Synergistic Cytotoxicity and DNA Strand Break Formation by Bromodeoxyuridine and Bleomycin in Human Tumor Cells

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ABSTRACT

5-Bromo-2'-deoxyuridine (BrdUrd) is a thymidine analogue whose cellular effects are related to its incorporation into DNA. BrdUrd is a known radiosensitizing agent that could potentially enhance the activity of chemotherapeutic agents that interact directly with DNA. Therefore we studied the interaction of BrdUrd and bleomycin in a human head and neck squamous carcinoma cell line, SQ20B. Using a colony-forming assay and analyzing results by the median-effect method, we have shown that there is synergistic cytotoxicity between BrdUrd and bleomycin. Synergism is evident when BrdUrd is administered prior to bleomycin or when the two drugs are applied simultaneously and is evident at a variety of BrdUrd:bleomycin concentration ratios. Alkaline elution of DNA from cells exposed to BrdUrd and bleomycin demonstrated greater single strand break formation than expected from the individual single strand break frequencies induced by each drug alone. BrdUrd did not affect the rate of repair of bleomycin-induced single strand breaks or the formation of double strand breaks. Although the mechanism of this interaction at the molecular level is unclear, our studies suggest that a direct interaction of bleomycin with BrdUrd-substituted DNA may be the cause of the synergism of these two agents.

INTRODUCTION

5-Bromo-2'-deoxyuridine is a synthetic analogue of thymidine whose cellular effects stem primarily from its incorporation into DNA (1). Although BrdUrd can competitively inhibit enzymes of nucleoside metabolism such as ribonucleotide reductase and thymidine kinase, its cytotoxic effects are directly correlated with the extent to which it replaces thymidine in DNA (1–3). In addition, incorporation of BrdUrd into DNA induces radiosensitization and photosensitization, although the mechanism of these effects has not been fully elucidated (4, 5).

Bleomycin is a mixture of glycopeptide antibiotics isolated from Streptomyces verticillus. It is widely used in cancer chemotherapy and, in combination with other drugs, is particularly effective against carcinomas of the head and neck, lymphomas, and germ cell tumors (6). The principal active component, bleomycin A2, has an aminoterminal tripeptide that binds to DNA resulting in a DNA-bleomycin-Fe2+O2 complex (7–9). Subsequent reduction of oxygen to reactive species then damages the DNA, causing chromosomal breaks and deletions as well as SSB and double strand breaks (8–10). The latter are generally believed to be the primary mechanism of cytotoxicity. Thus, bleomycin damages DNA in a way which resembles ionizing radiation, i.e., by generation of highly reactive oxygen radical species within the cell.

One potentially useful application of BrdUrd may be its ability to potentiate the actions of certain chemotherapeutic agents as well as radiation. Recently, enhancement of the toxicity of four unrelated chemotherapeutic agents (melphalan, doxorubicin, cisplatin, and neocarzinostatin) by both BrdUrd and iododeoxyuridine has been demonstrated in Chinese hamster V79 cells (11). We conducted an in vitro study of the interaction of BrdUrd and bleomycin in a human tumor cell line the objectives of which were (a) to evaluate the cytotoxic effects of BrdUrd, bleomycin, and their combination, and to determine whether synergistic, additive, or antagonistic effects occurred, (b) to examine the effects of BrdUrd on bleomycin-induced DNA strand break formation and repair, and (c) to correlate the biochemical effects with the observed cytotoxic effects of the combination.

MATERIALS AND METHODS

Cell Line

SQ20B, a squamous cell carcinoma cell line derived from a patient with recurrent carcinoma of the larynx following radiation therapy, has been described previously (12). The cells are maintained as a monolayer in RPMI 1640 medium (Hazeltone Research, Denver, PA) containing 2 mM L-glutamine, 0.4 µg/ml hydrocortisone, penicillin, 100 units/ml, streptomycin, 100 units/ml, and 20% fetal calf serum under a humidified atmosphere of 5% CO2 in air at 37°C. Under these conditions the population doubling time is 24 h, and the cells vigorously form keratin pearls.

Drugs

BrdUrd (Sigma Chemical Co., St. Louis, MO) powder was dissolved in 0.9% NaCl solution to a concentration of 100 µM and stored in polypropylene tubes under refrigeration (4°C). Bleomycin (Bristol-Myers Co., Syracuse, NY) powder for clinical use was dissolved in 0.9% NaCl solution to a concentration of 500 µunits/ml and stored in polypropylene freezing tubes at −70°C. Under these conditions the drugs were stable (<10% loss of activity) by spectroscopic analysis and cytotoxicity for at least six months.

