

Structure–Activity Analysis of the Potentiation by Aminothiols of the Chromosome-Damaging Effect of Bleomycin in G₀ Human Lymphocytes

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The radioprotective aminothiols 2-[(aminopropyl)amino] ethanethiol (WR-1065) and cysteamine (CSM) potentiate the induction of chromosomal damage by the radiomimetic compound bleomycin (BLM) in G₀ human lymphocytes. To investigate the mechanism of potentiation, we measured the clastogenic activity of BLM in the cytokinesis-block micronucleus assay in the presence and absence of amines, thiols, and aminothiols. The hydroxy analog of WR-1065, 2-(3-aminopropylamino) ethanol (WR-OH), potentiates BLM only slightly, indicating the critical nature of the thiol group. As thiols, WR-1065 and CSM may donate electrons for the activation of Fe⁺²-BLM or for the regeneration of Fe⁺²-BLM from inactive Fe⁺³-BLM. The amines putrescine, spermidine, and spermine all potentiate BLM, but they are weaker potentiators than the aminothiols, and they are effective only at high concentrations. Their activity, like that of WR-OH, is probably a consequence of conformational al-

teration of DNA. Dithioerythritol (DTE) and 2-mercaptoethanol (2-ME), thiols lacking an amino group, are less effective potentiators of BLM than are the aminothiols. The thiol group of WR-1065 and CSM is therefore essential, but insufficient, for explaining the strong enhancement of BLM activity. The cationic nature of CSM and WR-1065, conferred by the amino groups, evidently concentrates the active thiol function at the site of BLM action on DNA. As expected on this basis, the diamine WR-1065 is a more effective potentiator of BLM than is the monoamine CSM, whereas cysteine and N-acetylcysteine (NAC), which lack a net positive charge, potentiate BLM only weakly. These studies suggest that potentiation of the clastogenic action of BLM by aminothiols can be explained by the combination of a thiol-mediated redox mechanism and an amine-mediated targeting of the thiol function to DNA. Environ. Mol. Mutagen. 37: 117–127, 2001. © 2001 Wiley-Liss, Inc.

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INTRODUCTION

The drug bleomycin (BLM) is a mixture of closely related glycopeptide antibiotics, primarily bleomycins A₂ and B₂, isolated from *Streptomyces verticillus* [Povirk and Austin, 1991; Burger, 1998]. BLM has found use in the treatment of cancers of the head and neck, squamous cell carcinomas, testicular cancer, and some lymphomas [Hay et al., 1991; Stubbe et al., 1996]. The cytotoxicity of BLM depends on its induction of DNA damage [Hay et al., 1991; Stubbe et al., 1996; Burger, 1998]. BLM is genotoxic in assays for the induction of DNA damage, point mutations, recombination, chromosome aberrations, and micronuclei in diverse organisms, including bacteria and phage, fungi, *Drosophila*, and mammals [Vig and Lewis, 1978; Povirk and Austin, 1991; Hoffmann et al., 1993a, 1995; Povirk, 1996].

To cause DNA damage, BLM must associate with DNA and be activated [Povirk and Austin, 1991; Kane and Hecht, 1994; Povirk, 1996; Stubbe et al., 1996; Burger, 1998]. The

initial step is the binding of Fe⁺²-BLM to DNA by hydrophobic and ionic interactions mediated by its bithiazole moiety and C-terminus [Kane et al., 1994]. Activation occurs through addition of oxygen to Fe⁺²-BLM, followed by a one-electron reduction [Povirk, 1996; Stubbe et al., 1996; Burger, 1998], giving rise to a species called “activated bleomycin,” whose probable structure is a ferric hydroperoxide (HOO-Fe⁺³-BLM) [Stubbe et al., 1996; Burger, 1998]. The metal-binding portion of BLM, which determines the sequence selectivity of strand scission, seems to

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be oriented in the minor groove of DNA [Kane et al., 1994], where activated BLM specifically abstracts a hydrogen from the 4' position of deoxyribose, forming a free radical [Stubbe et al., 1996; Burger, 1998].

The addition of oxygen to the free radical at 4' gives rise to a peroxy radical whose decomposition causes the release of a base propenal and a strand break with 5'-phosphate and 3'-phosphoglycolate ends [Povirk and Austin, 1991; Stubbe et al., 1996; Burger, 1998; Charles and Povirk, 1998]. About 10% of the strand breaks induced by BLM are double-strand breaks, and they are induced with single-hit kinetics [Charles and Povirk, 1998]. After a single-strand break occurs at a primary cleavage site, the same molecule of BLM is apparently reactivated *in situ* and cleaves the complementary strand, resulting in a blunt-ended double-strand break [Steighner and Povirk, 1990; Stubbe et al., 1996; Charles and Povirk, 1998].

BLM is said to be radiomimetic because its induction of genetic damage resembles that of ionizing radiation. The primary features are S-phase independence and the induction of chromosome-type aberrations in G₀ cells [Povirk and Austin, 1991]. Treatment of G₀ lymphocytes with BLM causes dose-dependent increases in the frequency of chromosome aberrations [Dresp et al., 1978] and micronuclei [Hoffmann et al., 1993a].

