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CYTOSTATIC SYNERGISM BETWEEN BROMODEOXYURIDINE, BLEOMYCIN, CISPLATIN AND CHLORAMBUCIL DEMONSTRATED BY A SENSITIVE CELL KINETIC ASSAY

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Abstract—Bromodeoxyuridine/Hoechst flow cytometry was used to analyse the interference of common cytostatic agents with cell activation and cell cycle progression of human B-cell lines. Bleomycin impaired both cell activation and G2 transit, the latter effect being oxygen dependent. The DNA alkylating agents cyclophosphamide, chlorambucil and mitomycin C caused G2 arrest, whereas cisplatin arrested cells in both the S and G2 phase of the cell cycle. Vinblastin interfered with mitosis, but in addition arrested cells in all phases of the cell cycle. The growth inhibitory action of bleomycin, cisplatin and chlorambucil was dependent upon the bromodeoxyuridine (BrdU) concentration in the culture medium. No interaction was found between BrdU and cyclophosphamide, mitomycin C and vinblastin. The cell cycle kinetic mechanism of the interaction between BrdU and bleomycin, cisplatin and chlorambucil was a potentiation of the G2 arrest. In conclusion, BrdU may be useful in clinical chemotherapy as a chemosensitizer for selected cytostatic agents.

DNA staining and subsequent flow cytometric analysis of a cell suspension allows cells to be resolved into the G1, S and G2/M compartments of the cell cycle. This is a powerful tool for elucidating the cell kinetic mechanism of putative cytostatic agents [1–4]. However, this approach only enables analysis of how cells traverse a single cell cycle. No information can be obtained as to the fraction of cells irreversibly arrested in a cell cycle compartment, nor can the pattern of traverse through two or more cycles be resolved.

Bromodeoxyuridine (BrdU†) labeling and subsequent staining with the Hoechst-33258 DNA dye overcomes this limitation [5–7]. This method has been successfully applied to assess the effect of 4-hydroxynonenal and cumene hydroperoxide upon the minimal and mean cell cycle compartment duration, and to determine the fraction of cells irreversibly arrested in each cell cycle phase [8]. However, BrdU is not an inert base analog. BrdU incorporation into the DNA enhances cellular susceptibility to irradiation [9, 10]; likewise interactions between BrdU and elevated oxygen concentration [11], the superoxide generator paraquat [12] and bleomycin [13, 14] have been reported. Here we evaluate cell cycle disturbances induced by six common cytostatic agents and define their interaction, if any, with BrdU incorporated into the cellular DNA.

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† Abbreviations used: BrdU, bromodeoxyuridine; BM, bleomycin; MMC, mitomycin C; CDDP, *cis*-dichlorodiaminoplatinum (II); CP, cyclophosphamide; CA, chlorambucil; VB, vinblastin; FBS, foetal bovine serum.

MATERIALS AND METHODS

Drugs. Bleomycin sulfate (BM), mitomycin C (MMC), vinblastin (VB), BrdU, deoxycytidine, Hoechst 33258 dye and ethidium bromide were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Cyclophosphamide (CP), chlorambucil (CA) and *cis*-dichlorodiaminoplatinum(II) (CDDP) were from Aldrich Chemie (Steinheim, F.R.G.). All other reagents were from Merck (Darmstadt, F.R.G.) at the highest purity obtainable.

Cells and culture. Epstein-Barr virus transformed human B (EBL) cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% of pretested and heat-inactivated foetal bovine serum (FBS) (Gibco, Karlsruhe, F.R.G.). After 4 days of culture without medium replenishment cells are synchronized in the G1 phase of the cell cycle [7]. Experiments were initiated by resuspending EBL cells in fresh culture medium containing 10% FBS at a density of 5×10^4 cells per mL. Each culture was supplemented with 50–250 μM of BrdU and deoxycytidine, and the drugs to be analysed were added to the final concentrations indicated below. Cells were cultured at 37° in humidified incubators equipped with sensors regulating carbon dioxide and oxygen concentration in the atmosphere such that 5% CO_2 and 5% O_2 (v/v) were obtained. Air was replaced by adding CO_2 and N_2 from pressurized gas flasks. All culture flasks were carefully wrapped in aluminium foil to avoid exposure to light of short wavelengths. After 62 hr of culture, cells were harvested under illumination with red light only. Until analysis, cells were stored in the dark at −20° in culture medium supplemented with 10% FBS and 10% dimethylsulfoxide.

