubation at 37°. Cultures at this stage were treated with FUra or X-irradiation.

Synchronous Cultures. Synchronous cell populations were obtained by mitotic collection. All procedures were carried out at 37°. The mitotic frequency of harvested cells was in the range of 80 to 95%. The methods of synchronization and autoradiography were identical to those described previously (18, 20).

X-Irradiation. X-Rays were obtained from an X-ray unit operated at 200 kVp and 20 ma, with added filtration of 0.5 mm copper and 0.5 mm aluminum. The dose rate was 96 rads/min. All irradiations were performed at room temperature. In the split-dose experiment, dishes were returned to the CO₂ gas chamber between irradiations.

FUra Treatment. FUra was supplied by Hoffmann-LaRoche Ltd., Basel, Switzerland. The drug was diluted with fresh growth medium to the desired concentrations immediately before use. The medium containing FUra was added immediately after irradiation (postirradiation treatment) or removed immediately before irradiation (preirradiation treatment). The medium containing FUra was aspirated after treatment; the cells were

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³ The abbreviation used is: FUra, 5-fluorouracil.

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rinsed twice with 4 ml of Hanks' balanced salt solution and incubated with complete medium.

Survival Assay. Cultures were incubated for 14 days at 37° in a humidified CO₂ gas incubator. After incubation, colonies were fixed with 10% buffered formaldehyde and stained with 1% methylene blue. Colonies composed of 50 or more cells were scored as having arisen from surviving cells. Plating efficiencies in most of our experiments were about 80%. The survival fractions were determined from 2 or more repeat experiments in which each survival value was obtained from 2 or 3 dishes. The survival curve parameters (D_0 and D_q) were calculated by least-squares regression analysis. D_0 is the X-ray dose to reduce survival by e^{-1} on the straight portion of the curve. D_q is the initial shoulder width of the curve and is defined as the X-ray dose at which the straight portion of the curve cuts the dose axis.

RESULTS

Effect of FURa on X-Ray Survival Curve. X-Ray survival curves for L-cells treated with or without FURa after irradiation are shown in Chart 1A. The radiation survival curve without exposure to drug had a wide shoulder, followed by an exponential portion in the range of 400 to 900 rads.

The parameters of the X-ray survival curve, determined from the data in the range of 400 to 900 rads, were: D_0 , 126 rads; D_q , 264 rads. The parameters of the curve for postirradiation treatment with FURa (2.5 µg/ml) for 24 hr were: D_0 , 118 rads; D_q , 129 rads. Survival values for the combined treatment have been corrected for the amount of cell killing caused by FURa alone. The survival fraction of unirradiated cells after treatment with FURa (2.5 µg/ml) for 24 hr was 0.73. The results indicated that postirradiation treatment with FURa markedly reduced the width of the shoulder (D_q) without significantly altering the slope (D_0) of the survival curve.

Chart 1B shows the survival curves for X-ray and for preirradiation treatment with FURa (2.5 µg/ml) for 24 hr. The combined effect was smaller than that with postirradiation treatment. The shoulder of the survival curve without exposure to

FURa in this case was much larger than that shown in Chart 1A. The increased shoulder of the control curve may be attributed to cell multiplicity at the time of irradiation, because the cells were dividing during the 24 hr of incubation. These results indicated that postirradiation treatment with FURa enhanced cell killing to a much greater extent than did preirradiation treatment.

Enhanced Killing Effect as a Function of Drug Concentration and Duration of Treatment. The enhancement of cell killing is shown in Chart 2 as a function of drug concentration for various exposure times. Cells were irradiated with a dose of 400 rads. Immediately preceding or following irradiation, the cells were treated with various concentrations of FURa for 3, 6, 12, and 24 hr, respectively. Survival fractions have been corrected for cell killing caused by each treatment with FURa alone. Chart 2A represents the X-ray survivals which were observed in preirradiation treatment with FURa. The maximal enhanced killing was obtained at a concentration of 20 µg/ml or more when cells were treated with various concentrations of drug for fixed times (3, 6, and 12 hr). It was also noted that, in preirradiation treatment with a fixed concentration of FURa, the duration of treatment beyond 3 hr did not produce any further enhancement. Chart 2B represents the results obtained from postirradiation treatment with FURa. The enhancement similarly depended on the drug concentration detected in preirradiation treatment. The cytotoxic effect of X-rays (400 rads), however, was markedly enhanced with increase in the duration of FURa treatment, when cells were treated with a fixed concentration of FURa for varying times following irradiation. In order to determine how long the enhancement would last, cells were irradiated with a dose of 400 rads and immediately treated with FURa (1.0 and 2.5 µg/ml) for various times up to 90 hr (Chart 3). The enhancement increased gradually and reached a plateau approximately 48 to 72 hr after irradiation. Although the interpretation of these data was difficult because of the change in cell multiplicity during a long treatment with the drug, the results suggested that the enhancement lasted for a long period after irradiation.