Radioprotectors are compounds that reduce the biological effects of ionizing radiation, including lethality, mutagenicity, and carcinogenicity. WR-1065 is a member of a heavily studied group of radioprotectors, the aminothiols [Purdie, 1979; Purdie et al., 1983; Milas et al., 1984; Grdina et al., 1985, 1995; Fahey, 1988; Schwartz et al., 1988; Littlefield and Hoffmann, 1993; Littlefield et al., 1993]. WR-2721 (*S*-2-(3-aminopropylamino) ethylphosphorothioic acid) is a phosphorylated form of WR-1065 whose radioprotective action in mammals depends on its being dephosphorylated to the free thiol WR-1065 by membrane-bound alkaline phosphatase [Purdie et al., 1983; Smoluk et al., 1988a; Hospers et al., 1999]. WR-2721, also called amifostine, Ethylol, and ethiofos [Glover et al., 1988; Grdina et al., 1991; Kurbacher and Mallmann, 1998], has been used clinically to minimize damage to normal tissues in cancer radiotherapy [Glover et al., 1988; Hospers et al., 1999]. Protective effects with genotoxic chemicals led to the use of WR-2721 as a protective agent in cancer chemotherapy [Glover et al., 1988; Kurbacher and Mallmann, 1998; Hospers et al., 1999]. WR-2721 treatment also offers the prospect of reducing the risk of secondary tumors induced by radiation and chemotherapy [Grdina et al., 1991; Capizzi, 1999; Hospers et al., 1999].

Because BLM is a radiomimetic drug, WR-1065 might be expected to protect against its toxic, mutagenic, and clastogenic effects. This is the case in some systems [Nagy and Grdina, 1986; Hoffmann et al., 1995] but not in others. The difference among systems apparently depends on physiological conditions, most notably oxygen tensions [Hoff-

mann et al., 1995]. WR-1065 [Hoffmann et al., 1993b, 1994] and the related aminothiol radioprotector CSM [Hoffmann and Littlefield, 1995] both potentiate the induction of chromosomal damage by BLM in G₀ human lymphocytes. Several mechanisms have been proposed to explain this potentiation. As thiols, these compounds may provide electrons for the activation of Fe⁺²-BLM or for the regeneration of Fe⁺²-BLM from inactive Fe⁺³-BLM [Hoffmann et al., 1994]. As a diamine, WR-1065 binds to DNA [Smoluk et al., 1988a; Sy et al., 1999] and may alter DNA conformation so as to facilitate BLM action. Widening of the minor groove of DNA, which is the likely site of BLM binding [Strekowski et al., 1989; Kane et al., 1994; Stubbe et al., 1996; Burger, 1998] and the location of the 4' position of deoxyribose [Burger, 1998], may give BLM better access to its target.

In this study we explore the mechanism by which the aminothiols WR-1065 and CSM potentiate the clastogenicity of BLM in G₀ lymphocytes. To do so, we measured the effects of a series of amines and thiols on the activity of BLM in the cytokinesis-block micronucleus assay. Micronucleus assays use membrane-bound bodies containing chromosome fragments or whole chromosomes as an indicator of chromosomal damage or aneuploidy, respectively [Fenech and Morley, 1985; Heddle et al., 1991; Kirsch-Volders et al., 2000]. Aneuploidy is probably inconsequential as a contributor to micronuclei in this study since BLM is a known clastogen, treatments were restricted to cells in G₀, and WR-1065 was previously shown by metaphase analysis to potentiate the induction of chromosome-type aberrations by BLM under these conditions [Hoffmann et al., 1994]. In the cytokinesis block assay [Fenech and Morley, 1985, 1986; Fenech, 1993, 1997, 2000] cytochalasin B is used to inhibit cytoplasmic division, thereby making cells that have undergone a single nuclear division recognizable because they are binucleate. The technique avoids confusion that might otherwise result from differences in cellular proliferation kinetics. The compounds whose effects on the clastogenicity of BLM were evaluated are shown in Figure 1.

MATERIALS AND METHODS

Chemicals

Bleomycin (BLM; Chemical Abstracts Number [CAS] 11056-06-7; approximate molecular weight 1420) was obtained from Sigma Chemical (St. Louis, MO) as bleomycin sulfate (CAS 9041-93-4); vials of 15 units were dissolved in 2.2 ml sterile distilled water, and the solution (4 mg/ml) was frozen at -20°C in 250- μ l aliquots. WR-1065 (CAS 31098-42-7) was obtained from Ms. Nancita Lomax (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD). Cysteamine (CSM; 2-mercaptoethylamine hydrochloride; CAS 156-57-0), dithioerythritol (DTE; CAS 6892-68-8), 2-mercaptoethanol (2-ME; CAS 60-24-2), putrescine dihydrochloride (CAS 333-93-7), spermidine trihydrochloride (CAS 334-50-9), spermine tetrahydrochloride (CAS 306-67-2), L-cysteine hydrochloride monohydrate (CAS 7048-04-6), and *N*-acetylcysteine (NAC; CAS 616-

