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Synergistic Interactions of Ethidium Bromide and Bleomycin On Cellular DNA and Growth Inhibition

Michael J. Agostino, Ralph J. Bernacki and Terry A. Beerman*

Grace Cancer Drug Center, Roswell Park Memorial Institute,
New York State Department of Health,
666 Elm Street, Buffalo, NY 14263

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Bleomycin is an anti-tumor agent whose cytotoxicity is related to the introduction of both single-stranded and double-stranded breaks in cellular DNA. In an assay using isolated nuclei, low levels of ethidium bromide substantially increased bleomycin induced release of nuclear chromatin. Treatment of mouse L1210 leukemia cells in vitro with low levels of ethidium bromide followed 1 hr later by bleomycin produced a synergistic effect that was 8 fold greater than that expected from the additive cytotoxicity of each drug alone. Interestingly, when the order of drug addition was reversed the drug synergism was much reduced (2 fold). The combination of DNA unwinding and strand scission agents may represent a novel and rational approach to the chemotherapy of cancer.

Work in this laboratory and others suggests that the binding of intercalating drugs to chromatin causes a partial unwinding of nucleosomal structure. Evidence for this is generated from studies using the enzymatic probe micrococcal nuclease which is normally restricted to attacking the linker region of nucleosomes. Incubation of chromatin with ethidium bromide, a classical intercalating agent, renders the core DNA of nucleosomes susceptible to micrococcal nuclease attack, reflecting the unwinding of DNA away from the histone octomer (1,2,3). Studies from our laboratory show that the anthracycline intercalation drugs adriamycin and daunorubicin have an even stronger tendency to enhance micrococcal digestion of both isolated and nuclear chromatin (3).

Bleomycin, an anti-tumor agent, introduces both single-stranded and double-stranded breaks in cellular DNA (4). Like micrococcal nuclease, studies

*To whom correspondence should be addressed.

Abbreviations used: HEPES, N-2-hydroxyethyl- piperazine-N'-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetra-acetic acid, disodium salt; ID_{50}, concentration of drug which inhibits cell growth by 50%.

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have shown that bleomycin preferentially cleaves chromatin in the linker region of the nucleosome repeat unit (5,6). We then reasoned that chromatin that is preincubated with ethidium bromide will be made more susceptible to bleomycin digestion. To test this hypothesis, drug combinations were examined in a molecular assay in which we measured bleomycin activity on isolated nuclei preincubated with ethidium bromide. Ethidium bromide caused a substantial increase in bleomycin induced release of nuclear chromatin. Furthermore, this drug combination was tested in a cytotoxicity assay by the sequential addition of both agents to mouse L1210 leukemia cells. When the cells were pretreated with ethidium bromide and then incubated with bleomycin, the cytotoxicity was measured to be much greater than that expected of simple additive behavior. However, reversing the order of drug addition resulted in only a slight increase in the cytotoxic index.

Materials and Methods

Cells and Nuclear Assay:

Mouse L1210 leukemia cells were grown in suspension cultures in 1640 media (10% fetal calf serum, 20 mM Hepes pH 7.3), and metabolically labeled overnight with [^{14}C]thymidine (.005μCi/ml). Nuclei were isolated from these cells as previously described (7). The standard assay for measuring the effects of ethidium bromide on nuclear chromatin degradation by bleomycin is as follows. All manipulations are performed in subdued light, and all incubations take place in the dark. 10 μg of nuclei (determined from OD_{260} in 0.1M NaOH after the RNA content is accounted for) in 10 mM Tris pH 7.8, 0.2 mM $CaCl_2$, are incubated at the appropriate ratio of ethidium bromide (Sigma). After 10 minutes at 37°C, the reaction mixtures are placed in ice water. Preincubation with intercalation drugs for time periods longer than 10 min did not alter the results of the experiments (unpublished results). Dithiothreitol (0.5 mM final concentration), bleomycin (obtained from Bristol, freshly diluted in water, and at final concentrations ranging from 0.05-0.4 μg/ml), and ferrous ammonium sulfate (0.2 mM final concentration, from freshly prepared solutions) are added in rapid succession, for a final volume of 100μl. The digestions take place at 37°C for 15 minutes. The reaction is stopped by the addition of EDTA to a final concentration of 0.5mM and the samples were placed on ice for 10 min, then centrifuged in an Eppendorf Microfuge for 5 min. A 50μl aliquot of the supernatant was counted to determine the level of solubilized chromatin ([^{14}C]thymidine release).

