Amplification of Pepleomycin-Mediated DNA Cleavage and Apoptosis by Unfused Aromatic Cations†

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ABSTRACT: An important approach to improve chemotherapy of members of the bleomycin (BLM) family of antibiotics is to find compounds (amplifiers) that enhance the activity of BLM-mediated DNA cleavage and apoptosis. Using a DNA-sequencing technique and pulsed field gel electrophoresis, we have investigated whether BLM-mediated cleavage of isolated and cellular DNA is amplifed by three compounds (RW-12, LS-20, 1S-5Me) which have a conformationally flexible, unfused polyaromatic system and cationic side chain in the molecules. RW-12 enhanced most effectively both pepleomycin (PEM)-induced cytotoxicity and apoptosis. The order of the maximum enhancing effect of amplifiers on PEM-mediated DNA damage is RW-12 > LS-20 > 1S-5Me. RW-12 amplified PEM-mediated DNA cleavage most effectively not only in vitro but also in cultured cells. We have reported that the order of the DNA binding constants of these compounds is RW-12 > LS-20 > 1S-5Me. In this study, we found a good correlation between PEM-mediated cleavage of isolated DNA and cellular DNA. These results suggest that BLM amplifiers bind to DNA and by doing so enhance drug-mediated DNA degradation, ultimately leading to apoptosis. The present study on amplifiers of anticancer agents shows a novel approach to the potentially effective anticancer therapy.

It is believed that bleomycin (BLM)[1]-mediated degradation of DNA in the presence of ferrous ion is the major mechanism by which BLM exerts its antitumor activity (*1, 2*). Pepleomycin (PEM) is a semi-synthetic bleomycin with cytostatic activity and less pulmonary toxicity than the natural bleomycin mixture. Accordingly, PEM appears to have a clinical advantage. It was of interest to evaluate potential enhancement of the biological activity of PEM in the presence of additional agents that bind to DNA.

The enhancement of BLM-induced fragmentation of DNA by intercalative drugs was first noticed by Bearden (*3*). Subsequently, Allen et al. (*4*) reported that certain unfused heterocyclic compounds amplified the antitumor activity of phleomycin, an anticancer drug closely related to BLM, in rats and mice. Recently, Strekowski (*5*) reported that the compounds containing pyrimidine and thiophene rings bind to DNA and their binding constants and the binding-induced DNA elongation correlate well with the respective activities as BLM amplifiers (*6*−*11*).

Most of the cytotoxic anticancer drugs in current use have been shown to induce apoptosis in susceptible cells (*12*−*15*). The fact that disparate agents, which interact with different targets, induce cell death with some common features (endonucleolytic cleavage of DNA, changes in chromatin condensation) suggests that cytotoxicity is determined by the ability of the cell to engage this so-called "programmed" cell death (*16*). Recently, we demonstrated a mechanism of apoptosis by DNA-damaging anticancer drugs (*12*). It has been strongly suggested that anticancer treatment may induce apoptosis in tumor cells and merely a cell cycle pause in their normal cell counterparts. Factors that increase the propensity for apoptosis may determine the therapeutic index whereby anticancer agents selectively destroy tumor cells. The present study on amplifiers of anticancer agents shows a novel approach to the potentially effective anticancer therapies.

In this study, to clarify whether amplifiers enhance the anticancer activity of PEM, we examined the cytotoxicity using human cultured cells. We used three compounds (RW-12, LS-20, 1S-5Me) which bind to DNA by a nonstandard intercalation mode (*6*). The structures of these compounds which have a conformationally flexible, unfused polyaro-

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[1] Abbreviations: BLM, bleomycin; PEM, pepleomycin; in this work, in agreement with a generally accepted annotation, the abbreviation BLM is used for a family of bleomycins including pepleomycin (PEM).

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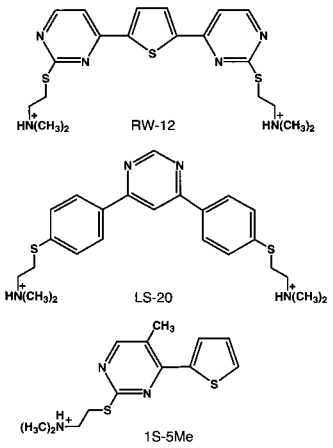

FIGURE 1: Structures of the BLM amplifiers RW-12, LS-20, and 1S-5Me. Dimethylamino groups of these compounds are fully protonated under physiological conditions.

matic system and at least one cationic side chain in the molecule are shown in Figure 1. These compounds have been studied previously as amplifiers of the natural BLM mixture (*5*−*11*). In addition, we investigated DNA strand breaks in HL-60 cells by using pulsed field gel electrophoresis. The relationship between cellular DNA damage and formation of the DNA "ladder", which is characteristic of apoptosis, was also examined. Furthermore, we determined the site specificity of PEM-induced DNA cleavage in the presence of amplifiers by using ^{32}P 5′-end-labeled DNA fragments of defined sequence obtained from human c-Ha-*ras*-1 protooncogene. We also investigated whether amplifiers have inhibitory effects on the DNA repair system.