Effect of FURa on 2-Dose Radiation Response. As shown in

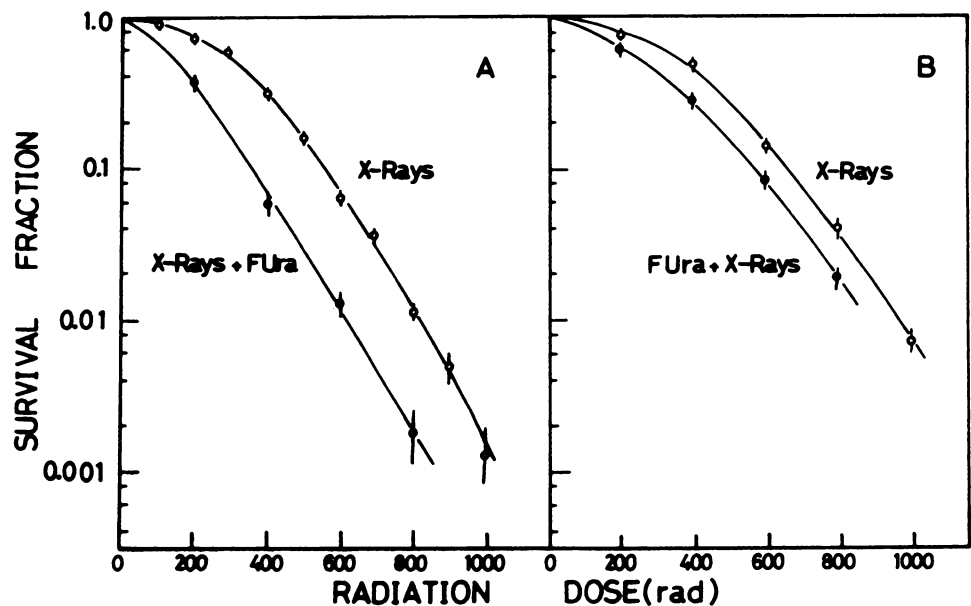


Chart 1. X-ray survival curves of L-cells. A, treatment with FURa (2.5 µg/ml) for 24 hr immediately following irradiation; B, treatment with FURa (2.5 µg/ml) for 24 hr immediately preceding irradiation. Bars, S.E.

Chart 2. Enhancement of cell killing as a function of FUra concentration for various exposure times. Cells were treated with various concentrations of FUra immediately preceding or following exposure to 400 rads. A, preirradiation treatment; B, postirradiation treatment. Survival fractions after treatment with FUra (20 $\mu\text{g}/\text{ml}$) for 3, 6, and 12 hr were 0.71, 0.58, and 0.21, respectively. \circ - -, survival level of 400 rads alone. Bars, S.E. Survival point without bars represents an average of triplicate dishes.

