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Inhibition of radiation-induced neoplastic transformation by β-lapachone

(error prone/potentially lethal DNA damage repair/DNA repair inhibition/topoisomerase I)

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ABSTRACT **β-Lapachone is a potent inhibitor of DNA repair in mammalian cells and activates topoisomerase I. We show that β-lapachone can prevent the oncogenic transformation of CHEF/18A cells by ionizing radiation. Potentially lethal DNA damage repair (PLDR) occurs while x-irradiated cells are held in medium containing low serum prior to replating. PLDR processes permitted survival recovery but also drastically increased the number of foci per plate (i.e., transformation) of CHEF/18A cells. By blocking PLDR with β-lapachone, both survival recovery and enhanced transformation were prevented. At equivalent survival levels, exposure of x-irradiated cells to β-lapachone resulted in an 8-fold decrease in the number of foci per dish as compared to the number of transformants produced after PLDR. Early PLDR-derived increases in transformation may be the result of error-prone genetic rearrangements dependent on topoisomerase I, which are thereby prevented by β-lapachone. β-Lapachone exposure decreased the rejoining of DNA strand breaks and produced additional double-strand breaks in x-irradiated cells during PLDR. The activation of topoisomerase I by β-lapachone may convert repairable single-strand DNA breaks into the more repair-resistant double-strand breaks, thereby preventing PLDR and neoplastic transformation. These results suggest a new direction for the development of anticarcinogenic agents.**

Many agents, including x-irradiation and antineoplastic drugs, are carcinogens. They damage DNA, creating a variety of lesions. A very large portion of this damage is repaired by normal cells (1, 2). Defective or incomplete repair of DNA lesions may be carcinogenic, since proposed defects in DNA repair processes have serious consequences, as illustrated by the cancer-prone diseases (3–5).

During a critical interval following damage to its DNA, a normal cell must adequately repair potentially lethal (and/or tumorigenic) lesions if it is to return to its normal phenotype. Failure (or inexact) repair of these lesions may result in either cell death or survival with incompletely repaired or misrepaired DNA, which may then lead to the production of a tumorigenic phenotype. Complete and accurate lesion repair by the cell will depend on the repair system used and optimizing such factors as the available time for repair, its repair capacity and cell-cycle position, and on the type and extent of initial DNA damage. If DNA lesions cannot be repaired correctly and completely, it may be beneficial to the organism to convert them into lethal events (cells containing such lesions would die rather than survive as transformants).

Certain drugs can interfere with DNA repair. They, like the genetic defects, increase the lethality of DNA damaging agents. Unlike the defective DNA repair in cancer-prone diseases, however, some drugs (i.e., most notably caffeine and 3-aminobenzamide) have been reported to decrease carcinogenicity (6–8). Such drugs may have promise not only for increasing the efficacy of chemotherapy (9), but also for decreasing carcinogenicity (10).

β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione) is a potent inhibitor of potentially lethal DNA damage repair (PLDR) (10–12). The mechanism of action of β-lapachone appears to be related to its activation of topoisomerase I, which suggests the involvement of this enzyme in the repair of DNA lesions (12, 13).

We demonstrate that a post-x-ray β-lapachone treatment results in a dramatic decrease in the neoplastic transformation of Chinese hamster embryo fibroblast (CHEF/18A) cells. β-Lapachone inhibits PLDR processes so as to convert repairable potentially carcinogenic DNA lesions into lethal events, thereby decreasing the overall number of transformed cells.

MATERIALS AND METHODS

Cell Culture Techniques. Stably diploid CHEF/18 cells were kindly donated by Ruth Sager (Division of Cancer Genetics, Dana–Farber Cancer Institute, Boston, MA) and subsequently subcloned to enrich for a cell culture with a low spontaneous oncogenic transformation frequency (i.e., CHEF/18A). Like CHEF/18 cells, the doubling time of CHEF/18A cells was 17.3 ± 0.4 hr in a 10% CO_2/90% air atmosphere at 37°C in Dulbecco's modified Eagle's medium (DME medium) (9). CHEF/18A cells were routinely found to be free of mycoplasma contamination (9, 14). They were stored in multiple vials in liquid nitrogen and a new vial of cells was used for each experiment.