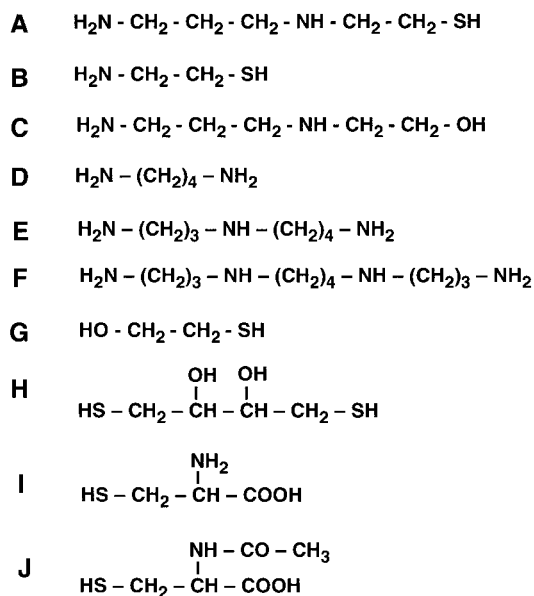


Fig. 1. The aminothiols WR-1065 (2-[(aminopropyl)amino] ethanethiol) (**A**) and cysteamine (**B**); the amines WR-OH (2-(3-aminopropylamino)-ethanol) (**C**), putrescine (**D**), spermidine (**E**), and spermine (**F**); the thiols 2-mercaptoethanol (**G**) and dithioerythritol (**H**); the sulfhydryl amino acid cysteine (**I**) and its amide derivative *N*-acetylcysteine (**J**).

91-1) were obtained from Sigma, and 2-(3-aminopropylamino)ethanol (WR-OH; CAS 4461-39-6) was obtained from Fluka Chemical (Buchs, Switzerland). All solutions except BLM were prepared fresh immediately before use.

Media

Lymphocytes were cultured in RPMI medium 1640 supplemented with 15% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and 2% phytohemagglutinin (PHA). The medium for washing cells was RPMI 1640 containing 10% FBS. PHA was from Murex Biotech (Dartford, UK); and penicillin-streptomycin, RPMI 1640, and FBS were from GIBCO (Grand Island, NY). Giemsa was from Biomedical Specialties (Santa Monica, CA).

Micronucleus Assay

The cytokinesis-block micronucleus assay was performed as described by Fenech and Morley [1985] with minor modifications as noted in Littlefield et al. [1989] and Hoffmann et al. [1993a,b]. Blood was collected from a healthy adult donor by venipuncture, and a lymphocyte-rich fraction (buffy coat) was prepared. Cells were pretreated with amines and thiols by mixing 1 ml buffy coat with 0.8 ml RPMI containing the chemical and 10% FBS. After 30 min at 37°C, treatment was initiated by adding BLM in 0.2 ml RPMI. Treatments were quenched after 2 hr at 37°C by adding 12 ml wash medium. The cells were washed three times and suspended in 10 ml RPMI culture medium. Cytochalasin B (6 $\mu\text{g}/\text{ml}$) was added to the cultures after 42 hr at 37°C, and the cells were harvested 24 hr later.

Preparation of Slides

Cultures were terminated after 66-hr incubation by adding 4 ml cold fixative (3:1, methanol:acetic acid) per flask. The cells were harvested by

centrifugation, washed in fixative, and dropped onto slides. Slides were stained for 12 min with 10% Giemsa.

Scoring of Slides

A proliferation index (P.I.) was calculated as an indicator of cytotoxicity based on the first 300 cells of good morphology observed in screening a slide: $\text{P.I.} = [(1 \times \text{the number of mononucleate lymphocytes}) + (2 \times \text{the number of binucleate lymphocytes}) + (3 \times \text{the number of lymphocytes with } \geq 3 \text{ nuclei})]/300 \text{ cells scored}$. Micronuclei were counted in binucleate cells with intact cytoplasm on coded slides. The micronuclei that we scored did not overlap the main nuclei or each other. They were appropriately stained and did not approach the main nuclei in size. Fisher's exact test was used to evaluate the significance of differences in numbers of cells containing micronuclei [Richardson et al., 1989; Scott et al., 1990].

RESULTS

G_0 lymphocytes were treated with BLM alone or in combination with WR-1065 or its nonthiol hydroxy analog WR-OH. The data in Table I show that WR-1065 strongly enhances the induction of micronuclei by BLM, whereas the effect of the concurrent treatment with WR-OH is slight. Rather than being devoid of an effect, however, there is a significant increase in the frequency of micronuclei at 10 mM WR-OH, the highest concentration that was sufficiently nontoxic to analyze. The result for WR-1065 is consistent with earlier studies showing strong potentiation of BLM [Hoffmann et al., 1994]. The data for WR-OH indicate weak potentiation of BLM by the hydroxy analog.

Other amines that lack a thiol group also potentiate BLM in the micronucleus assay. Table II shows potentiation of BLM by the diamine putrescine, and Tables III and IV show potentiation by the polyamines spermidine and spermine, respectively. These amines exhibit little toxicity to the lymphocytes and are effective potentiators of BLM at high concentrations (>40 mM). They have little or no effect on BLM activity at the relatively low concentrations (2.5 to 10 mM) at which WR-1065 strongly potentiates its activity. Putrescine and the polyamines therefore seem more similar in activity to WR-OH, although the toxicity of the latter precludes studying its effect at equally high concentrations.