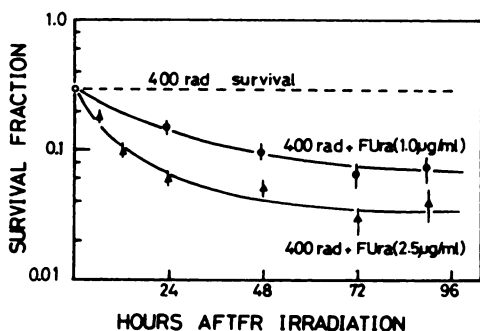
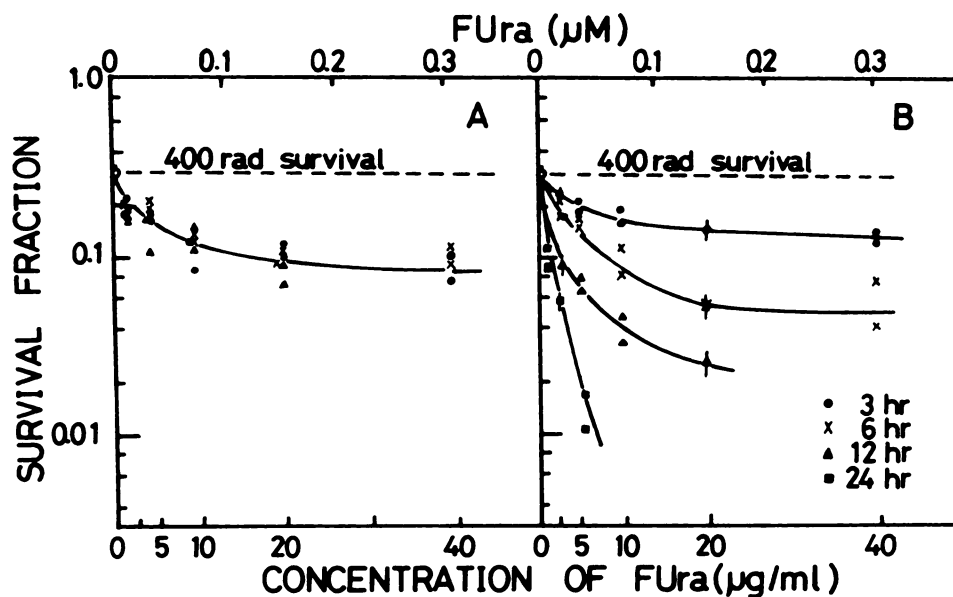


Chart 3. Cells were treated with FUra (1.0 and 2.5 $\mu\text{g}/\text{ml}$) for progressively longer times after 400 rads. Survival fractions have been corrected for cell killing caused by each treatment with FUra alone. Survival fractions after treatment with FUra (2.5 $\mu\text{g}/\text{ml}$) for 24, 48, 72, and 90 hr were 0.73, 0.60, 0.30, and 0.098, respectively. \circ - -, survival level of 400 rads alone. Bars, S.E.

Chart 1A, postirradiation treatment with FUra markedly reduced the width of the shoulder in the X-ray survival curve, suggesting a loss of the ability of a cell to repair sublethal radiation damage. To test this point, cells were treated with FUra during the interval between fractionated X-ray doses. Since a maximal enhanced effect was obtained at a concentration of 20 $\mu\text{g}/\text{ml}$ (Chart 2), this concentration of FUra was administered. Chart 4A shows survival curves for L-cells treated with FUra alone and with FUra following 400 rads irradiation as a function of time. The treatment with FUra following irradiation produced additional killing, indicating the development of an enhanced cytotoxic effect. Chart 4B represents the survival curves of fractionated X-ray treatment in the presence or absence of FUra between irradiations. The survival fraction of untreated cells increased up to 3 hr after the first dose of 400 rads. When FUra was present between 2 doses, the survival fractions decreased as compared to those of untreated cells. This result, however, does not suggest the suppression of repair from sublethal radiation damage. Because FUra toxicity and interaction of X-ray and FUra damages developed and gradually decreased the survival with increase in the duration of FUra treatment (Chart 4A), these factors must

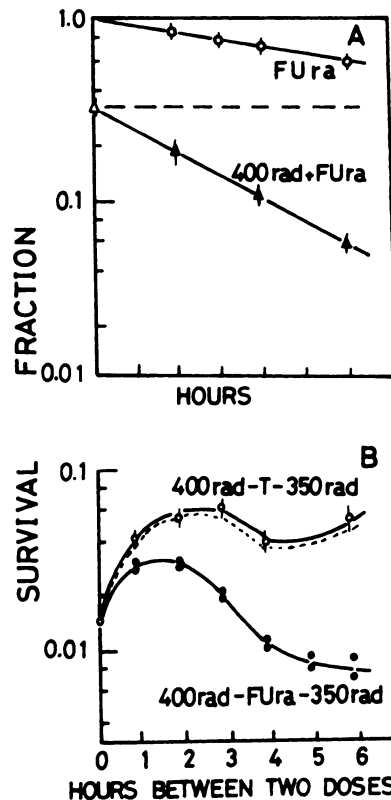
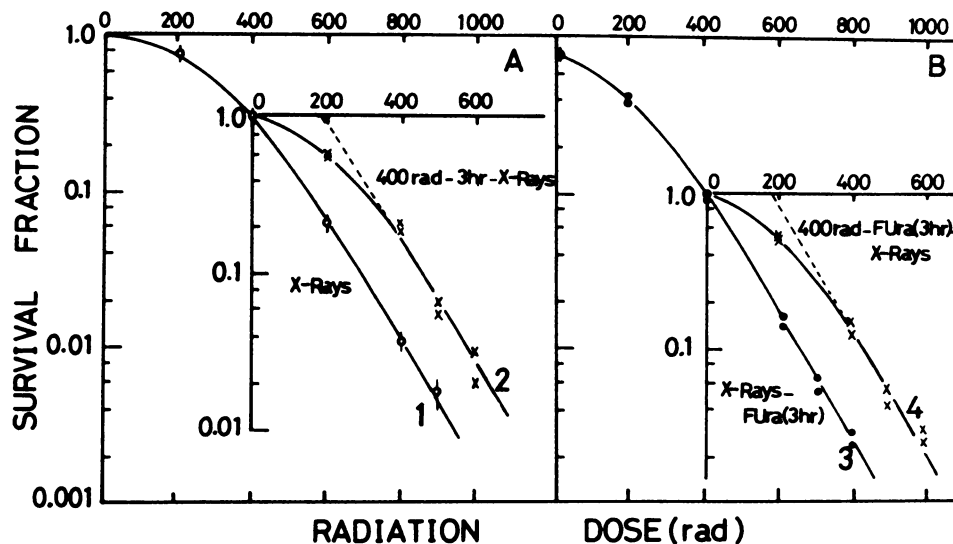