Survival and Transformation Assays. CHEF/18A cells (1×10^5) were plated onto 25-cm^2 tissue culture flasks (Falcon) and fed every other day until confluent cultures (<5% [^{3}H]thymidine-labeled nuclei; see ref. 15) were obtained. Cells were x-irradiated as described (9). One sample of x-irradiated cells was plated immediately after x-ray exposure (i.e., no posttreatment) to prevent PLDR. Posttreatments were applied for 5 hr (unless otherwise indicated) at 37°C in DME medium containing 0.2% fetal calf serum (0.2% FCS-DME medium) ± 4 μM β-lapachone.

To determine survival, cells were treated with trypsin and serially diluted in 10-fold increments to 50 cells onto 60-mm tissue culture dishes (Falcon) with 10–20% FCS-DME medium as described (9). All data represent a combination of at least three separate experiments. All conditions were completed in duplicate and each duplicate was assayed twice to assess survival and transformation. After 10 days of growth, colony-forming ability was scored (9). The plating efficiency of CHEF/18A cells was 61% ± 9%. X-ray survival values

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Abbreviations: DSB, double-stranded DNA break; SSB, single-stranded DNA break; FCS, fetal calf serum; PLDR, potentially lethal DNA damage repair.

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were corrected for variations in initial viable cells plated and for variations due to drug exposure or prolonged confluence-arrest of unirradiated cells.

To determine transformation, plates derived from the conditions described above were continually examined for confluency. A minimum of 10 days was allowed to achieve confluence and to allow expression of transformed phenotypes. At confluence, the medium was removed and replaced with low serum medium (i.e., 2.0% FCS-DME medium); cells were fed every 3rd day thereafter. After 14–21 days, foci corresponding to transformed phenotypes were scored under a dissecting microscope as described (16, 17). Transformed colonies were selected for (*i*) ability to grow in medium containing low serum, (*ii*) loss of contact inhibition (i.e., cells piled on one another in foci), and (*iii*) transformed morphology corresponding to previously described tumor-forming phenotypes (16, 17).

Filter Elution Analyses. A modification of the method of Bradley and Kohn (18) was used for neutral and alkaline filter elutions. CHEF/18A cells (1×10^5) were plated onto tissue culture plates (150 × 15 mm) (LUX, Miles Scientific). On day 2 of exponential growth, cells were labeled with [*methyl*-^{14}C]thymidine (0.15 μCi/ml) (specific activity, 52 mCi/mmol; 1 Ci = 37 GBq; New England Nuclear) until they reached confluence (usually 10 days later). CHEF/18A reference cells were labeled with [*methyl*-^{3}H]thymidine (1.5 μCi/ml) (specific activity, 78 Ci/mmol; New England Nuclear) for 3 or 4 days. Labeled cells were cooled on ice and x-irradiated (9). ^{14}C-labeled cells (1×10^6 cpm) were mixed with an equivalent number of ^{3}H-labeled reference cells (x-irradiated at 2000–4000 cGy) and analyzed by neutral or alkaline elutions (18, 19). Results represent the mean of at least two independent experiments completed in duplicate. Data are graphed as % ^{14}C-labeled experimental DNA retained vs. % ^{3}H-labeled reference DNA retained on the filters as described (19, 20).

RESULTS

β-Lapachone Blocks Transformation at Various X-Ray Doses. The transformation background in untreated control cells was 0.9 ± 0.05 foci per dish for every 6.6×10^6 cells present per dish. X-irradiation of CHEF/18A cells increased the number of foci per dish up to 20-fold (Fig. 1*A*). Scatter within a typical experiment is illustrated by individual data points; curves were drawn by eye. Plating serial dilutions of x-irradiated or unirradiated CHEF/18A cells of up to 10,000:1 yielded statistically identical numbers of foci per plate, and were therefore combined to calculate average foci formed per dish (Table 1). Similar results (i.e., equivalent foci per plate irrespective of the number of cells initially plated) have been reported (21, 22), indicating that this assay of transformation determines the percentage of transformants in any given final population of cells rather than the absolute number of transformants initially plated.