MATERIALS AND METHODS

Materials. Restriction enzymes (*Bam*HI, *Ava*I, X*ba*I, P*st*I, and *Hin*dIII) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Restriction enzymes, *Eco*RI, *Sma*I, *Apa*I, and *Sty*I, were from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). DTPA was from Dojindo Laboratories (Kumamoto, Japan). Acrylamide, $FeSO_4(NH_4)_2SO_4$, bisacrylamide, and piperidine were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RW-12 (*10*), LS-20 (*7*), and 1S-5Me (*8*) were prepared according to the methods described previously.

Cell Lines. Human myelogenous leukemic cell line HL-60 was grown in RPMI 1640 (GIBCO BRL, Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal calf serum (Whittaker Bioproducts, Walkersville, MD) at 37 °C under 5% CO_2 in a humidified atmosphere. Cells were incubated with 5 μM PEM after pretreatment with a10 μM sample of each amplifier for 30 min and harvested at the indicated time. Cell viability was assayed by trypan blue staining.

Detection of Cellular DNA Damage by Pulsed Field Gel Electrophoresis. For the determination of DNA strand breaks, the treated cells were washed twice with RPMI 1640 and once with PBS and resuspended in 150 μL of PBS. The cell suspension was solidified with agarose, followed by incubation with proteinase K according to the method described previously (*17, 18*). Electrophoresis was performed in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) by the pulsed field electrophoresis system (CHEF-DRII DNA megabase electrophoresis system, Bio-Rad, Hercules, CA) at 200 V at 14 °C. The switch time was 60 s for 15 h followed by a 90 s switch time for 9 h. The DNA in the gel was visualized using ethidium bromide.

Detection of DNA "Ladder" Formation. After the treated cells were washed, as described for pulsed field gel electrophoresis, the cells were disrupted in a solution containing 10 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100. Nuclei were pelleted by centrifugation at 1000*g*. The nuclei were then extracted in a solution containing 10 mM Tris-HCl (pH 7.6), 0.4 M NaCl, 1 mM EDTA, and 1% Triton X-100 and centrifuged at 12000*g* to separate the nucleoplasm from the high-molecular-weight chromatin. Nucleoplasm was incubated with RNase (final concentration 20 μg/mL) for 1 h and then with proteinase K (final concentration 100 μg/mL) for 2 h at 37 °C. DNA was purified with phenol−chloroform and precipitated with 2.5 volumes of ethanol and analyzed by conventional electrophoresis according to the method described previously (*12*).

Preparation of ^{32}P 5′-End-Labeled DNA Fragments Obtained from the c-Ha-ras-1 Protooncogene. DNA fragments were prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 protooncogene (*19, 20*). A singly labeled 261 base pair fragment (*Ava*I* 1645−X*ba*I 1905) and a 337 base pair fragment (*Pst*I 2345−*Ava*I* 2681) were obtained according to the method described previously (*19, 20*). The asterisk indicates ^{32}P-labeling, and nucleotide numbering starts at the *Bam*HI site (*21*).

Detection of Damage to Isolated DNA Induced by PEM in the Presence of Amplifiers. The reaction mixture contained the ^{32}P 5′-end-labeled 337 base pair fragment (*Pst*I 2345−*Ava*I* 2681), 50 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. Where indicated, 1 μM PEM, 1 μM $FeSO_4(NH_4)_2SO_4$, and/or amplifiers were added. After incubation at 37 °C for 5 min, the treated DNA fragments were electrophoresed on an 8% polyacrylamide, 8 M urea gel (12 × 16 cm), and the autoradiogram was obtained by exposing X-ray film to the gel.

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam−Gilbert procedure (*22*) using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). A laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech, Uppsala, Sweden) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Human DNA Polymerase β Inhibition Assay. The inhibitory effect of amplifiers on the activity of human DNA polymerase β, which is an important DNA repair-related enzyme, was examined using a Human Beta-Polymerase kit (Trevigen, Inc.). The reaction mixture contained [α-^{32}P]dATP (111 TBq/mmol), control DNA (0.5 mg/mL λDNA/*Hin*dIII), 1mM dCTP, 1mM dGTP, 1mM dTTP, amplifier, and 2.5 units of β polymerase enzyme in 10 μL of REC Buffer 8. After incubation at 37 °C for 2 h, the radiolabeled DNA product was electorophoresed on a 0.8% agarose gel, and the autoradiogram was obtained by exposing X-ray film to the gel.