Isobologram Analysis:

L1210 leukemia cells were suspended at $5x10^4$ cells/ml in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 20 mM HEPES buffer, 50 μg/ml gentamycin and drug(s) to a final volume of 2 ml per tube. Ethidium bromide (at $1x10^{-8}$M, $5x10^{-7}$ M, $1x10^{-6}$ M, $2x10^{-6}$ M, $4x10^{-6}$ M and $8x10^{-6}$M) was added to duplicate cell cultures 1 hour preceding the addition of bleomycin. Following this 1 hour preincubation at 37°, bleomycin was added at $2.5x10^{-6}$ M, $5x10^{-6}$ M, $1x10^{-5}$ M, $2x10^{-5}$M, $4x10^{-5}$ M and $8x10^{-5}$ M.

The cell cultures were incubated for another 48 hours and cellular growth (inhibition) was assessed by counting cell number with a Coulter electronic cell counter. Initial cell inocula were subtracted from the final cell counts and the percentage of control (no drugs added) cellular growth was calculated. The growth inhibition data were analyzed and plotted using a Hewlett-Packard microcomputer and each data point in the isobologram represents the fraction of each agents' ID_{50} (concentration of drug which inhibited cell growth by 50%) which together resulted in 50% cell growth inhibition (8).

RESULTS

Micrococcal nuclease digestion of nuclear chromatin can be enhanced by the presence of intercalating agents (1,2,3). Possible ethidium bromide stimulation of bleomycin digestion of nuclear chromatin was assayed by measuring bleomycin activity over a wide range of ethidium bromide/DNA ratios. Bleomycin alone digested 23% of nuclear chromatin (Fig. 1). Extremely low levels of ethidium bromide (<3 ethidium bromide molecules/1000 DNA bases) have little or no effect on bleomycin induced cutting. However, increased levels of ethidium bromide (5-10 molecules/1000 DNA bases) caused a definite enhancement of bleomycin activity, reaching a peak stimulation of 26%

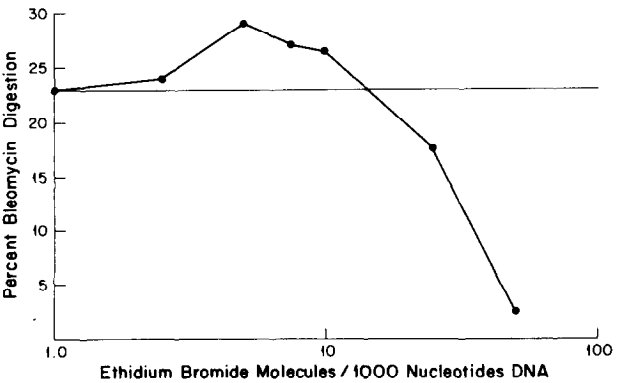

Figure 1 - The effects of Ethidium Bromide/DNA ratio on bleomycin digestion of nuclear chromatin. The horizontal line indicates 23% digestion, which is the level produced by bleomycin in the absence of ethidium bromide. These are the data points from a single experiment. The degree of stimulation and the ethidium bromide concentration for optimum digestion varied slightly in each experiment, but the pattern of ethidium bromide stimulation and inhibition was consistent. Ethidium bromide alone caused no more than 2% strand scission across the entire range of concentrations utilized (data not shown).

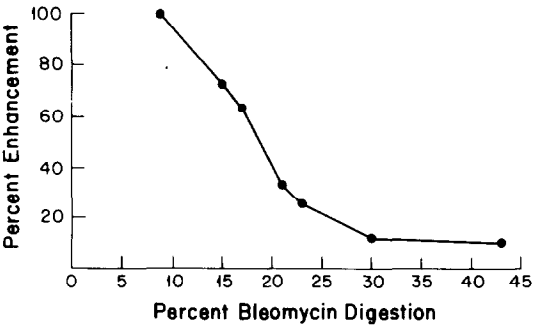

Figure 2 - Ethidium bromide enhancement varies with the level of bleomycin digestion. See the legend of figure 1 for materials and methods. Nuclei were incubated with the narrow stimulatory range of ethidium bromide concentrations (see figure 1) and digested with a wide range of bleomycin levels. Each data point represents the maximum digestion observed within an experiment of 5 different ethidium bromide/DNA base ratios and a single bleomycin level.

over bleomycin alone. Higher concentrations of ethidium bromide inhibited bleomycin activity.