At each x-irradiation dose, those CHEF/18A cells that carried out PLDR produced a dramatically higher number of foci per dish than cells given no time to repair (Fig. 1*A*). For example, following 500 cGy, the number of transformants per dish increased from a basal level of 0.9 ± 0.05 for unirradiated cells to 21 ± 4.2 for x-irradiated CHEF/18A cells given no posttreatment. The transformation frequency nearly doubled for a similar x-irradiated population of cells that was allowed to carry out PLDR, producing 39.6 ± 3.1 transformants per dish. This increase is similar to results previously observed with C3H 10T½ cells (23). The survival of x-irradiated CHEF/18A cells following an identical posttreatment increased from 2.2% ± 0.1% for cells given 500 cGy and no posttreatment to 12% ± 1.1% for cells that carried out PLDR (Fig. 1*B*).

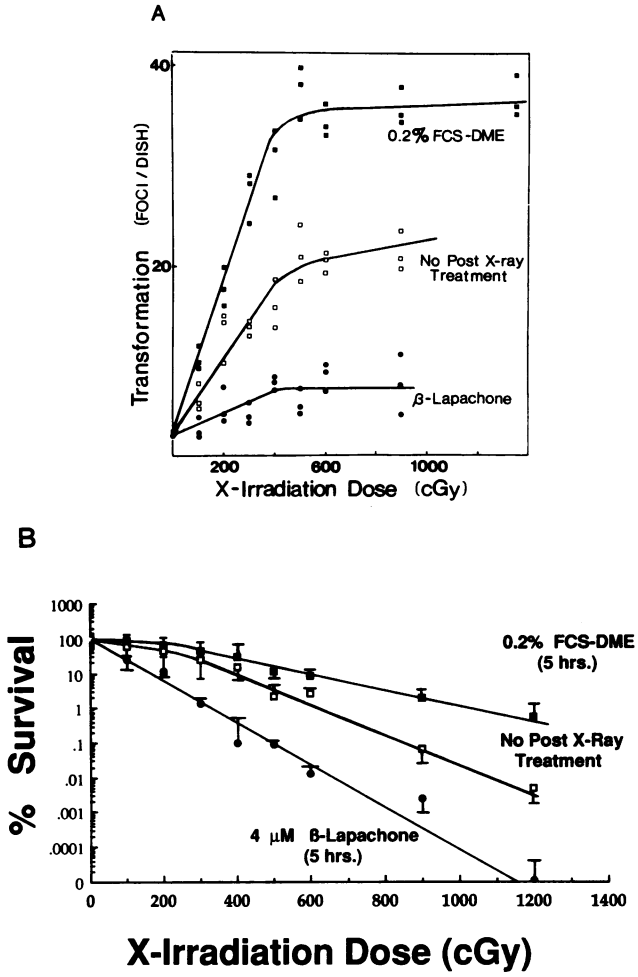

FIG. 1. The effect of β-lapachone on the transformation and survival of x-irradiated CHEF/18A cells. Confluence-arrested CHEF/18A cells were either unirradiated or treated with various doses of x-irradiation. One set of plates was treated with trypsin immediately after x-irradiation and replated to prevent PLDR. Two other sets were given a 5-hr post-x-ray treatment of 0.2% FCS-DME medium with or without 4 μM β-lapachone. Both neoplastic transformation (*A*) and survival recovery (*B*) were assayed as described. Post-x-ray treatment conditions were as follows: □, no post-x-ray treatment; ■, 0.2% FCS-DME medium; ●, 4 μM β-lapachone in 0.2% FCS-DME medium.

The addition of β-lapachone during PLDR reduced both transformation (Fig. 1*A*) and survival recovery (Fig. 1*B*) of confluence-arrested CHEF/18A cells. The transforming effects of PLDR were not only nullified by β-lapachone but were decreased to values below those for cells given no posttreatment. The reduction in the number of transformants per dish by β-lapachone held true even when equivalent survival levels were compared. For example, the survival level was 12% following either 500 cGy and a low serum (i.e., 0.2% FCS-DME medium) holding period or 200 cGy when β-lapachone was present during the holding period (Fig. 1*B*). The numbers of transformants per dish were 39.6 ± 3.1 and 5.0 ± 0.7, respectively (after background subtraction) (Fig. 1*A*); this represented nearly an 8-fold reduction.