Cell staining and flow cytometry. After thawing,

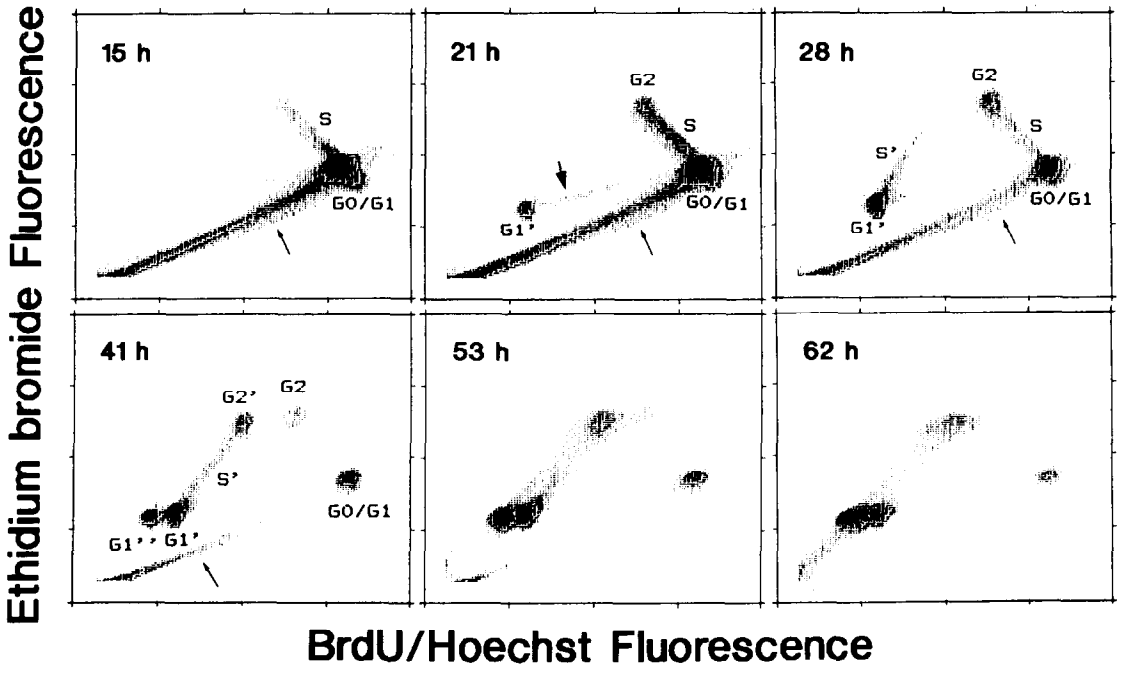

Fig. 1. Bivariate Hoechst/ethidium bromide cytogram of EBL cultures continuously labeled with 100 μM BrdU for the indicated time intervals. The abscissa displays BrdU-quenched Hoechst fluorescence, while the ordinate shows un-quenched ethidium bromide fluorescence. The thin arrow indicates a trail of cells with disintegrating chromatin. The thick arrow in the 21 hr panel depicts a trail of cells, which incorporated BrdU during part of their first S phase.

cell pellets were resuspended in a staining buffer containing 1.2 μg of Hoechst 33258 and 2.0 μg of ethidium bromide per mL of buffer [7]. Flow cytometric analysis was performed with an epi-illumination system of conventional design (ICP 22, Ortho Diagnostic Systems, Raritan, NJ, U.S.A.) equipped with a mercury arc lamp (HBO 100, Osram, Berlin, F.R.G.). Bivariate cytograms of Hoechst and ethidium bromide fluorescence were digitalized and recorded with a PDP 11/23 microcomputer (Digital Equipment Corporation, Maynard, MA, U.S.A.). Cells belonging to three successive cell cycles can be distinguished due to BrdU quenching of Hoechst fluorescence [5–7]. By electronic selection of the signal clusters representing each cell cycle, rotation and deconvolution, conventional cell cycle distributions were obtained, which were fitted with a standard cell cycle algorithm (Phoenix Flow Systems, San Diego, CA, U.S.A.). The cell numbers in each cell cycle were normalized to the percentage of original cells by correcting for the number of cell divisions by which cells originated.