Colony-forming Assay

Two methods of drug exposure were used.

Method 1. Logarithmically growing SQ20B cells were exposed to BrdUrd 0.05–4.0 µM in the dark for 48 h. Following drug exposure, the medium was aspirated, monolayers were washed twice with drug-free RPMI 1640, and then detached by a 5-min incubation in 0.25% trypsin-0.02% EDTA (Hazelton Research, Lenexa, KS). A single cell suspension was prepared and the cells were then plated in quadruplicate in 35- × 10-mm tissue culture dishes (Corning Glass Works, Corning, NY), at a density of 103–4 × 104 cells/dish and incubated in the dark for 24 h to allow attachment and growth. Bleomycin was then added to the media to a final concentration of 0.25–10 µunits/ml for 4 h, after which the cells were washed twice and drug-free media were replaced in the dishes. The cells were then allowed to incubate in the dark for 8–10 days to allow macroscopic colonies to form. Quadruplicate dishes containing control cells (not drug treated) and cells treated with each drug alone were made in parallel. All manipulations were performed under dim yellow safelight. At the end of the incubation period, colonies (>50 cells) were fixed and stained with 2% crystal violet in 95% ethanol and counted. Plating efficiencies of drug-treated versus control cells were determined.

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Method 2. Logarithmically growing SQ20B cells, plated in quadruplicate, were exposed to BrdUrd, 0.5–10 μM and bleomycin, 0.05–1 μunit/ml simultaneously for 24 h, following which they were washed twice and incubated according to method 1. Similarly, dishes containing control cells and cells treated with each drug alone were made in parallel.

Analysis of Combined Drug Effects

Colony-forming assay results were analyzed according to the median effect principle described by Chou and Talalay (13, 14). Unlike the fractional product and isobologram methods, the medium effect method allows analysis of combined drug effects independent of individual drug kinetics (first-order or higher order) and independent of the mechanism of drug interaction (mutually exclusive or nonexclusive) (14, 15, 16).

The principle is described by the equation

\[ \ln\left( \frac{1}{SF} - 1 \right) = m \cdot \ln\left( \frac{D}{Dm} \right) \]

where SF is the surviving fraction of colonies compared to control for each dose, D, of drug, Dm is the median-effect dose (i.e., the dose producing a surviving fraction of 0.5), and m is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve. A plot of \( y = \ln(1/SF - 1) \) versus \( x = \ln(\text{dose}) \) (a median-effect plot) linearizes the dose-effect relationship with the slope m and the x-intercept at ln(Dm). m and Dm were calculated for each drug alone in each experiment and for each drug combination at a fixed concentration ratio.

The interaction of the two drugs was quantitated by determining a CI for each fixed concentration ratio at surviving fractions of 0.75, 0.5, 0.25, 0.1, according to the equation

\[ CI = \frac{D_x}{D_{x1}} + \frac{D_y}{D_{y2}} + \frac{D_{x1}D_y}{D_{x2}D_{y2}} \]

where \( D_{x1}, D_{y2} \) are the doses of drugs 1 and 2, respectively, which when used alone cause a SF of x; \( D_x, D_{y2} \) are the cytotoxic contributions of drugs 1 and 2, respectively, in the mixture calculated from the known dose ratio of the two drugs, and the dose, \( D_{x2}, D_{y2} \) of the mixture required to cause a SF of x. Thus, if the ratio of drugs 1 and 2 in the mixture is \( P/Q \), then

\[ D_1 = \frac{D_{x2} - D_{x2} - D_x}{P + Q} \]

and

\[ D_2 = \frac{D_{x1} - D_{x1} - D_y}{P + Q} \]

Finally a CI versus SF plot was generated for all values of SF between 0.05 and 0.95. Then, when CI < 1, synergy is indicated; when CI = 1, summation is indicated; when CI > 1, antagonism is indicated.

