Bromodeoxyuridine: a comparison of its photosensitizing and radiosensitizing properties

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The photo- and radiosensitizing properties of bromodeoxyuridine (BUdR) were assessed in vitro using the 9L rat brain tumor cell line. Pretreatment of 9L cells with 10 μM BUdR for 24 hours followed by irradiation with ultraviolet (UV) light resulted in a dose-enhancement ratio of 3.8:1 compared with UV radiation alone. X-radiation of BUdR-pretreated cells produced a dose-enhancement ratio of 1.7:1. Alkaline elution analysis of deoxyribonucleic acid (DNA) from cells treated with BUdR and UV irradiation showed the presence of DNA single-strand breaks and DNA-protein cross-links. Analysis of DNA from cells treated with BUdR and then x-irradiated showed no increase in DNA single-strand breaks compared with cells treated with x-radiation alone; no DNA-protein cross-links could be detected. The possible clinical relevance of these findings is discussed.

KEY WORDS • phototherapy • radiation sensitizers • bromodeoxyuridine • gliosarcoma • rat

Bromodeoxyuridine (BUdR) is a thymidine analog that is incorporated into cellular deoxyribonucleic acid (DNA) during DNA replication and repair. Cellular DNA containing BUdR is more sensitive to ionizing radiation (x-rays) than is native DNA. While the exact mechanism of radiosensitization caused by incorporation of BUdR into cellular DNA is not clearly understood, it is probably not related to secondary effects such as inhibition of DNA polymerase or inhibition of enzymes involved in thymidine synthesis.

Serum BUdR levels as high as 10 μM have been measured in patients receiving continuous intravenous infusions of 0.8 gm/sq m/day of BUdR for 4 days. Toxicity is limited to reversible bone marrow suppression and dermal tissue reactions. Bromodeoxyuridine is an ideal drug for the treatment of malignant cerebral neoplasms because it is incorporated only into the DNA of cells that are actively undergoing DNA synthesis; therefore, rapidly cycling tumor cells should incorporate much more BUdR than relatively quiescent normal brain cells, and subsequent irradiation may selectively kill tumor cells with relative sparing of normal brain. The radiosensitizing properties of BUdR are currently being explored in Phase II trials for the treatment of malignant gliomas. Bromodeoxyuridine is also a potent photosensitizing agent.

The effect on cell survival of irradiation with ultraviolet (UV) light and x-rays after incorporation of BUdR into the DNA of 9L rat brain tumor cells has been examined. The effect of combination treatment on cellular DNA was examined using the alkaline elution assay. Compared with irradiation alone, incorporation of BUdR into 9L cell DNA followed by irradiation caused an increase in cell kill and in the number of DNA single-strand breaks; irradiation with UV light caused the formation of DNA-protein cross-links, whereas x-irradiation did not produce these lesions.

Materials and Methods

Monolayer Cell Cultures

Monolayer cultures of 9L rat brain tumor cells were grown at 37°C in complete medium consisting of Eagle’s minimum essential medium supplemented with newborn calf serum (100%), nonessential amino acids, and gentamicin (50 μg/ml). Under these conditions, the plating efficiency is 40% to 60%, and the population doubling time is about 18 hours. The number of cells initially seeded for each experiment was chosen to...
assure log phase growth for the duration of BUdR treatment.

**Cell Treatment**

The cells were incubated with 10 μM BUdR for 24 hours. To prevent photolysis of BUdR, all procedures were performed in the dark or under yellow light. Cells to be irradiated with UV light were grown in Petri dishes. Before irradiation, the cells were washed with cold phosphate-buffered saline minus the phenol red indicator. They were then irradiated with the Petri dish lids off under a germicidal lamp* that generates more than 95% of its output at 254 nm. Under the conditions used, the fluence was 0.6 joule/sq m/sec. Cells for x-radiation were exposed at a dose rate of 1.4 Gy/min using an x-ray machine (280 kV peak, 20 mA, half-value layer (HVL) = 2 mm Cu).† The cells were kept on ice after either treatment.

**Removal of Cells from Plastic Surfaces**

For removal of cells from 75-sq cm flasks or Petri dishes, the complete medium was decanted and the cells were rinsed with 3 ml of STV (Saline A containing 0.05% trypsin and 0.02% versene). After the rinse was decanted, another 3 ml of STV was added and the cells were incubated at 37°C for 5 minutes. Enzymatic action was stopped by the addition of 10 ml complete medium, and the suspension was transferred to a 50-ml plastic centrifuge tube. The flask or dish was rinsed with 5 ml complete medium that was added to the centrifuge tube. Gentle pipetting produced a single-cell suspension.