Chart 4. Effect of FUra on recovery from sublethal radiation damage. A, survival curve for various incubation times with FUra (20 $\mu\text{g}/\text{ml}$) alone and 400 rads plus FUra (20 $\mu\text{g}/\text{ml}$). Δ - -, 400-rad survival level. B, survival fractions after fractionated doses (first dose, 400 rads; second dose, 350 rads) with or without FUra (20 $\mu\text{g}/\text{ml}$) between doses. ----, corrected survival level. T, time. Bars, S.E. Survival point without bars represents an average of triplicate dishes.

be taken into account for each survival value. When each survival value (Chart 4B, closed circle) is corrected by these factors, each corrected survival value virtually overlapped that of fractionated X-ray treatment. The enhanced killing may result from interaction between FUra and X-ray damages other than sublethal radiation damage. The results suggested that the

Chart 5. Effect of FUra on the shoulder of fractionated survival curve. A: Curve 1, single doses of X-rays; Curve 2, first dose of 400 rads followed by graded second doses of X-rays 3 hr later; B: Curve 3, single dose of X-rays immediately followed by FUra (20 µg/ml) for 3 hr; Curve 4, first dose of 400 rads followed by FUra (20 µg/ml) for 3 hr and by graded second doses of X-rays. Bars, S.E. Survival point without bars represents an average of triplicate dishes.



repair of sublethal radiation damage was not suppressed by FUra. To confirm this point further, another type of dose fractionation experiment was carried out (Chart 5). The initial radiation dose, the concentration of FUra, and the interval between 2 doses were fixed at 400 rads, 20 µg/ml, and 3 hr, respectively. The second dose was varied from 200 to 600 rads. In Chart 5A, Curve 1 indicates the survival curve obtained with single graded doses of X-rays. Curve 2 shows the fractionated survival curve determined after 3 hr of incubation following a first dose of 400 rads. In Chart 5B, Curve 3 shows the single dose survival curve of the cells treated with FUra for 3 hr following irradiation. Curve 4 was obtained from the cells which were treated with FUra for 3 hr following a first dose of 400 rads and then irradiated with the second dose. An equal shoulder width (D_0) was noted in Curves 2 and 4. This result indicated that postirradiation treatment with FUra did not suppress the recovery from sublethal radiation damage.

Age-dependent Response to Combined Treatment. An experiment was designed to determine whether or not the enhanced cytotoxic effect in the combined treatment depended on cell age. Synchronous cells were irradiated with a fixed dose of 500 rads and immediately treated with FUra (20 µg/ml) for 3 hr. The combined treatment was done at 2, 6, 10, 15, or 17 hr after mitotic collection, representing G₁, G₁-S, mid-S, S-G₂, and G₂, respectively (Chart 6). The progression of harvested cells through the cell cycle had been monitored by autoradiogram. The cells were labeled with [³H]thymidine (Radiochemical Centre, Amersham, England), 0.5 µCi/ml, for 20 min. The times of irradiation were determined from these data (Chart 6B). The result is shown in Chart 6A. Administration of FUra at each age caused a slight reduction in survival fraction, and a greater reduction was observed after irradiation alone, especially at G₁-S. Assuming that there was no enhancement in the combined treatment, survival would fall along the broken line as calculated by multiplying fractional survivals obtained with each treatment. The survival values of the combined treatment, however, were more decreased than the level of the broken line. This indicates that combined treatment enhanced cell killing at all ages throughout the cell cycle, but that the enhancement was greater when cells were irradiated in S