Alkaline Elution of DNA

This procedure was performed according to the method of Kohn et al. (17). Logarithmically growing SQ20B cells were incubated in 0.06 μCi/ml [14C]thymidine (Amersham, Arlington Heights, IL) for 48 h to label DNA. Then, after washing twice, incubation was continued in BrdUrd, bleomycin, or both for 24 h, analogous to colony-forming assay method 2 above. At the end of drug exposure, monolayers were rinsed, trypsinized, agitated, and chilled immediately on ice. Two × 10^6 SQ20B cells, together with an internal standard of 5 × 10^6 L1210 cells previously labeled with [3H]thymidine and irradiated with 500 rads, were placed on ice-cold 1.0-μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA). Cells were immediately lysed with 5 ml of 0.025 M EDTA-2% SDS, pH 9.7. After connection of the filter apparatus to the pump tubing, 2 ml of lysis solution containing 0.5 mg/ml of proteinase-K (Boehringer Mannheim Biochemicals, Indianapolis, IN) were added and pumped through slowly over 1 h, followed by 40 ml of eluting solution (0.1 M tetrapropyl-ammonium hydroxide-0.02 M EDTA-0.1% SDS, pH 12.1). Alkaline elution was undertaken at a speed of 2 ml/h and 2-h fractions were collected for 10–16 h. Filters were then removed and treated as described (17) to depurinate and detach DNA. All collected fractions and filters were then mixed with 4 volumes of aqueous counting solution (Amersham, Arlington Heights, IL) containing 0.7% glacial acetic acid and were counted in a Packard Tri-carb Model 4530 liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) using a dual-label counting mode. Data were analyzed and plotted as the percentage of [14C]DNA remaining on the filter versus the percentage of internal standard [3H]DNA. An elution rate constant for each experiment was calculated as the mean slope of the elution curve over the first 2 fractions collected. Single strand break frequency as rad-equivalents was calculated from this value using a previously determined linear relationship between elution rate constant and radiation dose for SQ20B cells (r = 0.99; data not shown).

Neutral Elution of DNA

This procedure was performed by the method of Bradley and Kohn (18), except that elution was performed at pH 7.2 with 0.05 M Tris-HCl-0.05 M glycine-0.025 M disodium EDTA-2% SDS. SQ20B cells were labeled, drug treated, and applied to filters, and fractions were collected and analyzed as in the alkaline elution experiments. Results were plotted as a function of elution time.

RESULTS

A number of cytotoxicity experiments have been performed according to method 1 to analyze the interaction of BrdUrd and bleomycin, each experiment testing each drug alone and combinations at two to four fixed concentration ratios. The median-effect dose, Dm, for BrdUrd was 1.05 ± 0.50 (SD) μM (n = 8). The median-effect dose for bleomycin was 1.13 ± 0.35 μunits/ml (n = 12) (Fig. 1). Table 1 and Fig. 2 show colony-forming assay results and a median-effect plot, respectively, from a single experiment.

The parameters, m and Dm, which describe the median-effect plots for each experiment are shown in Table 2. The observed interexperiment variability is likely to be due to biochemical
and/or kinetic differences of the cells at different times; however, within each experiment results are consistent.

The composite CI versus SF plot for the data from one such experiment (shown in Table 1 and Fig. 2) is presented as Fig. 3. Synergism is apparent at all tested concentration ratios at high effect levels (SF < 0.5), but there is evidence of ratio dependence at lower effect levels. More synergism is apparent with the 2:1 concentration ratio than with higher or lower ratios. This finding suggests that for this particular schedule of administration in this in vitro cellular system, the 2:1 concentration ratio is optimal. Combination indices at a range of effect levels for other experiments, shown in Table 3, lend support to this contention; clearly the 2:1 and 1:1 concentration ratios are more synergistic than 4:1 or 1:2 concentration ratios at low surviving fractions.

Drug interactions were also analyzed following exposure of cells to BrdUrd and bleomycin simultaneously for 24 h (method 2). This schedule was studied in order to correlate the cellular effects (colony-forming assay) with the DNA effects (DNA strand break formation), since alkaline elution of radiolabeled cells treated according to method 1 was not technically feasible. Using method 2, the median-effect dose of BrdUrd was 4.24 ± 2.14 μM (n = 2) and the median-effect dose for bleomycin was 0.23 ± 0.05 μunits/ml (n = 2). Representative results of two experiments are shown in Table 2B and Fig. 4. The 5:1 concentration ratio is synergistic at all effect levels, and the 1:1 concentration ratio is synergistic when the surviving fraction is less than 0.5, once again suggesting that synergism between BrdUrd and bleomycin is dependent on the relative concentrations of the two drugs in the mixture.