**Cell Survival Assay**

Petri dishes (60 mm) were prepared 24 hours in advance of the cell survival assay by seeding heavily irradiated (with 40 Gy) feeder cells into 4 ml of growth medium. The optimum number of feeder cells (5 × 10^4) had been determined in preliminary experiments. After plating of the treated cells, the dishes were incubated for 2 weeks at 37°C in a humidified atmosphere containing 5% CO_2/95% air, after which they were fixed, stained with 0.125% crystal violet in methanol, and counted. Plating efficiencies were determined by dividing the number of colonies on the plates by the number of cells seeded. Surviving fractions were calculated as the plating efficiency of treated cells divided by the plating efficiency of untreated cells.

**Alkaline Elution Assay**

After treatment, the cells were removed from the flasks as single-cell suspensions and placed on ice; 5 × 10^6 cells were then deposited on polycarbonate filters (2 μm pore size) and lysed with a solution containing 2 M NaCl, 0.04 M Na_2_ EDTA (ethylenediaminetetra-

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* Sterilamp, Model G3OT9, manufactured by Westinghouse Corp., Pittsburgh, Pennsylvania.
† Modified Vanguard Therapy Unit manufactured by Picker X-Ray Machines, Cleveland, Ohio.

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The colony-forming efficiency assay was used to assess the effect of sequential treatment of log phase 9L cells with BUdR followed by UV irradiation or x-radiation. The concentration of BUdR used (10 μM) is the maximum concentration that has been found in the serum of humans after continuous intravenous infusion of the drug. Twenty-four hours after UV irradiation alone, however, combination treatment caused increased cell kill with a dose enhancement ratio

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† Tritiated BUdR obtained from New England Nuclear, Boston, Massachusetts.
of 3.8:1 at the 50% survival level (Fig. 1A). Compared with x-radiation alone, combination treatment caused increased cell kill with a dose enhancement ratio of 1.8:1 at the 50% survival level (Fig. 1B). At approximately isoeffective doses of UV light and x-rays (18 joule/sq m/sec and 4 Gy, respectively), BUdR pretreatment caused an approximately 2-log increase in cell killing by UV and an approximately twofold increase in killing by x-rays.

Alkaline Elution Analysis of DNA Single-Strand Breaks

Irradiation with UV alone or pretreatment for 24 hours with 10 μM BUdR caused only a few DNA single-strand breaks. Lesions in BUdR-treated cells are probably alkaline-labile sites and not true single-strand breaks; the appearance of strand breaks decreased progressively for elution analysis of solutions with decreasing pH (12.4 to 11.8, data not shown). Compared with each agent alone, combination treatment with BUdR and UV irradiation produced a large number of single-strand breaks (Fig. 2A). In addition, the DNA elution profile for cells treated with BUdR and UV irradiation had two components. The initial component eluted quickly, indicating that a portion of the DNA had a large number of single-strand breaks; the second component eluted more slowly and contained fewer apparent strand breaks. Moreover, the first component eluted more rapidly than any portion of the DNA from cells pretreated with BUdR and then x-irradiated (Fig. 2B).

Pretreatment of cells for 24 hours with 10 μM BUdR followed by x-radiation caused more single-strand breaks than the same dose of x-rays alone (Fig. 2B).

The elution profile of DNA from cells treated with BUdR and x-irradiated at a given dose is quite similar to the profile found for x-radiation alone at twice the given dose.

To determine the distribution of single-strand breaks in total DNA compared with breaks in newly synthesized DNA that contained BUdR, 0.01 μM of tritiated BUdR was added to cell cultures at the start of the incubation period. As shown in Fig. 3, DNA contained tritiated BUdR eluted with the fast component in both the UV-irradiated (Fig. 3A) and x-irradiated cells (Fig. 3B); the difference was slight for x-irradiated cells. Single-strand breaks appeared to be more evenly distributed between DNA synthesized before and after the addition of BUdR in the x-irradiated cells.