The induction of micronuclei by BLM in the presence and absence of the thiol 2-ME, which is the non-amino-hydroxy analog of CSM, is shown in Table V. The data indicate weak potentiation of BLM by 2-ME. Data from concurrent treatments with CSM show that CSM enhances the induction of micronuclei by BLM more strongly than does 2-ME. The result for CSM is consistent with our earlier studies of this compound [Hoffmann and Littlefield, 1995]. While CSM potentiates BLM at modest concentrations (e.g., 10 mM), 2-ME caused significant increases in the frequency of BLM-induced micronuclei only at concentrations at least fivefold higher.

Table VI shows data for cotreatments with BLM and DTE, which is a dithio compound lacking an amino group. The toxicity of DTE to the lymphocytes limits the range of

TABLE I. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of WR-OH or WR-1065

BLM (μg/ml)	WR-OH (mM)	WR-1065 (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	0	1.81	17/2000	17	0.8
50	0	0	1.85	41/2000	46	2.3
0	0	5	1.77	27/2000	27	1.4
0	0	10	1.67	20/2000	22	1.1
50	0	2.5	1.48	106/2000***	149	7.4
50	0	5	1.47	358/2000***	545	27.2
50	0	10	1.19	64/240 ^a ***	109	45.4
50	2.5	0	1.79	42/2000	49	2.4
50	5	0	1.74	41/2000	49	2.4
50	10	0	1.39	70/2000**	88	4.4

^aFew binucleate cells in good condition at this toxic dosage.

**Significance of difference from BLM treatment without WR-OH or WR-1065: Fisher's exact test $P < 0.01$.

***Significance of difference from BLM treatment without WR-OH or WR-1065: Fisher's exact test $P < 0.001$.

TABLE II. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of Putrescine

Bleomycin (μg/ml)	Putrescine (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	1.40	14/1000	16	1.6
25	0	1.50	14/1000	15	1.5
25	5	1.50	16/1000	24	2.4
25	10	1.48	25/1000	36	3.6
25	20	1.46	34/1000**	35	3.5
25	40	1.43	38/1000**	48	4.8
25	80	1.40	118/1000***	163	16.3
25	160	1.37	92/1000***	118	11.8
0	0	1.62	8/1000	10	1.0
0	120	1.46	11/1000	11	1.1
75	0	1.54	27/1000	31	3.1
75	20	1.58	27/1000	33	3.3
75	40	1.47	39/1000	48	4.8
75	80	1.36	115/1000***	159	15.9
75	120	1.33	83/1000***	104	10.4

**Significance of difference from BLM treatment without putrescine: Fisher's exact test $P < 0.01$.

***Significance of difference from BLM treatment without putrescine: Fisher's exact test $P < 0.001$.

concentrations that can be analyzed, in that there were no binucleate lymphocytes at 7.5 mM. The data show potentiation of BLM at the highest testable DTE doses. Though statistically significant, the increases are small compared to those observed for WR-1065 and CSM.

Figure 2 summarizes the results on enhancement of the genetic activity of BLM in G₀ lymphocytes by 2-ME, putrescine, spermidine, and spermine, and it compares them to dose-response curves from earlier studies of WR-1065 [Hoffmann et al., 1994] and CSM [Hoffmann and Littlefield, 1995]. The comparison supports the interpretation that simple amines and thiols are not as effective potentiators of BLM as are aminothiols. Less extensive tests of WR-1065 and CSM in the current study support the same interpretation. However, not all aminothiols are as effective as WR-

1065 or CSM. The data in Table VII show minimal potentiation of BLM by the sulfhydryl amino acid cysteine and its amide derivative NAC.

DISCUSSION

Various agents affect the genetic activity of BLM, causing either potentiation of its effects or antimutagenesis. Mutagenic, clastogenic, and DNA-damaging effects of BLM were previously reported to be potentiated by acetaldehyde, 3-aminobenzamide, caffeine, ethanol, hydroxyurea, isoptin, hyperthermia [Povirk and Austin, 1991], β-carotene [Salvadori et al., 1994; Gentile et al., 1998], heterocyclic aromatic intercalating agents [Strekowski et al., 1991; Snyder, 1998], triethylenetetramine [An and Hsieh, 1992], and

TABLE III. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of Spermidine

Bleomycin (μg/ml)	Spermidine (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	1.85	13/1000	14	1.4
25	0	1.78	24/1000	29	2.9
25	40	1.71	17/1000	20	2.0
25	60	1.56	85/1000***	123	12.3
25	80	1.64	102/1000***	142	14.2
25	100	1.60	160/1000***	231	23.1
0	0	1.62	8/1000	10	1.0
75	0	1.54	27/1000	31	3.1
75	20	1.56	26/1000	34	3.4
75	40	1.45	32/1000	40	4.0
75	80	1.30	143/1000***	187	18.7
75	120	1.32	77/1000***	99	9.9
0	80	1.78	23/1000	26	2.6
0	100	1.70	14/1000	16	1.6

***Significance of difference from BLM treatment without spermidine: Fisher's exact test $P < 0.001$.

TABLE IV. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of Spermine

Bleomycin (μg/ml)	Spermine (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	1.65	9/1000	11	1.1
0	40	1.53	8/1000	8	0.8
0	80	1.58	10/1000	10	1.0
25	0	1.51	12/1000	14	1.4
25	20	1.50	25/1000*	30	3.0
25	40	1.50	69/1000***	94	9.4
25	60	1.40	122/1000***	199	19.9
25	80	1.33	156/1000***	225	22.5
75	0	1.54	27/1000	31	3.1
75	20	1.55	30/1000	32	3.2
75	40	1.43	58/1000***	71	7.1
75	80	1.19	152/1000***	198	19.8

*Significance of difference from BLM treatment without spermine: Fisher's exact test $P < 0.05$.