In order to investigate the nature of the interaction between BrdUrd and bleomycin, we performed alkaline and neutral elution for SSB and DSB, respectively, of SQ20B cells exposed to one or both drugs. Analysis was performed only in cells treated according to method 2, since method 1 would have resulted in considerable dilution of radiolabel in DNA during the long drug exposure period prior to elution (simultaneous BrdUrd and [3H]thymidine treatment was also not feasible since this would have resulted in inhibition of incorporation of BrdUrd by thymidine). BrdUrd-treated DNA showed evidence of alkali lability manifest as an increasing DNA elution rate with time, as previously described (19). The initial elution rate, a measure of the SSB frequency in BrdUrd-treated DNA not modified by alkali, was very low with the BrdUrd concentrations used in this study, and increasing BrdUrd concentration up to 4 μM resulted in a less-than-proportional increase in SSB frequency. In contrast to BrdUrd, bleomycin-treated DNA eluted at a constant rate, indicative of random DNA SSB formation. Over the dose range studied, bleomycin caused a linear increase in SSB formation with increasing dose.

As shown in Table 4 and Fig. 5, simultaneous treatment of SQ20B cells with BrdUrd and bleomycin at several doses resulted in clear-cut enhancement of SSB formation. Various dose combinations over the range of 1–4 μM BrdUrd and 0.5–5 μunits/ml bleomycin have been studied; in each case additive or greater-than-additive SSB formation was demonstrable. No particular concentration ratio appeared clearly more effective at inducing SSB.

Both the absolute amount of DNA damage produced and its persistence are related to cellular cytotoxicity (20–23). Therefore we studied the rate of repair of drug-induced DNA SSB by incubating cells in drug-free media for various times after drug treatment and prior to elution. A 1-h drug-free incubation resulted in an apparent slight increase in bleomycin-induced SSB, whereas incubation for longer periods resulted in progressively decreased SSB frequency, such that by 6 h after bleomycin washout approximately 50% of SSB had been repaired (data not shown). BrdUrd affected only the initial frequency of SSB, not the rate of repair as evaluated by alkaline elution.

Neutral elution of DNA from drug-treated cells, to assess the effect of BrdUrd on bleomycin-induced DSB formation, showed that BrdUrd in the doses used in this experiment had essentially no effect on DSB formation (see Fig. 6). Treatment of cells with BrdUrd alone resulted in an elution rate identical to control cells, whereas bleomycin resulted in an elution profile which was curvilinear and concave upwards, suggestive of either nonrandom DSB formation or heterogeneous sensitivity of SQ20B cells to bleomycin. This phenomenon has been observed in other in vitro cell systems with bleomycin (18).

DISCUSSION

Using the median effect principle, we have clearly demonstrated synergistic cytotoxic effects for the combination of BrdUrd and bleomycin in human tumor cells. Synergism is seen with two different schedules of administration and a number of different drug concentration ratios.
A. Drug exposure according to method I

Control. Ratios, fixed concentration of BrdUrd (>1 μM) : bleomycin (>units/ml).

In order to exclude the possibility of a cytotoxic interaction between the two drugs and the possibility that BrdUrd may be incorporated into DNA repair patches after bleomycin damage, we also chose to include a drug-free interval prior to bleomycin exposure. Using this schedule of administration, synergistic cytotoxicity was evident. Since bleomycin causes DNA strand breaks we wanted to investigate the effects of BrdUrd on bleomycin-induced strand break formation and repair, and the relationship these effects may have to the synergistic cytotoxicity. However, the lengthy drug-exposure periods make these effects difficult to investigate. Therefore, we undertook more limited series of colony-forming assays using simultaneous drug exposure for 24 h (method 2). Again, synergistic cytotoxic effects were demonstrated. We then chose to evaluate the effects of this administration schedule on DNA strand breaking. The studies described here indicate an enhancement of SSB formation for the combination of BrdUrd and bleomycin compared to that expected from addition of the isolated SSB frequencies of each drug alone.

Although our data allow us to speculate about the possible mechanisms of the interaction between BrdUrd and bleomycin, further studies will be necessary to define the actual mechanism(s) more accurately. To begin with, there may be increased SSB frequencies of each drug alone. * Mean ± SEM of at least three determinations.