Alkaline Elution Analysis of DNA-Protein Cross-links

To determine if DNA-protein cross-links were formed by combination treatments, alkaline elution analysis was performed in the presence and absence of proteinase K. Elution profiles for cells treated with BUdR alone or UV irradiation alone were the same with or without the presence of proteinase K in the lysis solution (Fig. 4). However, DNA from cells pretreated

![Fig. 1. Cytotoxicity produced by combined treatment with bromodeoxyuridine (BUdR) and ultraviolet (UV) light or x-rays. 9L cells were grown in 10 μM BUdR for 24 hours. The cells were then irradiated for various lengths of time with UV light at a rate of 0.6 joule/sq m/sec (A) or with various doses of x-rays (B). The BUdR treatment produced a 90% cell kill. In A, circles = cells not treated with BUdR, but irradiated with UV, and squares = cells treated with BUdR followed by UV irradiation. In B, circles = cells not treated with BUdR, but x-irradiated, and squares = cells treated with BUdR and then UV-irradiated.](image)

![Fig. 2. Deoxyribonucleic acid (DNA) single-strand breaks produced by bromodeoxyuridine (BUdR) and ultraviolet (UV) light or x-rays. A: Alkaline-elution profiles from DNA of 9L cells treated for 24 hours with 10 μM BUdR and irradiated with 18 joule/sq m of UV light. Open circles = untreated cells; open squares = cells treated with 18 joule/sq m of UV light alone; closed squares = cells treated with 10 μM BUdR alone for 24 hours; closed circles = combined treatment with BUdR and UV light. B: Alkaline-solution profiles from DNA of 9L cells treated for 24 hours with 10 μM BUdR and increasing doses of x-radiation. Closed squares = cells treated with 10 μM BUdR alone; open diamonds = cells treated with 1 Gy x-ray radiation alone; closed diamonds = cells treated with 1 Gy x-ray radiation combined with BUdR; open triangles = cells treated with 2 Gy x-ray radiation alone; closed triangles = cells treated with 2 Gy x-ray radiation combined with BUdR; open circles = cells treated with 4 Gy x-ray radiation alone; closed circles = cells treated with 4 Gy x-ray radiation combined with BUdR.](image)
Photosensitizing and radiosensitizing properties of BUdR

with BUdR and UV-irradiated eluted more slowly when proteinase K was absent from the lysis solution, indicating the presence of DNA-protein cross-links. The calculated cross-linking factor is 0.24. In contrast, the presence or absence of proteinase K in the lysis solution had no effect on elution profiles for combination treatment with BUdR and x-radiation (Fig. 4B), which suggested that no DNA-protein cross-links were formed.

Discussion

The results reported here show that BUdR is a radiosensitizer of 9L cells in vitro. Compared with the cell-killing effects of radiation alone, pretreatment of cells with BUdR followed by irradiation caused increased cell kill, with a dose enhancement ratio of 1.8:1 for x-rays and of 3.8:1 for UV light. Thus, BUdR is much more effective as a photosensitizing agent.

Erikson and Szybalski compared the cell-killing properties of various halogenated thymidine analogs, including BUdR, in combination treatment protocols with UV light or x-rays. Their results suggested that BUdR is superior as a radiosensitizer, although this comparison was not made in their report. In contrast, Ben-Hur and Elkind found that in Chinese hamster cells pretreated with BUdR, cell kill is greater for subsequent UV irradiation than for x-irradiation. These results are very similar to those reported here.

Ultraviolet irradiation alone caused no DNA strand breaks, but single-strand breaks could be detected after pretreatment with BUdR alone. Preliminary results suggest, however, that these lesions are alkaline-labile sites and are not true strand breaks. Alkaline-labile sites have been found in other cell lines treated with BUdR.

Pretreatment with BUdR followed by UV irradiation causes single-strand breaks in a number of cell lines in vitro. Our results show that UV irradiation of BUdR-pretreated cells causes a large number of single-strand breaks in the portion of DNA containing BUdR. Using the alkaline elution assay, a direct correlation was found between the amount of DNA in the fast-eluting component of DNA and the length of time cells are pretreated with BUdR (C Raffel, et al., in preparation). Irradiation with UV alone produces few, if any, single-strand breaks in cellular DNA. While the thymidine dimer is the most common lesion found in UV-irradiated cellular DNA, this lesion is rarely found in UV-irradiated cells pretreated with BUdR. The decrease in the number of dimers formed probably reflects the random substitution of BUdR for thymidine.