β-Lapachone Prevents Increases in Transformation During Early Error-Prone PLDR. Previous data with C3H 10T½ cells suggested that the early phase of PLDR, which starts 1–2 min after x-irradiation, was error prone and that the slow phase of PLDR, which starts 1–4 hr after x-irradiation (depending on cell type), was error-free (23). We investigated the effect of β-lapachone on both transformation (Fig. 2*A*) and survival recovery (Fig. 2*B*) of x-irradiated (400 cGy) confluence-

Table 1. Radiation-induced transformants observed as a function of the initial number of cells plated

Post-x-ray (400 cGy) treatment	Transformants, foci per dish				
	500,000 plated	50,000 plated	5000 plated	500 plated	50 plated
None	18.6 ± 3.2	21.5 ± 2.6	21.7 ± 2.5	21.0 ± 3.7	23.0 ± 4.1
0.2% FCS-DME for 5 hr	36.2 ± 8.3	31.0 ± 1.4	30.8 ± 4.6	34.2 ± 9.3	33.5 ± 7.7
4 μM β-lapachone for 5 hr	4.6 ± 2.2	4.8 ± 1.9	7.9 ± 4.2	3.0 ± 1.6	5.2 ± 0.7

Post-x-ray treatments and transformation (foci per dish) assays are described in Fig. 1 and *Materials and Methods*. Numbers represent the mean ± SE of six separate dilutions from three experiments completed in duplicate as summarized in Fig. 1*A*.

arrested CHEF/18A cells during increasing times of PLDR. During the first 10 hr of PLDR, the number of transformants per dish more than doubled, increasing from 16.3 ± 2.1 (no posttreatment) to 35.4 ± 0.8. Some variability in the enhancement of transformation due to PLDR was observed between experiments (compare Figs. 1*A* and 2*A* at 400 cGy), since a similar peak in oncogenic transformants appeared only 5 hr after x-irradiation in Fig. 1*A*. This variation may be due to the intermittent feeding (every 3 hr) of the CHEF/18A cells in Fig. 2*A*, which could impede the second, slow phase of PLDR.

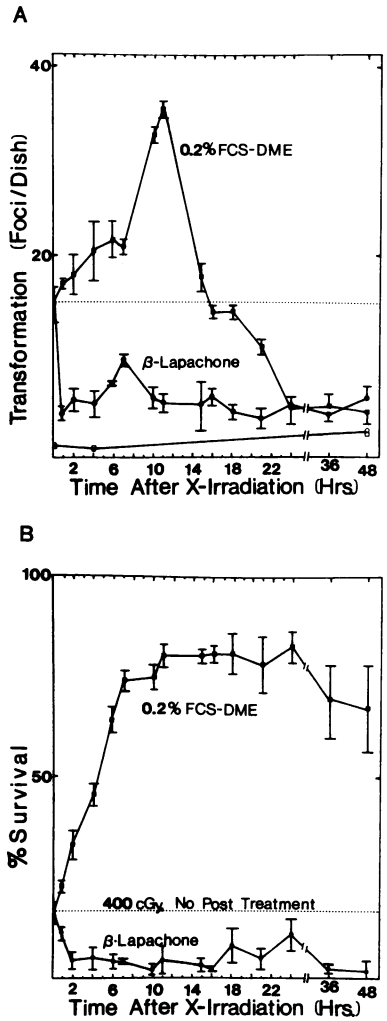

FIG. 2. Blockage of the PLDR-derived augmentation of neoplastic transformation and survival recovery by β-lapachone. Confluence-arrested CHEF/18A cells were treated with 400 cGy as described in Fig. 1, except that various lengths (up to 48 hr) of post-x-ray treatments in 0.2% FCS-DME medium with (●) or without (■) 4 μM β-lapachone were given; cells were fed every 3 hr to improve plating efficiencies. Both neoplastic transformation (*A*) and survival recovery (*B*) were determined. Survival levels for x-irradiated cells were corrected for losses due to drug exposure and/or confluence-arrest. Unirradiated cells were exposed to 0.2% FCS-DME medium with (○) or without (□) 4 μM β-lapachone for up to 48 hr (*A*).

When x-irradiated CHEF/18A cells were given longer times to repair (e.g., 48 hr) the number of foci per dish gradually decreased to 4.8 ± 0.7, a level only slightly greater than that of similarly treated unirradiated cells (Fig. 2*A*). X-irradiated C3H 10T½ cells showed similar initial increases in transformation during the first 4 hr of PLDR and subsequent lowering of transformation (nearly to the level of unirradiated cells) when longer PLDR holding was allowed (23). The survival of x-irradiated CHEF/18A cells held in low serum medium gradually increased from 16.0% ± 1.5% (no posttreatment) to >70% after 48 hr of PLDR.