We next studied the degree of ethidium bromide stimulation as the level of bleomycin digestion was varied. Nuclei were digested over a wide range of bleomycin levels and across the narrow stimulatory range of ethidium bromide (see Fig. 1). The degree of ethidium bromide stimulation is greatest at low levels of bleomycin digestion (Fig. 2). For example, bleomycin activity at 9% digestion is stimulated to 18% (100% enhancement) in the presence of ethidium bromide while higher levels of bleomycin (e.g., 21% digestion) are enhanced by only 30%. In each case peak stimulation occurs in the presence of only 5-10 ethidium bromide molecules/1000 DNA bases.

Since the cytotoxicity of bleomycin has been correlated with the introduction of single- and double-stranded DNA breaks and ethidium bromide can increase the strand scission activity of bleomycin, the sequential treatment of cells with ethidium bromide and bleomycin would perhaps lead to greater cytotoxicity. The ability of these two agents to inhibit L1210 cell growth was evaluated in a series of experiments in which the ID_{50} (concentration of agent capable of decreasing cell growth by 50%) of drug

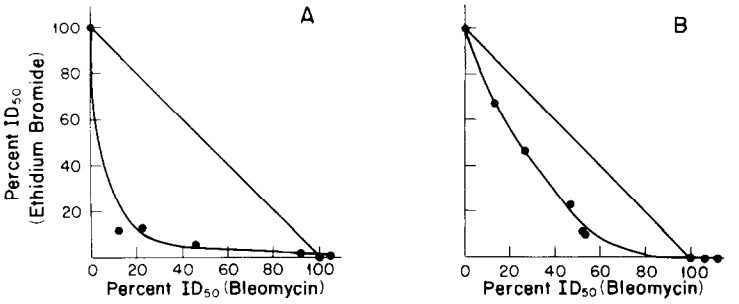

Figure 3 - Isobologram Analysis of Combinations of Ethidium Bromide and Bleomycin

(A) Ethidium bromide was added 1 hour prior to the addition of bleomycin. In this experiment the 100% ID_{50} for ethidium bromide alone was 1.5×10^{-6} M and the 100% ID_{50} for bleomycin alone was 2×10^{-5} M. Note the strong synergism of this sequential combination of agents

(B) Bleomycin was added 1 hour prior to the addition of ethidium bromide. The 100% ID_{50} for ethidium bromide was 4.0×10^{-6} M and the 100% ID_{50} for bleomycin was 1.9×10^{-5} M. Note that a reversal in the order of drug additions resulted in a reduction of the synergistic response. These experiments were performed on at least three separate occasions resulting in similar findings and a representative isobologram is presented above for each experimental condition.

combinations was determined. Isobologram analysis was employed to determine the type of interaction caused by the two drug combinations.

Mouse L1210 leukemia cells were exposed to ethidium bromide and bleomycin in the two orders of sequential treatment. Results of pretreatments of L1210 cells with ethidium bromide for 1 hour followed by bleomycin addition clearly indicated a strong synergistic inhibition of cell growth with these two drugs. The data points in the isobologram were well below the diagonal line that represents additive behavior and are therefore indicative of an extremely synergistic type of growth inhibition (Fig. 3A). The data also suggest that the more synergistic combinations for the two agents tend to be those where both drugs levels were low. This is consistent with the isolated nuclei experiments which indicated maximum enhancement of DNA strand scission events at reduced levels of both drugs.

The results of pretreatment of L1210 cells with bleomycin for 1 hour followed by ethidium bromide indicated that this drug regimen was synergistic as well, but substantially less than that observed with the ethidium bromide pretreatment. Simultaneous addition of ethidium bromide and bleomycin also resulted in a decreased synergistic effect (data not shown).