The proposed mechanism for strand breakage in BUdR-substituted DNA is thought to involve a uracil radical formed by photolytic cleavage of the uracil-bromine bond. The uracil radical abstracts the hydrogen on C'-2 of an adjacent ribose, which leads to scission of the phosphodiester backbone of DNA. The most convincing evidence for this mechanism is the presence of a uracil moiety at the 3' terminus of all DNA fragments obtained from photolytic cleavage of DNA in cells pretreated with BUdR and UV irradiation. The presence of free-radical inhibitors decreases the number of single-strand breaks.

![Fig. 3. Single-strand breaks in deoxyribonucleic acid (DNA) synthesized after the addition of bromodeoxyuridine (BUdR).](image1)

![Fig. 4. Deoxynucleoside acid (DNA)-protein cross-links produced by bromodeoxyuridine (BUdR) and ultraviolet (UV) light or x-radiation.](image2)
X-rays alone produced a large number of strand breaks of 9L cells, but the number of strand breaks in the DNA at 9L cells pretreated with BUdR was approx- imately twofold greater; thus, pretreatment with BUdR essentially doubles the effect of a given dose of x-rays. The mechanism of x-ray-induced single-strand breaks is more complex than the mechanism for UV light. The first step involves the formation of hydroxyl radicals that react indiscriminately with labile sites on DNA; abstraction of ribose hydrogens leads to single-strand breaks. It is possible that hydroxyl radicals may abstract a bromine and lead to BUdR-specific strand breaks. The distribution of x-ray-induced strand breaks found in BUdR-substituted DNA is only slightly increased in the portion of DNA containing BUdR (Fig. 3B). This suggests that the mechanisms of formation of single-strand breaks is different for x-rays and UV light.

Ultraviolet irradiation but not x-radiation causes the formation of DNA-protein cross-links in 9L cells pretreated with BUdR. At high fluences, UV irradiation alone can cause DNA-protein cross-links, but at the relatively low fluences used in these experiments no significant DNA-protein cross-linking was detected. The presence of DNA-protein cross-links in UV-irradiated cells pretreated with BUdR has been reported but not quantified. No DNA-protein cross-links were formed in 9L cells pretreated with BUdR and x-irradiated. Differences in the dose enhancement ratio for UV- and x-radiation may be related to the formation and repair of DNA-protein cross-links. Repair of the single-strand breaks for BUdR-UV irradiation combination treatment was found to occur much more rapidly than repair of the DNA-protein cross-links, which has been observed in other cell lines. Therefore, the much greater photosensitizing properties of UV irradiation are probably related to the induction of DNA-protein cross-links in BUdR-pretreated cells. Protein-DNA lesions may inhibit repair sterically, or repair enzymes may be cross-linked to DNA and thereby inactivated.

There have been reports of DNA double-strand breaks in other cell lines treated in combination with BUdR and irradiation. In Escherichia coli, a single double-strand break is lethal. Induction of double-strand breaks in cells treated with BUdR and UV irradiation apparently involves both single- and double-photon events. Recently, Kinsella, et al., induced double-strand breaks in cells treated with BUdR or iododeoxyuridine and then x-irradiated the cells at doses five and 15 times greater than used in our experiments. They reported that the kinetics of repair of double- and single-strand breaks were similar. In the present studies, nearly 100% cell kill was achieved by pretreatment of 9L cells with 10 μM BUdR for 24 hours followed by irradiation with these high doses of x-rays. These results suggest either that DNA double-strand breaks are responsible for cell death (even at the low levels at which they occur), or that for x-radiation at lower doses other lethal events occur before double-strand breaks appear. Experiments are in progress to determine which of these possibilities is correct.

These results may have clinical relevance. They suggest that BUdR may have a role as a photosensitizer in the treatment of malignant brain tumors. Bromodeoxyuridine can be incorporated in the cycling cells of malignant lesions by treating patients with BUdR before surgical resection. Ultraviolet irradiation of the surface of the tumor cavity created by resection may selectively kill cells in the critical brain adjacent to tumor and prevent or delay recurrences. The incorporation of BUdR into cells that cannot be UV-irradiated may increase the sensitivity of these cells to x-rays delivered during adjuvant radiation therapy. Radiosensitization could be enhanced by administering additional doses of BUdR before and during radiation therapy. Because UV irradiation cannot penetrate too deeply into brain tissue, phototherapy may be most applicable to tumors such as medulloblastoma, where a gross total resection of tumor is possible.

References

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