B. Drug exposure according to method 2

Table 2 Parameters of median effect plots (median doses, Dm and slopes, m) derived from colony-forming assay results after exposure to BrdUrd and/or bleomycin

<table>
<thead>
<tr>
<th>Drug</th>
<th>4:1</th>
<th>2:1</th>
<th>1:1</th>
<th>1:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdUrd</td>
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<td>1.08</td>
<td>1.13</td>
<td>1.28</td>
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<tr>
<td>Bleomycin</td>
<td>1.72</td>
<td>1.19</td>
<td>1.24</td>
<td>1.36</td>
</tr>
</tbody>
</table>

Table 3 Combination indices at various dose effect levels (survival fractions) for combinations of BrdUrd and bleomycin

Table 4 DNA single strand break frequencies (expressed as rad-equivalents) in SQ20B cells treated with BrdUrd and/or bleomycin

When this series of investigations was begun, we hypothesized that BrdUrd incorporation into DNA was necessary before synergism with bleomycin would occur. Hence our initial investigations involved a prolonged BrdUrd exposure to achieve incorporation. In order to exclude the possibility of a cyto-sis that BrdUrd incorporation into DNA was necessary be...
cells exposed to BrdUrd and/or bleomycin simultaneously for 24 h at various indicated concentrations. I', units.

Fig. 5. Representative alkaline elution pH 12.1 curves of DNA from SQ20B cells exposed to BrdUrd (BrdR) and/or bleomycin simultaneously for 24 h at various indicated concentrations. U, units.

Fig. 6. Representative neutral elution pH 7.2 curves of DNA from SQ20B cells exposed to BrdUrd and/or bleomycin simultaneously for 24 h at various indicated concentrations. U, units.

exposure method 2, would still allow incorporation of BrdUrd so that either of these mechanisms may occur. Moreover, BrdUrd-containing DNA may be more resistant to repair after damage by bleomycin than native DNA. Alternatively, the presence of BrdUrd at the time of DNA repair may result in delayed or inefficient repair. In our studies, there has been no evidence that BrdUrd slows the rate of repair of bleomycin-induced SSB. Hence either increased affinity or increased sensitivity of BrdUrd-substituted DNA to bleomycin may account for our findings. A prerequisite for almost all known effects of BrdUrd on cell function is its incorporation into DNA (1). Subsequent modification of RNA and protein synthesis may then occur (1). Hence other possible mechanisms of BrdUrd:bleomycin interaction include modification of uptake or cellular metabolism of bleomycin by BrdUrd. Our studies have not addressed these issues.

The observation that synergistic cytotoxicity and SSB enhancement varies with different BrdUrd:bleomycin concentration ratios suggests that an ideal concentration ratio may exist that provides optimal synergism. Similar observations have been made by others in studies of drug interactions (14). Our studies suggest that this ideal ratio is in the range of 2:1 [BrdUrd (µM):bleomycin (µunits/ml)] for method 1, and 5:1 for method 2.

In studying the effects of combination BrdUrd and bleomycin on DSB formation we did not find a substantial enhancement. This was an unexpected finding since DSB are often numerically related to SSB and are generally felt to be the cytotoxic lesions (18, 20, 25, 26). The neutral elution technique for evaluating DSB may be too crude to detect changes in DSB frequencies which could account for the observed synergism, especially since drug concentrations 5–10 times greater than those used in cytotoxicity experiments are necessary to allow DSB detection. Alternatively, SSB rather than DSB may account for the synergistic cytotoxicity.

Although we have shown that BrdUrd enhances the effect of bleomycin on DNA in human tumor cells, the mechanisms of interaction of these agents require further exploration. Analysis of the extent of thymidine replacement by BrdUrd in this system may allow further insights into the mechanism of BrdUrd:bleomycin interaction as well as possibly elucidating an optimal extent of replacement to achieve maximal synergism. Russo et al. (11) showed that BrdUrd or iododeoxyuridine pretreatment (to achieve 16% thymidine replacement) enhanced the cytotoxicity of melphalan, doxorubicin, cisplatin, and neocarzinostatin in Chinese hamster V79 cells. These four agents all interact with cellular DNA causing various nucleotide alterations including strand breaks and cross-links. The mechanism of enhancement was not elucidated in this study, and the study design did not exclude a direct cytoplasmic interaction of the two agents. However, the observation that BrdUrd enhances the cytotoxicity of a number of unrelated agents whose common cellular target is DNA also suggests that the interaction of these agents with BrdUrd-substituted DNA may be the cause of the synergistic cytotoxicity. In addition, this study (11) along with the data presented here indicates that radiosensitizers may enhance the cytotoxic effects of chemotherapeutic agents as well as radiation and suggests that other radiosensitizers might be explored for potential synergism with chemotherapeutic agents. Finally, these experimental data provide a basis for considering clinical evaluation of the combination of BrdUrd and bleomycin, perhaps primarily in bleomycin-sensitive tumors such as head and neck carcinomas. The drug concentrations used in this study are clinically achievable with acceptable toxicity (27, 28), but preliminary animal studies would be necessary to define the normal tissue toxicity of the combination.

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