RESULTS

Continuous exposure of a cell culture to BrdU and sequential analysis with bivariate Hoechst/ethidium bromide flow cytometry allows to delineate cell activation and cell cycle transit. Figure 1 shows bivariate cytograms obtained from a quiescent culture of EBL cells during an observation interval of 15 to 62 hr after growth stimulation. The abscissa displays BrdU-quenched Hoechst fluorescence (blue), while the ordinate shows unquenched ethidium bromide fluorescence (red). Each dot represents an individual cell; clusters arise where cells with similar fluorescence intensity concur. After 15 hr of growth stimulation in the presence of 100 μM BrdU most cells are still in the resting state (labeled as G0/G1). Some cells initiated BrdU incorporation into their DNA during S phase. Due to BrdU quenching of Hoechst fluorescence the trail of S phase cells moves to the left from the G0/G1 cluster. Since ethidium bromide fluorescence remains stoichiometric with cellular DNA content the S phase trail moves also upwards from G0/G1. The trail connecting the origin of the cytogram with the G0/G1 cluster indicated by the thin arrow may represent cells with disintegrating chromatin. The cytogram obtained after 21 hr of growth stimulation exhibits more cells in the S phase trail and a second cluster, which represents the G2 compartment of the first cell cycle. Some cells traversed mitosis and attained the G1 compartment of the second cycle (depicted as G1'). Since these G1' cells contain half the amount of DNA of G2 cells, their fluorescence intensity on both the Hoechst and ethidium bromide axis is halved. The short, thick arrow indicates a thin signal trail connecting the G0/G1 and the G1' clusters, which represents cells that incorporated BrdU only during part of their S phase. A second round of BrdU incorporation is initiated after 28 hr of growth stimulation. As a result, an S phase trail leaves the G1' cluster. Due to bifilary substitution

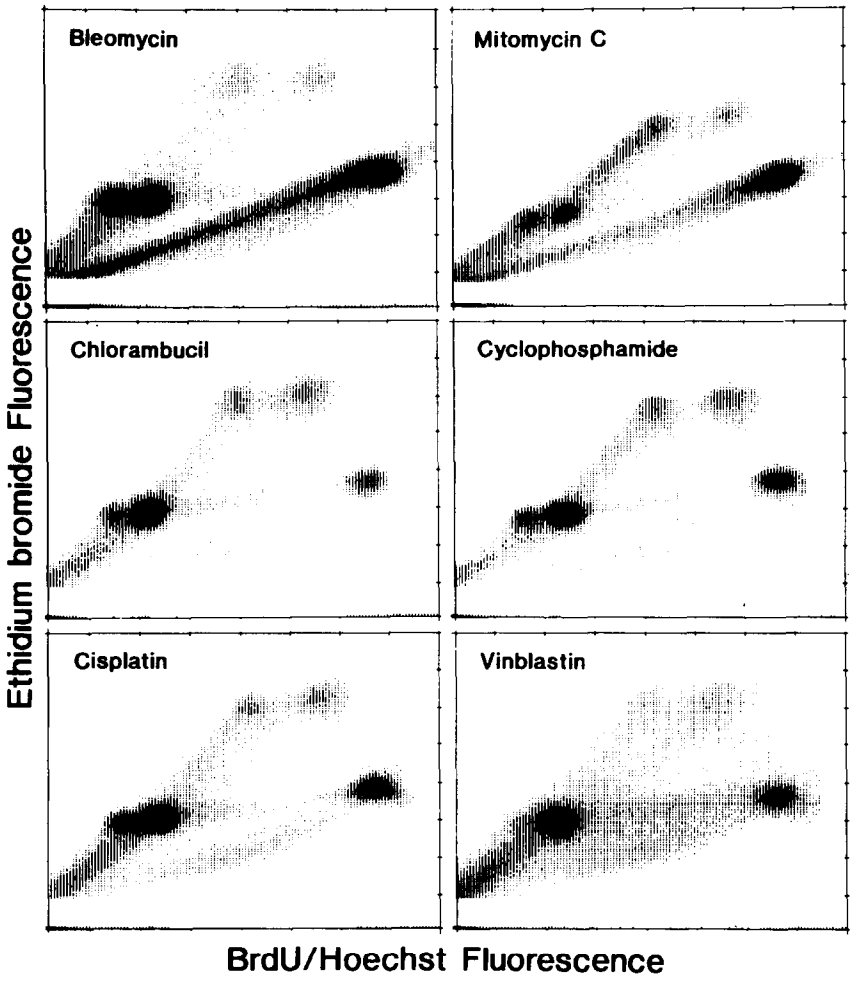

Fig. 2. Bivariate Hoechst/ethidium bromide cytogram of EBL cultures exposed during 62 hr to 2 mUnits BM per mL of culture medium, 10 ng/mL MMC, 5 μM CA, 2 mM CP, 0.5 μM CDDP or 5 nM VB. Note the difference in cell number in the G0/G1 and G2 clusters and in the trail of cells with disintegrating chromatin. For explanation see legend to Fig. 1.