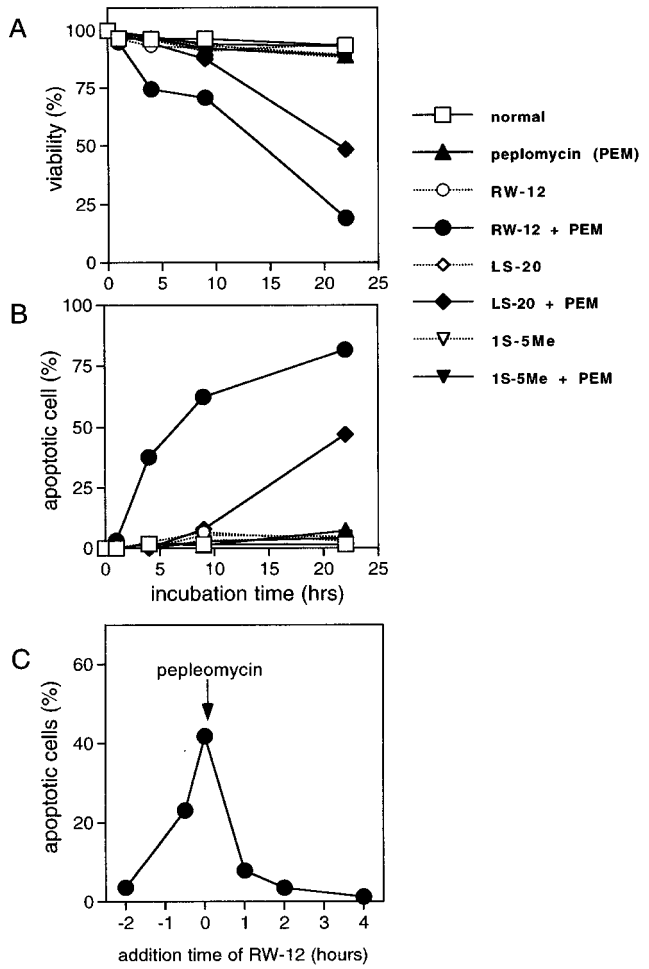

FIGURE 2: Cell viability and apoptosis of HL-60 treated with PEM in the presence of amplifiers. Cells were incubated with 5 μM pepleomycin after pretreatment with 10 μM each amplifier for 30 min. At the indicated time, the percentage of trypan blue-positive cells (A) and the percentage of cells showing condensed chromatin in the acridine orange staining (B) were determined. RW-12 was added at the indicated time relative to addition of PEM, and apoptotic cells were determined by acridine orange staining at 6 h incubation with PEM (C). PEM was added at time 0. These results were obtained from two independent experiments.

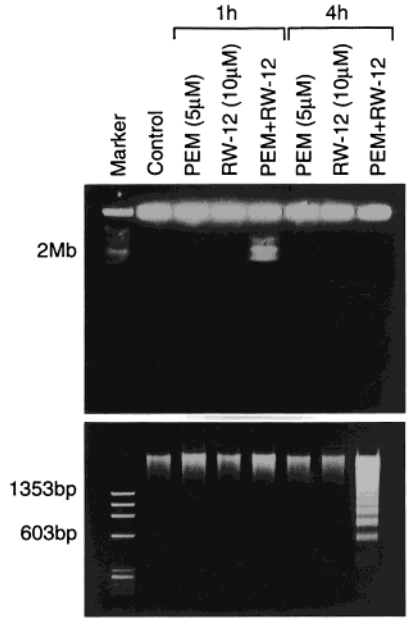

FIGURE 3: Effects of amplifiers on PEM-mediated DNA cleavage in HL-60 cells. After pretreatment with 10 μM RW-12 for 30 min, HL-60 cells were incubated with 5 μM PEM. Cells were harvested at the indicated time and divided into two aliquots. One aliquot was used for pulsed field gel analysis, and other aliquots were used for analysis of nucleosomal DNA fragmentation. Cells were prepared as agarose plugs, lysed, and subjected to pulsed field gel electrophoresis through a 1% agarose gel, as described under Materials and Methods. The gel was stained in ethidium bromide (top). Marker lane: size marker DNA (*Saccharomyces cerevisiae*) (top). The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described under Materials and Methods (bottom). Marker lane: size marker DNA (ΦX 174/*Hae*III digest) (bottom).