***Significance of difference from BLM treatment without spermine: Fisher's exact test $P < 0.001$.

hyperbaric oxygen [Cederberg and Ramel, 1989]. Agents reported to protect against the genotoxicity of BLM include ascorbic acid, chlorophyllin, galangin, 13-*cis*-retinoic acid, taurine [Gentile et al., 1998], butylated hydroxytoluene [Grillo and Dulout, 1997], the chelating agent EGTA, Zn⁺² [Tempel and Ignatius, 1993], and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl [An and Hsie, 1992]. The potentiating or protective effects of these agents on BLM activity undoubtedly encompass diverse mechanisms of action.

Whether amino thiols enhance the genotoxicity of BLM or protect against it depends on the assay and experimental conditions. Potentiation of the genetic activity of BLM has been observed with CSM [Hoffmann and Littlefield, 1995; Hoffmann et al., 1995], cysteine [Chatterjee and Jacob-Raman, 1993], glutathione [Chatterjee et al., 1989; Chattopadhyay et al., 1997], and WR-1065 [Hoffmann et al.,

1993b, 1994]. Protective effects have also been ascribed to CSM [Hoffmann et al., 1995], cysteine [Chatterjee and Jacob-Raman, 1993], and WR-1065 [Nagy and Grdina, 1986; Hoffmann et al., 1995, 1999]. Of the other amines and thiols that we have studied, putrescine, spermidine, and spermine have been shown to potentiate the degradation of DNA by BLM [Strekowski et al., 1989]. The reducing agents 2-ME and dithiothreitol (a diastereomer of DTE with similar chemical properties) enhance the induction of DNA strand breakage by BLM in vitro [Sausville et al., 1978]. These amines and thiols are also potentially antimutagenic, decreasing the genetic effects of mutagens under some conditions [Hartman and Shankel, 1990; DeFlora et al., 1994].

WR-1065 potentiates the clastogenicity of BLM in G₀ human lymphocytes [Hoffmann et al., 1993b, 1994] but

TABLE V. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of 2-Mercaptoethanol or Cysteamine

BLM (μg/ml)	2-ME (mM)	CSM (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	0	1.71	4/1000	4	0.4
0	0	20	1.82	10/1000	10	1.0
0	30	0	1.52	5/1000	5	0.5
0	60	0	1.54	6/1000	6	0.6
0	90	0	1.44	6/1000	7	0.7
25	0	0	1.75	25/1000	28	2.8
25	0	10	1.69	65/1000***	77	7.7
25	0	20	1.68	119/1000***	155	15.5
8.3	0	0	1.74	19/1000	23	2.3
8.3	30	0	1.50	24/1000	29	2.9
8.3	60	0	1.74	17/1000	25	2.5
8.3	90	0	1.54	40/1000**	53	5.3
25	0	0	1.84	19/1000	22	2.2
25	15	0	1.81	21/1000	23	2.3
25	30	0	1.84	19/1000	24	2.4
25	60	0	1.76	31/1000	43	4.3
25	90	0	1.64	53/1000***	84	8.4
75	0	0	1.67	49/1000	63	6.3
75	30	0	1.55	41/1000	48	4.8
75	60	0	1.51	76/1000*	99	9.9
75	90	0	1.39	105/1000***	146	14.6

*Significance of difference from BLM treatment without 2-ME: Fisher's exact test $P < 0.05$.

**Significance of difference from BLM treatment without 2-ME: Fisher's exact test $P < 0.01$.

***Significance of difference from BLM treatment without 2-ME or CSM: Fisher's exact test $P < 0.001$.

TABLE VI. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of Dithioerythritol

Bleomycin (μg/ml)	DTE (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	1.91	6/1000	6	0.6
0	1.25	1.89	7/1000	7	0.7
0	2.5	1.85	8/1000	8	0.8
0	5	1.77	12/1000	12	1.2
25	0	1.88	18/1000	25	2.5
25	1.25	1.87	14/1000	15	1.5
25	2.5	1.82	31/1000	41	4.1
25	5	1.75	39/1000**	50	5.0 ^a
75	0	1.77	36/1000	44	4.4
75	2.5	1.76	74/1000***	100	10.0
75	5	1.61	77/1000***	97	9.7

^aA concurrent positive control with BLM at 25 μg/ml and 5 mM WR-1065 gave 24.0 micronuclei per 100 cells.

**Significance of difference from BLM treatment without DTE: Fisher's exact test $P < 0.01$.

***Significance of difference from BLM treatment without DTE: Fisher's exact test $P < 0.001$.

reduces its mutagenicity in Chinese hamster V79 cells [Nagy and Grdina, 1986]. Studies in yeast under hypoxic and oxygenated conditions help reconcile this inconsistency by showing that the availability of oxygen is a critical factor in the nature of the interaction between BLM and amino-thiols [Hoffmann et al., 1995]. CSM and WR-1065 cause

concentration-dependent protection against the genotoxicity of BLM in yeast, but the antimutagenic effect is reversed if the yeast are supplied excess oxygen or if hypoxia is avoided by culture conditions [Hoffmann et al., 1995]. CSM enhances the induction of gene conversion by BLM in yeast under well-oxygenated conditions [Hoffmann et al., 1995].