of the DNA, the extent of Hoechst quenching is reduced and the S′ trail moves to the right rather than to the left. Hoechst fluorescence is diminished to a minor extent by this second round of BrdU incorporation. Therefore, the second G2 cluster (G2′) arrives left from the first G2 cluster in the 41 hr cytogram. In the 53 and 62 hr cytograms the image further develops as cells leave the first cell cycle and increasingly occupy the second and third cycle. The signal trail of cells with disintegrating chromatin progressively diminishes with culture time since these cells get diluted out by the dividing cell population. At 62 hr of growth stimulation, a fully developed cell cycle image is obtained: few cells are retained in the original G0/G1 cluster, and the first cycle S and G2 compartment are nearly devoid of cells. From this sequence of cytograms it is concluded that 62 hr represents an optimal observation period to analyse cytostatic action of drugs.

Figure 2 depicts bivariate cytograms obtained from quiescent cultures activated to grow in the presence of 100 μM BrdU and exposed to the six cytostatic agents tested at concentrations causing 50% growth inhibition. BM provokes 50% growth inhibition by increasing the fraction of cells retained in the G0/G1 cluster and to a minor extent by arresting those arriving in the G2 cluster of the first cycle. A signal trail from the G0/G1 cluster to the origin of the cytogram (which represents cells with disintegrating chromatin) is conspicuous in the BM cytogram. The DNA alkylating agents CA and CP cause an increase of cells in the first G2 phase, whereas MMC and CDDP exposed cells accumulate in both the G0/G1 and G2 compartment of the first cell cycle. VB elicited cell accumulation in all phases of the first cell cycle.

In Fig. 3 dose–effect profiles for all six cytostatic agents are depicted. The percentages of cells in the G0/G1, S and G2 compartment of the first cycle are added up at each concentration tested. Thus, an increasing distance between successive curves denotes an accumulation of cells in that particular