RESULTS

Cytotoxicity of HL-60 Treated with PEM in the Presence of Amplifiers. As shown in Figure 2A, amplifiers enhanced cytotoxic effects of PEM. Among the three compounds, RW-12 showed the strongest effect. Cell death occurred as early as 4 h after treatment with PEM plus RW-12, and approximately 80% of the cells died at 22 h after treatment, whereas PEM alone or RW-12 alone showed little effect on cell viability. Similar results were obtained by acridine orange staining, which clearly distinguished between viable cells and cells showing the condensed chromatin staining characteristic of apoptosis (Figure 2B). In addition, RW-12 was added at various times relative to addition of PEM (Figure 2C). Treatment of cells with RW-12 and PEM at the same time most effectively induced apoptosis.

Cellular DNA Damage and Internucleosomal DNA Fragmentation Induced by PEM in the Presence of Amplifiers. To confirm whether accelerated death of PEM plus amplifier-treated HL-60 cells was associated with cellular DNA damage and subsequently the typical DNA ladder formation characteristic for apoptosis, we analyzed genomic DNA. As shown in Figure 3, DNA fragments of 1−2 Mb were clearly detected as early as 1 h after addition of PEM in the presence of RW-12. At 4 h after treatment, however, the high molecular weight DNA fragments disappeared, and instead 180−200 base pair oligonucleosomal DNA ladder fragments, which are associated with apoptosis, were detectable. PEM alone and RW-12 alone did not effectively induce oligonucleosomal DNA fragmentation as well as high molecular weight DNA damage under the conditions tested. These results show that amplifiers can enhance DNA damage by PEM in cells, resulting in chromosomal DNA fragmentation and subsequent induction of apoptotic death.

Enhancing Effects of Amplifiers on PEM-Mediated DNA Damage. Polyacrylamide gel electrophoretic analysis was used to estimate the effect of amplifiers on PEM-mediated cleavage of DNA fragments. Figure 4 shows the effect of amplifier concentration on PEM-mediated cleavage of ^{32}P-labeled DNA fragment obtained from human c-Ha-*ras*-1 protooncogene (*21*). For RW-12, the maximum enhancing effect on the PEM-mediated DNA cleavage in the presence of Fe(II) was observed at the concentration of 20 μM, whereas 1S-5Me showed maximum enhancement at 500 μM (Figure 4). LS-20 showed maximum enhancement at 50 μM (data not shown). The enhancing effects of all the amplifiers decreased with increasing concentrations. Similar effects of amplifiers were observed with other DNA fragments.

Site Specificity of DNA Cleavage Induced by PEM in the Presence of Amplifiers. For the measurement of sequence-

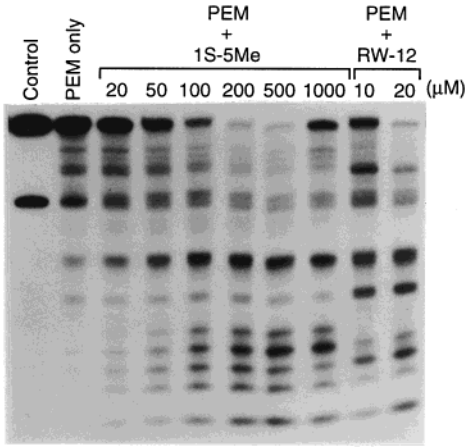

FIGURE 4: Autoradiogram of the ^{32}P 5′-end-labeled DNA fragment cleaved by PEM in the presence of amplifiers. The reaction mixture contained the ^{32}P 5′-end-labeled 337 base pair fragment (*Pst*I 2345−*Ava*I* 2681), 50 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. Where indicated, 1 μM PEM, 1 μM $FeSO_4(NH_4)_2SO_4$, and/or amplifier (1S-5Me or RW-12) were added. After the incubation at 37 °C for 5 min, the treated DNA fragments were electrophoresed on an 8% polyacrylamide, 8 M urea gel (12 × 16 cm), and the autoradiogram was obtained by exposing X-ray film to the gel.

specific DNA cleavage, ^{32}P 5′-end-labeled DNA fragments incubated with PEM and Fe(II) in the presence of the amplifiers were electrophoresed, and the autoradiogram was obtained. The autoradiogram was scanned with a laser densitometer (Figure 5). The DNA cleavage sites were determined by using the Maxam−Gilbert procedure (*22*). Control digestions, performed in the absence of amplifiers, revealed that 5′-GC-3′ and 5′-GT-3′ sequences (underscoring: the cleaved nucleotide) are the sites most extensively cleaved by PEM. The result is in excellent agreement with that previously reported (*2*, *23*, *24*) for BLM. When the DNA molecules were incubated with RW-12 prior to addition of PEM, a dramatic increase was observed in the cleavages at 5′-GC-3′ and 5′-GT-3′ sequences containing A·T, especially at 5′-AGCT-3′ (Figure 5). The enhancing effects of other amplifiers on the same cleavage patterns were observed at a higher concentration than that of RW-12 (data not shown).