The addition of 4 μM β-lapachone during all times of PLDR not only inhibited survival recovery (Fig. 2*B*) and blocked PLDR-derived early phase increases in the number of transformants per dish after x-irradiation (Fig. 2*A*) but lowered the number of foci per dish to levels below those observed for no post-x-ray treatment. β-Lapachone decreased the number of foci per dish nearly 6-fold as compared to the level observed with x-irradiated CHEF/18A cells given no posttreatment (Fig. 2*A*). Prolonged post-x-ray treatment with β-lapachone resulted in only a slight increase to 7.7 ± 0.2 foci per dish at 10 hr and only a moderate increase (i.e., 5.0 foci per dish) compared to the unirradiated background levels (0.9 ± 0.05 foci per dish). Exposure of unirradiated CHEF/18A cells to 4 μM β-lapachone for up to 48 hr did not increase the number of transformants per dish beyond the observed increase in background levels (from 0.9 ± 0.5 foci per dish to 2.1 ± 0.6) upon confluent holding. Similar spontaneous increases in transformation due to prolonged confluence-arrest have been observed (24, 25).

β-Lapachone inhibited the survival recovery of x-irradiated CHEF/18A cells in a manner similar to that described for human melanoma cells (12). The survival of unirradiated CHEF/18A cells decreased to 32% ± 1.3% after 48 hr of exposure to β-lapachone as compared to 71% ± 2.6% for control cells exposed to 0.2% FCS-DME medium; survival values in Fig. 2*B* have been corrected for the respective decreases in plating efficiencies of unirradiated cells.

β-Lapachone Increased the Number of Double-Stranded DNA Breaks (DSBs). Previous research in our laboratory (12) suggested that β-lapachone increased the lethal effects of x-rays by converting repairable x-ray-induced single-stranded DNA breaks (SSBs) into the possibly more repair-resistant DSBs via the activation of mammalian topoisomerase I by β-lapachone (12).

We examined the effect of β-lapachone on the production and repair of both total [as measured by alkaline elution at pH 12.0 (Fig. 3*A*)] and double-stranded [as measured by neutral elution at pH 7.0 (Fig. 3*B*)] x-ray-induced DNA breaks in CHEF/18A cells. After x-irradiation, CHEF/18A cells repaired both SSBs and DSBs during 5 hr of PLDR as shown by the increase in [^{14}C]DNA retained on the filter as compared to the retained DNA from cells given no post-x-ray

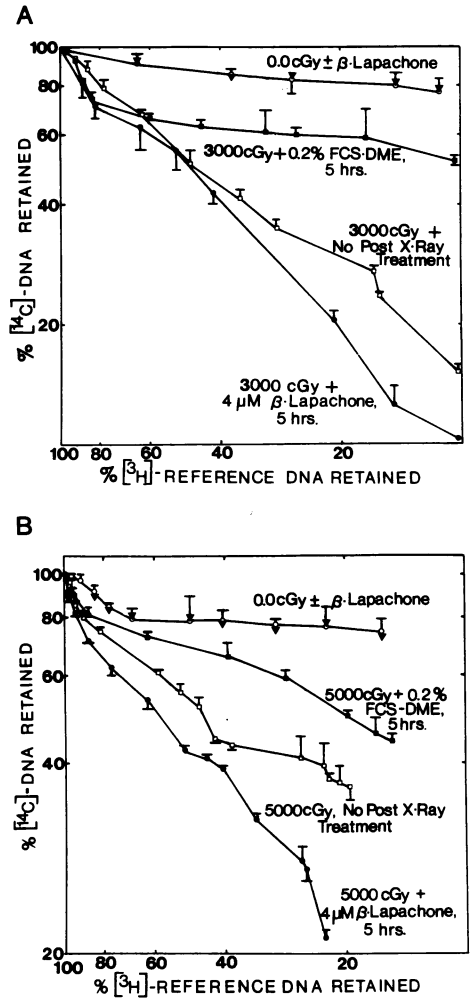

FIG. 3. Inhibition of total DNA break rejoining (*A*) and increased production of DSBs (*B*) by β-lapachone. Confluence-arrested CHEF/18A cells were either unirradiated or x-irradiated and analyzed by alkaline elution at pH 12.0 (*A*) or neutral elution at pH 7.0 (*B*). Unirradiated CHEF/18A cells were exposed to 0.2% FCS-DME medium with (▼) or without (○) 4 μM β-lapachone for 5 hr.