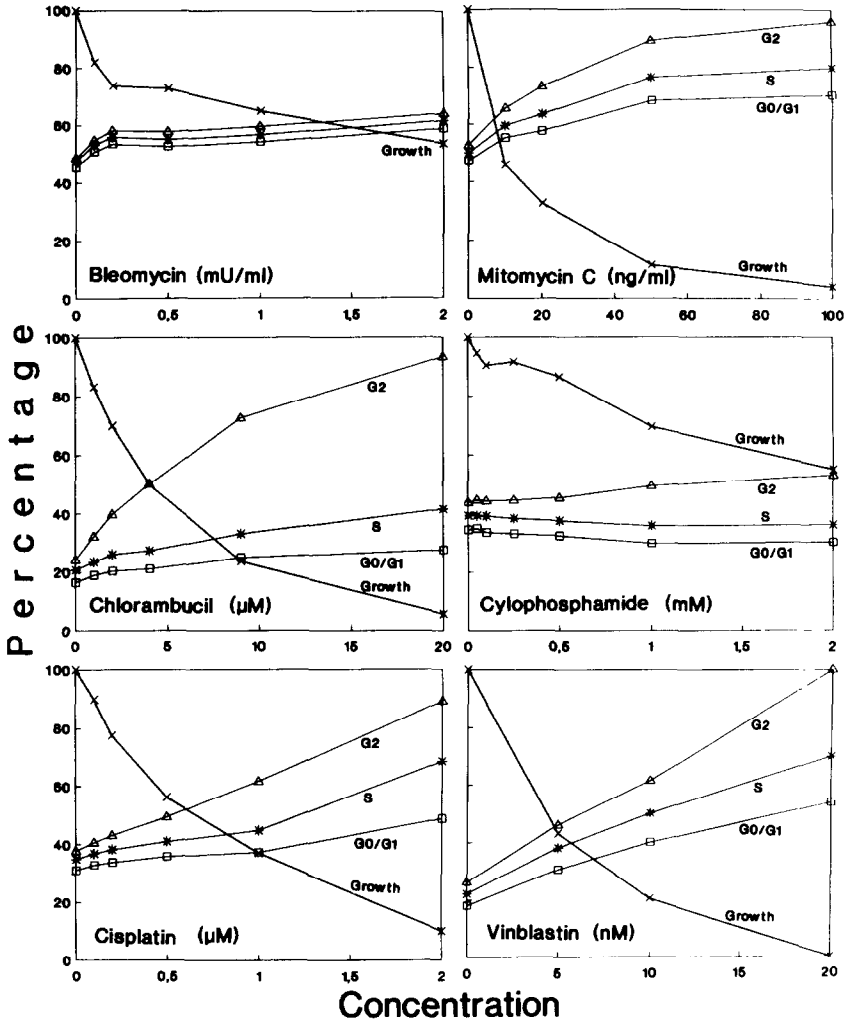

Fig. 3. Dose–effect profiles of BM, MMC, CA, CP, CDDP and VB at 100 μM BrdU. The ordinate displays relative increase in cell number (growth). Increasing distance between two successive curves denotes dose dependent accumulation of cells in a particular cell cycle compartment. All values are the mean of a typical experiment performed in triplicate. Standard deviations did not exceed the size of the symbols.

compartment, whereas a constant distance represents no accumulation of cells with increasing dose. The total number of cells cannot exceed 100%, which puts a limit to the value obtained by the curve denoting the sum of the G0/G1, the S and the G2 compartment. In keeping with the initial inspection of the cytograms, we observed retention of cells in the G0/G1 and to a minor extent in the G2 compartment of the first cycle with increasing doses of BM. At 20% (v/v) oxygen, as opposed to our standard 5% oxygen system, BM causes a much stronger growth inhibition (ID_{50} of 0.4 mUnits/mL vs 2.1 mUnits/mL at 5% oxygen). This oxygen enhancement of growth inhibition is mainly due to an increased accumulation of cells in the G2 phase (result not shown). The DNA alkylating agents MMC, CP and CA caused a dose dependent increase of cells in the G2 compartment, whereas CDDP exposed cells accumulate in the G0/G1, S and G2 phases of the cell cycle. The spindle poison VB elicited a dose dependent cell accumulation in all compartments of the cell cycle. The DNA alkylating agents CP and CA show a mild oxygen dependency of growth inhibition, whereas CDDP and VB elicited no difference in cell cycle disturbance at 20% vs 5% oxygen (v/v). MMC elicited a slightly stronger growth inhibition at 5% vs 20% (v/v) oxygen concentration (results not shown).

To investigate a putative interaction between BrdU and the six cytostatic agents, we varied both the concentration of BrdU and the cytostatic agent and calculated which combinations cause 50% growth inhibition. Figure 4 displays the iso-effect curves thus derived. BM, CA and CDDP show hollow iso-effect curves with BrdU, indicative of a synergism [15]. In contrast, MMC, CP and VB exhibit no interaction with BrdU, as their iso-effect curves are straight lines.