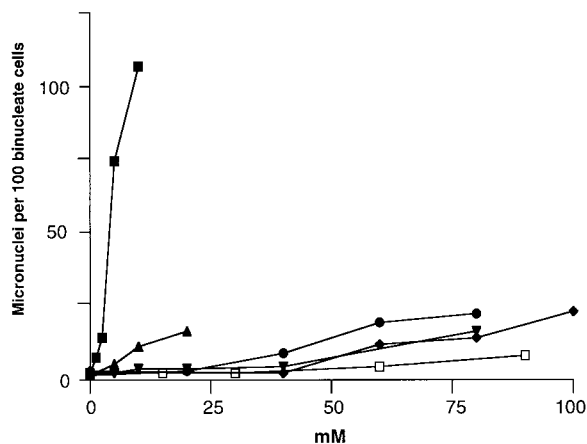


Fig. 2. Potentiation by amines and thiols of micronucleus induction by BLM (25 $\mu\text{g}/\text{ml}$) in G_0 human lymphocytes. Responses in the presence of putrescine (\blacktriangledown), spermidine (\blacklozenge), spermine (\bullet), and 2-ME (\square) are compared to those observed with WR-1065 (\blacksquare) [Hoffmann et al., 1994] and CSM (\blacktriangle) [Hoffmann and Littlefield, 1995] under the same conditions.

The potentiation of BLM by amino thiols is apparently limited to assays with sufficient oxygen, such as those in fresh G_0 lymphocytes. WR-1065 [Hoffmann et al., 1993b, 1994] strongly potentiates BLM in G_0 lymphocytes. The concentration of BLM required to cause a given level of chromosomal damage in the presence of WR-1065 is as much as 500-fold lower than that in its absence [Hoffmann et al., 1994]. CSM also substantially increases the genetic damage induced by BLM but is not as strong a potentiator as is WR-1065 [Hoffmann and Littlefield, 1995].

The potentiation of BLM by WR-1065 and CSM could be explained by either the thiol or the amino groups of these compounds. As thiols, they may act as electron donors for the activation of Fe^{+2} -BLM or reduction of Fe^{+3} -BLM [Hoffmann et al., 1994, 1995]. BLM activation requires Fe^{+2} -BLM, oxygen, and an additional electron, which may be provided by an intracellular reducing agent or by a second molecule of Fe^{+2} -BLM in a disproportionation reaction [Kane and Hecht, 1994]. The amino thiol may serve as an alternate electron source for this activation. Inactive Fe^{+3} -BLM is formed in the interaction of activated BLM with DNA [Povirk and Austin, 1991]. As reducing agents, amino thiols may regenerate Fe^{+2} -BLM by reducing Fe^{+3} -BLM. Alternatively, acting as amines, amino thiols may potentiate BLM through their association with DNA. WR-1065 and CSM bind to DNA [Liquier et al., 1983; Smoluk et al., 1986, 1988a]. This binding, like that of polyamines [Feuerstein et al., 1986], may cause widening of the minor groove [Strekowski et al., 1989; Sy et al., 1999], thereby increasing the access of BLM to the site where it abstracts the 4'-hydrogen. Rather than being alternatives, the thiol and amine functions may also act in concert, such that the amino groups bring the thiol group to its site of action on DNA.

Potentiation of the clastogenic action of BLM by gluta-

thione [Chatterjee et al., 1989; Chattopadhyay et al., 1997] can be explained similarly to the thiol-based mechanism proposed for WR-1065 [Hoffmann et al., 1994, 1995]. However, glutathione does not concentrate near DNA because it is not cationic at physiological pH [Newton et al., 1992; Zheng et al., 1992]. Moreover, the diverse physiological roles of glutathione suggest that indirect mechanisms of potentiation may also be involved. Though treatment with WR-1065 has been found to cause an elevation in intracellular glutathione, radioprotection by WR-1065 does not appear to be a consequence of the elevated glutathione concentrations [Grdina et al., 1995].

Data on radioprotection can provide insight into the basis for the potentiation of BLM. Amino thiols protect against ionizing radiation by scavenging hydroxyl radicals, transferring hydrogen to DNA radicals, and causing hypoxia near DNA [Purdie et al., 1983; Schwartz et al., 1988; Smoluk et al., 1988a; Hospers et al., 1999]. Other mechanisms that may contribute to radioprotection by WR-1065 are its causing increased synthesis of radioprotective polyamines [Mitchell et al., 1998], protecting sulfhydryl enzymes [Hospers et al., 1999], affecting enzymes of DNA synthesis [Grdina et al., 1994], and facilitating DNA repair [Grdina et al., 1999; Hospers et al., 1999]. Radioprotective effects of the polyamines spermine [Chiu and Oleinick, 1997] and spermidine [Newton et al., 1997] can be ascribed to scavenging of hydroxyl radicals and the compaction or stabilization of chromatin. Since WR-1065 and CSM bind to DNA [Liquier et al., 1983; Smoluk et al., 1986, 1988a], they may also alter the configuration of chromatin in ways that modulate clastogenic activity.