Discussion

Present chemotherapeutic approaches in the treatment of cancer include the combination of two or more drugs, administered sequentially or simultaneously, depending on established regimens that are often arrived at empirically. Work in this department has shown that, in vitro, the vast majority of drug combinations are pharmacologically antagonistic while few combinations are strongly synergistic (9). Drug combinations are usually composed of agents whose biochemical targets differ. Our observations demonstrate that the activity of a DNA interactive agent such as a strand scission drug can be enhanced by the presence of a second DNA interactive drug that modulates the structure of cellular DNA.

A model for this approach is the interaction of two well characterized DNA reactive drugs. Ethidium bromide, a classical unwinding agent, is a planar molecule that intercalates between the DNA bases causing the helix to unwind (10). Bleomycin is a strand scission agent that causes both single- and double-stranded breaks in the linker region of chromatin. The unwinding action of ethidium bromide would likely cause chromatin to assume a configuration that renders the DNA more susceptible to attack by bleomycin. As shown in Fig. 1, ethidium bromide both enhanced and inhibited the quantity of strand scission events caused by bleomycin, depending on the ethidium bromide/DNA ratio. Inhibition was observed at high ratios of ethidium bromide, most probably due to steric interference with the action of bleomycin. But at lower levels of intercalator, approximately 5 to 10 molecules/1000 bases of DNA, enhancement of bleomycin scission was observed. It is difficult to imagine why as few as 5 molecules/1000 bp of DNA could increase the amount of bleomycin cutting by as much as 100% (Fig. 2) unless

ethidium bromide and bleomycin are acting on common regions of chromatin. Localized binding of ethidium bromide could cause limited areas of extensive unwinding of the DNA helix creating DNA sites that are preferentially digested by bleomycin.

The enhancement effect of ethidium bromide as a function of the level of bleomycin digestion suggests that ethidium bromide is unable to stimulate high levels of bleomycin digestion to the same extent as that seen with low levels of digestion.(e.g. stimulation of 12% vs 100%, respectively). Although several interpretations are possible, the simplest explanation is that ethidium bromide renders a domain of chromatin preferentially sensitive to bleomycin attack, and that this domain is progressively digested as bleomycin levels increase. At low levels of bleomycin, much of this chromatin fraction is digested via ethidium bromide mediated unwinding, and maximum stimulation is seen. However, at higher levels of bleomycin digestion, little of this fraction remains, and significant stimulation is not possible. Judging by the data in figure 2, this domain is approximately 10% of total nuclear chromatin (i.e. 10% is the maximum absolute amount of chromatin then can be made sensitive to bleomycin digestion). Efforts are now underway to determine the nature of this chromatin fraction.

The isobologram data clearly show a synergistic growth inhibition relationship between ethidium bromide and bleomycin. Bleomycin's cytotoxicity is directly correlated with the introduction of single- and double-strand breaks to DNA (4). The synergistic cytotoxicity seen when cells are incubated with both agents is likely due to the unwinding properties of ethidium bromide causing chromatin to become more susceptible to the strand scission activity of bleomycin. The nuclear assay demonstrates that ethidium bromide does increase the bleomycin induced release of soluble chromatin. Ethidium bromide pretreatment of cells (Fig. 3A) at low concentrations of ethidium bromide (12% of the ethidium bromide ID_{50}) resulted in nearly an 8 fold synergistic cytoxicity. What should also be considered is the possibility that ethidium bromide is interfering with the repair of bleomycin induced damage to DNA

(11). This may account for the results appearing in Figure 3B where bleomycin pretreatment of cells resulted in synergism albeit at a lower level (2 fold).

Thus, we believe the 8 fold synergism seen with ethidium bromide pretreatment is predominantly a result of the altered bleomycin scission activity. Decreased repair of DNA damage may also be contributing to the cytotoxicity. In addition, bleomycin damage to DNA may be qualitatively different in the presence of the intercalating agent. There have been numerous studies using naked DNA in which ethidium bromide and other agents have been shown to alter the base recognition sequence of strand scission drugs (12,13). We are currently evaluating this possibility using nuclear chromatin.

Research of the interaction between DNA unwinding agents and strand scission drugs may well lead to new regimens of cancer treatment that are based upon drug interactions at the molecular level with cellular DNA. Results with other DNA unwinding agents look very promising as we have found other examples of synergistic relationships with bleomycin (unpublished data).

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