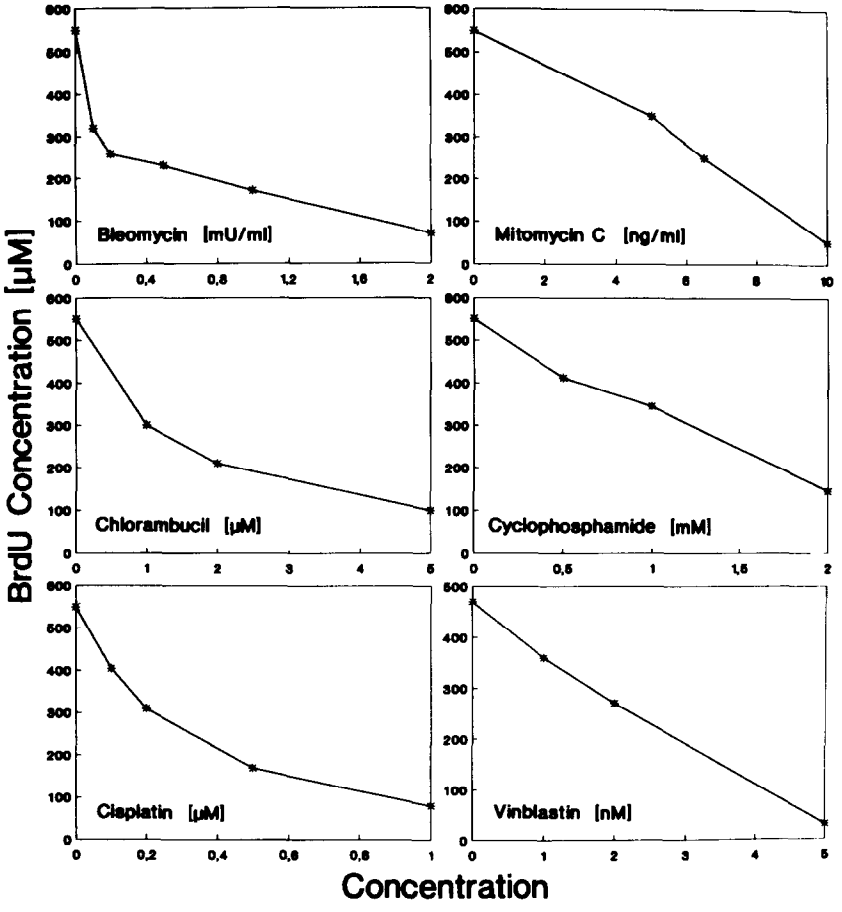

Fig. 4. Iso-effect curves of BM, MMC, CA, CP, CDDP and VB with BrdU. The curves represent the 50% growth inhibition level.

Since the interaction between the superoxide generator paraquat and BrdU was most distinctive at the level of the G2 compartment [12], we determined the extent of G2 arrest as a function of BrdU concentration with all six cytostatic agents at drug concentrations provoking 20 to 50% growth inhibition. Figure 5 displays the fraction of cells in the G2 phase of the first cycle as a function of BrdU concentration in a control culture and in cultures exposed to two concentrations of a given cytostatic agent. Bleomycin, at 0.2 mUnits per mL culture medium, causes an increase in G2 arrest from 2.1% at 50 µM BrdU to 5.4% at 250 µM BrdU. CA shows a BrdU-dependent G2 arrest at both concentrations tested, whereas CP caused a BrdU-dependent G2 arrest at 1 mM only (Fig. 5). Also, MMC and CDDP elicit a BrdU-dependent G2 arrest at the two concentrations tested. VB, not showing any interaction with BrdU at the level of the iso-effect curve (Fig. 4), exhibited a G2 arrest regardless of BrdU concentration in the medium (Fig. 5).

DISCUSSION

Our results show that BrdU labeling followed by bivariate Hoechst/ethidium bromide flow cytometry affords valuable information as to the cell kinetic mechanism of cytostatic agents. This method allows, for the first time, to assess both exit of cells from the G0/G1 compartment and progression of cells through three successive cell cycles. In keeping with literature data [3, 4, 16, 17] we observed a G2 arrest with CA, CP, MMC and CDDP; the latter agents also caused, in addition to a G2 arrest, some S phase arrest. BM strongly affects exit from the quiescent state (G0/G1), but also elicits some G2 arrest.

Our control experiment aiming at excluding BrdU interference with the cell kinetic effects of the cytostatic agents failed to do so in the case of BM, CDDP and CA. It stands to reason that BrdU, being a clinical radiosensitizing agent [9, 18], may also enhance the sensitivity of DNA to free radical attack. A thymidinyl-like species has been detected in BrdU substituted DNA after irradiation [19, 20]. Both elevated oxygen concentration and paraquat show synergism with BrdU [11, 12]. BM, which is believed to act via a free radical attack on the DNA [21], shows elevated DNA strand breakage in BrdU substituted DNA [14]. This may account for the synergism between BrdU and BM that we observe at the cell kinetic level in the present study (Fig. 5).