Cellular uptake and intracellular distribution are factors that can affect the biological activity of amino thiols. Relatively high water:octanol partition coefficients of WR-1065 and, to a lesser extent, CSM [Newton et al., 1996a] suggest that their passive uptake into cells might be a limiting factor. However, active mechanisms can facilitate uptake. WR-33278, the disulfide oxidation product of WR-1065, can enter cells by a polyamine transport system and be reduced intracellularly to WR-1065 [Mitchell et al., 1995; Newton et al., 1996a]. Radioprotection against γ -rays in V79 Chinese hamster cells depends on the charge (Z) of thiol compounds [Aguilera et al., 1992], such that WR-1065 ($Z = +2$) was effective at the lowest cellular thiol concentration, followed by CSM ($Z = +1$), 2-ME ($Z = 0$), and 3-mercaptopropionate ($Z = -1$). Preferential uptake is unlikely to explain the effectiveness of WR-1065, in that the latter three compounds were taken up by the cells roughly equally, and all were taken up better than was WR-1065 [Aguilera et al., 1992]. Moreover, the potencies with which the thiols protected naked plasmid DNA from strand breakage were similar: WR-1065 > CSM > dithiothreitol ($Z = 0$) > 2-ME > 3-mercaptopropionate [Zheng et al., 1992]. If uptake of WR-1065 is limited in cellular systems, the limitation may be offset by intracellular distribution, in that

TABLE VII. Induction of Micronuclei in G₀ Human Lymphocytes by Bleomycin in the Presence of Cysteine or N-Acetylcysteine

BLM ($\mu\text{g/ml}$)	Cysteine (mM)	NAC (mM)	Proliferation index	Micronucleate cells/binucleate cells scored	Total micronuclei	Micronuclei per 100 cells
0	0	0	1.53	8/1000	8	0.8
25	0	0	1.61	6/1000	6	0.6
25	2.5	0	1.44	14/1000	15	1.5
25	5	0	1.56	9/1000	9	0.9
25	10	0	1.60	13/1000	20	2.0
25	20	0	1.67	27/1000***	31	3.1
25	0	0	1.50	10/1000	10	1.0
25	0	15	1.46	20/1000	27	2.7
25	0	30	1.48	26/1000*	34	3.4
25	0	60	1.37	30/1000**	36	3.6

*Significance of difference from BLM treatment without NAC: Fisher's exact test $P < 0.05$.

**Significance of difference from BLM treatment without NAC: Fisher's exact test $P < 0.01$.

***Significance of difference from BLM treatment without cysteine: Fisher's exact test $P < 0.001$.

concentrations of WR-1065 within V79 cell nuclei were higher than those of either CSM or 2-ME [Newton et al., 1996b].

Comparing effects of WR-OH and WR-1065 provides information on which portion of the WR-1065 molecule is most responsible for the potentiation of BLM because WR-OH differs from WR-1065 only in having a hydroxyl group in place of the thiol group (Fig. 1). Thus, WR-OH seems as likely as WR-1065 to bind to DNA and induce conformational changes, but it lacks its reducing potential. Table I shows that WR-OH potentiates BLM much less effectively than does WR-1065, causing only a slight increase in the frequency of micronuclei at the highest non-toxic dosage. This result suggests that the thiol group is critical in the potentiation of BLM by WR-1065, a finding compatible with a redox mechanism of potentiation. The increase in BLM-induced micronuclei at the highest concentration of WR-OH can be ascribed to the amine portion of the molecule.

This view is reinforced by studies of other amines that lack a thiol function. Putrescine, spermidine, and spermine all potentiate BLM (Tables II, III, and IV), but none of them is as potent as the aminothiols (Fig. 2), and they are ineffective potentiators at low concentrations. As a consequence of their positively charged amino groups, polyamines [Strekowski et al., 1989; Sy et al., 1999] and WR-1065 [Smoluk et al., 1988a; Sy et al., 1999] bind to DNA. Their binding in the major groove [Feuerstein et al., 1986] or in the minor groove [Sy et al., 1999] can cause widening of the minor groove [Strekowski et al., 1989; Sy et al., 1999], thereby affording BLM greater access to the 4' position of deoxyribose. We speculate that this mechanism is the most likely means of potentiation of BLM applicable to putrescine, spermidine, spermine, and WR-OH. It seems unlikely, however, to be a major contributor to the potentiation of BLM by WR-1065, in that the latter compound is more

potent, acts at lower concentrations, is toxic at higher concentrations, and apparently shares transport mechanisms with the other amines.

Thiols that lack an amino group also potentiate BLM. CSM and 2-ME (Fig. 1; Table V) are structural analogs, differing only with respect to the amino group. The weakness of BLM-potentiation by 2-ME relative to CSM demonstrates the importance of the amino group in potentiation of BLM by aminothiols. The dithiol DTE (Table VI) is too toxic to be studied at over 5 mM and cannot be directly compared to the other compounds on the basis of equal concentrations, but the data indicate that it causes modest potentiation of BLM.