treatment. A 5-hr post-x-ray treatment with 4 μM β-lapachone increased the final amounts of both total and DSBs and thereby decreased the amount of [^{14}C]DNA retained. The increase in total DNA strand breaks following a post-x-ray treatment with β-lapachone (Fig. 3*A*) could be due to the increased number of DSBs (Fig. 3*B*). The DNA from unirradiated CHEF/18A cells was unaffected by a 5-hr exposure to 4 μM β-lapachone (Fig. 3).

DISCUSSION

By what mechanism does β-lapachone act to enhance lethality and prevent the transforming effects of x-irradiation? We hypothesize that β-lapachone potentiates the lethality of DNA damaging agents (such as x-rays, which nick the DNA sugar–phosphate backbone) by converting initially repairable SSBs into the more repair-resistant DSBs via its activation of topoisomerase I (12). Once a SSB has been created by a DNA-damaging agent, topoisomerase I may lose its sequence-specific binding activity (26, 27). Topoisomerase I can preferentially bind to a SSB in simian virus 40 DNA and cut the intact DNA strand by itself (28). Thus, following its activation by β-lapachone, it might also produce a DSB from an x-ray-induced SSB. The enzyme might covalently attach to the newly created DSB in a DNA–protein complex in the presence of β-lapachone, in a mechanism similar to that proposed for camptothecin (29). The covalently bound protein near the DSB may then inhibit, or significantly delay, DNA lesion repair. Increased DSBs could dramatically decrease survival, since the presence of DSBs correlates well with lethal events following x-irradiation (19, 30–32).

Such a model would adequately explain (*i*) the increase in total SSBs and DSBs in the DNA of x-irradiated cells in the presence of β-lapachone (Fig. 3); (*ii*) the synergistic lethality between x-rays and β-lapachone occurring in both plateau-phase and exponential-phase mammalian cells, since topoisomerase I activity is not cell-cycle regulated (33); (*iii*) why β-lapachone synergized with x-rays and neocarzinostatin but not melphalan (12), since the latter agent is a bifunctional alkylator and does not readily induce SSBs (34); (*iv*) why β-lapachone does not decrease the survival of unirradiated cells, in which no SSBs are present; and (*v*) why a pretreatment with β-lapachone has little or no effect on the survival of subsequently x-irradiated cells (12).

A post-x-ray treatment with β-lapachone may modify a topoisomerase I activity so as to prevent initial genetic rearrangements, which would otherwise produce transformed phenotypes. Topoisomerase I has been proposed to function in both site-specific (35) and illegitimate (36, 37) recombinations. Alternatively, β-lapachone could accelerate the production of gross genetic rearrangements in damaged cells so that lethality results.

The data presented are consistent with the hypothesis that PLDR processes are error prone and can cause transformation (23). The immediate response of x-irradiated cells may be to initiate (or induce) PLDR processes that facilitate survival. During this initial response period, preferential repair of essential actively transcribed genes may take place compared to the genome as a whole, as found with UV-irradiation (1, 2). After 6–12 hr (repair time depends on cell type), there is a second phase of PLDR during which the number of foci per dish decreases. This later repair could allow error-containing cells enough time to completely repair the remaining DNA lesions and correct initial misrepairs created during the early phase of PLDR. The transformation experiments shown in Figs. 1*A* and 2*A* with CHEF/18A cells, which showed early error-prone and later error-correcting PLDR processes, are consistent with earlier data (23). Similar decreases in transformation following extensive PLDR have been reported with other cell lines (38). A holding period of up to 5 hr between two fractionated x-ray doses also increased transformation relative to an equivalent single dose, further suggesting the presence of transient error-prone PLDR processes in mammalian cells (39, 40). Thus, faulty or incomplete DNA repair occurring in early phases of PLDR following x-ray or other genetic insults may play a significant role in the early steps of carcinogenesis.

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