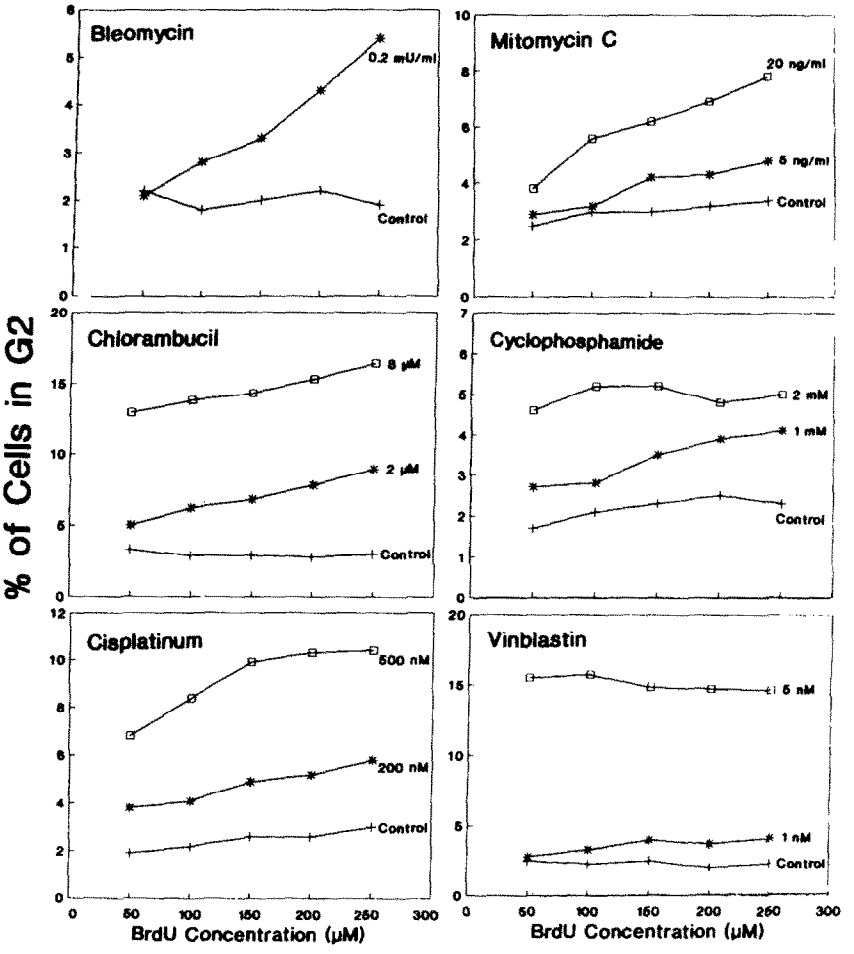

Fig. 5. BrdU-dependency of G2 arrest induced by BM, MMC, CA, CP, CDDP and VB. The bottom curve denotes the G2 arrest of a control culture, the middle curve represents a culture exposed to a drug concentration able to inhibit growth for 20 to 25% and the top curve shows a culture exposed to the concentration of drug provoking 50% growth inhibition. All values are the mean of a typical experiment performed in triplicate. Standard deviations did not exceed the size of the symbols.

The synergism between BrdU and CDDP or CA is in agreement with the reported enhancement of melphalan and CDDP toxicity by iododeoxyuridine incorporation into the DNA of V-79 cells [22]. This synergism cannot readily be explained by invoking a free radical mechanism. The acid test for involvement of free radicals is sensitivity of toxicity to oxygen. BM exhibits oxygen enhanced toxicity, whereas CDDP exerts an oxygen neutral effect [23]. The mild oxygen dependencies we found do not support a direct free radical mechanism, but may suggest an indirect involvement of oxygen free radicals. Both CDDP and CA are strong electrophiles, which may deplete cellular glutathione levels [24]. As a result the cell is devoid of its major defence against free radicals and lipid peroxidation may ensue. CDDP has indeed been reported to provoke lipid peroxidation [25, 26], but it is unclear whether this relates to the apparently lethal advent of DNA double strand breaks in CDDP treated L1210 cells [17]. Another possibility may be that during activation of CDDP and CA a strongly oxidizing species is being formed. This species may then preferentially attack the electron-rich BrdU moiety in the DNA. Further experimentation is needed to elucidate mechanistic aspects of the synergism between halogenated deoxyuridine and CDDP or CA.

MMC provoked a growth inhibition which was inversely related to oxygen concentration, as well as a BrdU dependent G2 arrest, but no synergism with BrdU. The chemical mechanism of MMC may involve reductive activation of its quinone function and subsequent redox cycling with molecular oxygen [27–30]. The sequinone species thus generated may exhibit addition chemistry to the DNA [28–30], but also the azaridine group is available for DNA adduction [27]. Our data do not allow to distinguish between these possibilities, but the inverse sensitivity to oxygen [23] supports reductive activation as the prime step in MMC toxicity.

In conclusion, BrdU incorporation into DNA

enhances cellular sensitivity to BM, CDDP and CA. Regardless of its mechanism, this result may render BrdU useful as a clinical chemosensitizer.

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