Our findings indicate that the combination of the thiol and amino functions, rather than either alone, makes CSM and WR-1065 effective in potentiating the clastogenicity of BLM. A thiol-based redox mechanism seems pivotal in the potentiation of BLM by aminothiols. The aminothiol may serve as an electron source for the activation of Fe^{+2} -BLM or for regenerating Fe^{+2} -BLM from inactive Fe^{+3} -BLM. The finding that Fe^{+3} -BLM cleaves naked DNA effectively when provided along with the thiol 2-ME [Povirk and Houlgrave, 1988] lends support to the latter hypothesis. Though primary, the thiol function is not sufficient to explain the strong potentiation of BLM by WR-1065 and CSM. Rather, the electrostatic association of these cationic thiols with DNA, mediated by their amino groups, concentrates the thiol group at the site of its action on the complex of BLM with DNA.

WR-1065 exists as a family of species whose composition depends on the ionization of its thiol and two amino groups; 67% of the compound is maximally protonated and carries a charge of +2 at pH 7.4, and 84% does so at pH 7 [Newton et al., 1992]. Thus, in our experiments, the distribution of species is heavily skewed to a charge of +2, as the pH is 7.0 or slightly less. The positive charge enables

WR-1065 to bind electrostatically to DNA [Smoluk et al., 1986, 1988a]. The greater charge of a diamine than that of a monoamine explains why WR-1065 concentrates near DNA more than does CSM [Smoluk et al., 1988a, 1998b] and is a more effective potentiator of BLM [Hoffmann and Littlefield, 1995]. Thus, the order of effectiveness in potentiating BLM in our study (WR-1065 > CSM > simple thiols) is as predicted from the tendency of counterions (cations) to concentrate near DNA and coions (anions) to be depleted near DNA [Smoluk et al., 1988b]. This view is reinforced by the finding (Table VII) that cysteine, which lacks the net positive charge of WR-1065 and CSM at physiological pH, potentiates BLM only weakly, as does its negatively charged amide derivative NAC.

The modulation of BLM activity by amino thiols is influenced by physiological conditions. Studies in yeast have shown that the potentiation of BLM by CSM occurs only under well-oxygenated conditions, whereas antimutagenic effects of amino thiols prevail under hypoxic conditions [Hoffmann et al., 1995]. If the same applies in lymphocytes, one might expect that the potentiation that we described in G₀ lymphocytes would be less prominent if oxygen were depleted in cultures of dividing cells. Preliminary experiments on cycling lymphocytes suggest that this may be the case (Hoffmann and Littlefield, unpublished data). The potentiation of BLM by amino thiols may have implications for chemotherapy if it occurs in intact mammals. In principle, potentiation of BLM in target tissues could allow effective DNA degradation at lower drug concentrations, whereas potentiation in nontarget tissues would be a potential hazard. However, predicting effects in vivo from studies in cultured cells and yeast is limited by the fact that hypoxic and oxygenated conditions both occur in vivo and may strongly influence the nature of the interactions.

Protective influences of amino thiols in modulating genotoxic treatments have been demonstrated both in laboratory animals and in human clinical exposures. Clinical applications have relied on WR-2721 (amifostine), whose biological activity depends on intracellular dephosphorylation to WR-1065 [Purdie et al., 1983; Smoluk et al., 1988a; Hospers et al., 1999]. WR-2721 reduces toxicity to nontarget tissues in radiotherapy [Capizzi, 1999; Hospers et al., 1999] and chemotherapy with cyclophosphamide, alkylating agents, and platinum compounds [Glover et al., 1988; Kurbacher and Mallmann, 1998; Capizzi, 1999; Hospers et al., 1999]. Other agents for which laboratory studies or early clinical trials offer promise of chemoprotection by WR-2721 include mitomycin C, anthracyclines, taxanes, 5-fluorouracil, and topoisomerase inhibitors [Kurbacher and Mallmann, 1998; Capizzi, 1999; Hospers et al., 1999]. WR-2721 seems to reduce the toxic effects of therapeutic agents in nontarget cells without compromising their effectiveness against tumor cells [Kurbacher and Mallmann, 1998; Grdina et al., 1999; Hospers et al., 1999].

Although amino thiols are often protective, they may

sometimes potentiate toxicity. WR-2721 has been reported to protect against the toxicity of the radical generator 6-hydroxydopamine in mice under some conditions but to potentiate it under others, the differences depending on strains and dosages [Schor, 1987, 1988]. On the basis of these findings, Schor [1987, 1988] urged caution in using WR-2721 with BLM. Findings that glutathione [Chatterjee et al., 1989], CSM [Hoffmann and Littlefield, 1995], and WR-1065 [Hoffmann et al., 1993b, 1994] all potentiate BLM in lymphocytes lend support to the possibility of potentiation. Recent studies suggest that WR-2721 can reduce the pulmonary toxicity of BLM in hamsters [Nici et al., 1998; Nici and Calabresi, 1999] and mice [Nici and Calabresi, 1999]. These findings are of special interest, in that pulmonary toxicity is an important limiting factor in therapy with BLM [Hay et al., 1991; Nici and Calabresi, 1999]. Though the potential benefits of using WR-2721 with BLM are considerable, the possibility of potentiation of BLM by amino thiols in some tissues calls for vigilance with respect to unexpected interactions. If occurring in vivo, potentiation would be most likely in tissues having relatively high oxygen tensions, and it could be either a property to be exploited or a risk to be avoided in formulating therapeutic strategies.

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