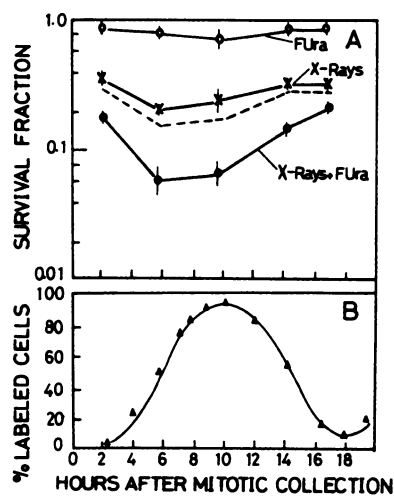


Chart 6. Age-dependent response to combined treatment. A, synchronized cells irradiated with 500 rads at each stage of the cell cycle and immediately treated with FUra (20 µg/ml) for 3 hr. - - -, survival level expected if there is no enhanced effect by combined treatment. B, percentage of labeled cells as a function of time after mitotic collection. Bars, S.D.

phase than in G₁ and G₂ phases. A similar pattern of enhancement was observed when synchronous cells were irradiated with 600 rads at each age and immediately treated with FUra (2.5 µg/ml) for 24 hr.⁴

DISCUSSION

The combined effect of FUra and X-irradiation was reported at the cellular level by Vietti et al. (19). They investigated the enhanced cytotoxic effect mainly relating to the interval between irradiation and FUra treatment. Their results indicated that a marked cytotoxic effect was observed when FUra was administered within 8 hr after irradiation. As the interval between radiation and drug treatment increased, loss of interaction occurred, and the enhanced effect was no longer demon-

⁴ Y. Nakajima, T. Miyamoto, M. Tanabe, I. Watanabe, and T. Terasima, unpublished experiment. Detailed data from this experiment are available on request.

strable at about 16 hr or later. Using human tumor cells in culture, Byfield *et al.* (4) recently stated that the radiosensitization effect by FUra resulted in reduction of slope (D_0) of survival curve.

The present study has demonstrated that the mode of interaction between X-ray and FUra damages enhances cell killing. Our results indicate that enhancement of the cytotoxic effect of FUra was greater for postirradiation treatment than for preirradiation treatment (Charts 1 and 2). For both pre- and postirradiation treatment, the degree of enhancement depended on the concentration of FUra (Chart 2). In the case of postirradiation treatment, the duration of treatment with FUra greatly contributed to enhancement (Chart 2B). This result suggests that X-ray-induced damage interacts with FUra damage for considerably long periods after irradiation (Chart 3). In the case of preirradiation treatment, however, the enhancement did not depend on exposure time (Chart 2A). This suggests that FUra-induced damage interacts with X-ray damage for only a short period after treatment with FUra.

The survival curve for graded doses of X-rays following treatment with FUra was characterized by a reduction in D_q dose (Chart 1A). The reduction in the width of the shoulder (D_q) of the X-ray survival curve may suggest suppression of recovery from sublethal radiation damage (8, 9) and/or decreasing the capacity of a cell to accumulate sublethal damage, as was seen with 5-bromodeoxyuridine (17). The present data have demonstrated that FUra did not suppress recovery from sublethal radiation damage (Chart 5 and 6). It is unlikely that FUra decreases the capacity of a cell to accumulate sublethal radiation damage, because the accumulation capacity of the cells was not affected by FUra at the time of irradiation for the postirradiation treatment. Based on the consideration of theoretical survival curves for various modes of interaction between X-rays and drug (7), the removal of shoulder by postirradiation treatment with FUra can be explained by an additive mode of interaction between FUra and X-ray damages. Belli and Piro (3) have reported an additive mode of interaction between Adriamycin and X-ray damages. Our result with synchronous cells revealed that the interaction of FUra and X-ray damages was greatest in the process of DNA synthesis (Chart 6).

The present findings may provide a useful combination schedule of X-radiation and FUra for the therapy of malignant tumors. The continuous infusion of FUra for a period up to 48 to 72 hr starting immediately after irradiation might be the most effective for tumor cell inactivation.

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