Effect of Amplifiers on the Activity of Human DNA Polymerase β. To clarify whether amplifiers influence the DNA repair system, the effects of amplifiers on the activity of human DNA polymerase β were examined. The activity of human DNA polymerase β was strongly inhibited by RW-12 at 30 μM (Figure 6). A similar result was obtained with quercetin, which was a strong inhibitor of DNA polymerase β (*25*). LS-20 exhibited weak inhibitory activity against the DNA polymerase β.

DISCUSSION

The present study demonstrates that unfused aromatic cations enhance both PEM-induced cytotoxicity and apoptosis, and among them RW-12 shows the strongest effect. These results suggest that the amplifier-enhanced cytotoxic effects of PEM and the enhanced cytotoxicity are due to induction of apoptosis. Of the three compounds studied, RW-12 most efficiently enhances DNA strand breaks in the cells treated with PEM. The order of the maximum enhancing effect of amplifiers on PEM-mediated DNA damage in isolated DNA fragments is RW-12 > LS-20 > 1S-5Me. Moreover, we found a good correlation between PEM-mediated cleavage of isolated DNA and cellular DNA. Wilson et al. (*6*) and Strekowski et al. (*10*) have reported that the order of the DNA binding constants of these compounds is RW-12 > LS-20 > 1S-5Me. All these compounds are nonstandard DNA intercalators, and the same order has been found for elongation of the DNA molecule upon intercalation, as measured by viscometric titrations (*6*, *9*, *10*). Therefore, there is not only a parallel behavior between the DNA binding constants of these unfused amplifiers and the extent of the DNA distortion upon binding, but the parallel behavior also involves the enhancement effect of the PEM-mediated DNA damage. This last feature also extends to the cell system.

It has been demonstrated that BLM binds preferentially to 5′-GC-3′ or 5′-GT-3′ sequence in the minor groove of DNA (*26*). The interaction specificity can be explained by assuming that (i) conformational changes of the DNA duplex are required for the stereochemical fit with a drug molecule upon complex formation and (ii) the DNA stereochemistry around the 5′-GC-3′ and 5′-GT-3′ sequences approximates better the final DNA conformation in the complex than local DNA structures around other base sequences (*5*). Evidence has been accumulating that relaxation of the DNA structure at the preferred binding sites of the drug or adjacent to the binding sites strongly facilitates interaction of the drug with DNA. It is known that a local distortion of the DNA double helix can be transferred several base pairs away from the original distortion site (*27*). Therefore, the sites of local distortion or unwinding in DNA may be particularly favorable for BLM to bind and to cleave (*23*, *28*). Williams and Goldberg (*29*) reported that BLM causes specific strand cleavage near DNA bulges. Suh and Povirk (*30*) suggested that the binding site required for BLM-induced DNA cleavage spans a region of approximately 2 or 3 bp in the minor groove, including the base associated with the sugar attacked and one or two bases to its 5′ side. Sucheck et al. (*31*) presented direct evidence for minor groove binding of the bithiazole moiety in the BLM, although several papers emphasized the importance of intercalation of BLM into DNA (*32*). The latter feature may cause relaxation of the DNA local structure for a stronger complex formation. It has also been demonstrated that binding of BLM with DNA increases with increasing temperature, and the maximum interaction occurs at temperatures at which kinks have been postulated to form in the B-helix (*5*). Similar kinks, almost certainly, are the result of the interaction of unfused intercalators with DNA (*5*). The discussed DNA binding agents may simply stabilize the helix in its distorted conformation, similar to that in the complex with the drug. The overall effect would be lowering of the energy of the BLM−DNA interaction, which, in turn, would result in the increase of the BLM affinity for DNA.

There is a good correlation between the BLM-mediated cleavage of isolated DNA and that of cellular DNA in the presence of amplifiers. These results indicate that the amplifiers bind to DNA and by doing so enhance drug-mediated DNA degradation. More specifically, the good correlation between BLM-mediated cleavage of isolated and cellular DNAs strongly suggests the direct action of amplifiers on the DNA conformation. This idea may be supported

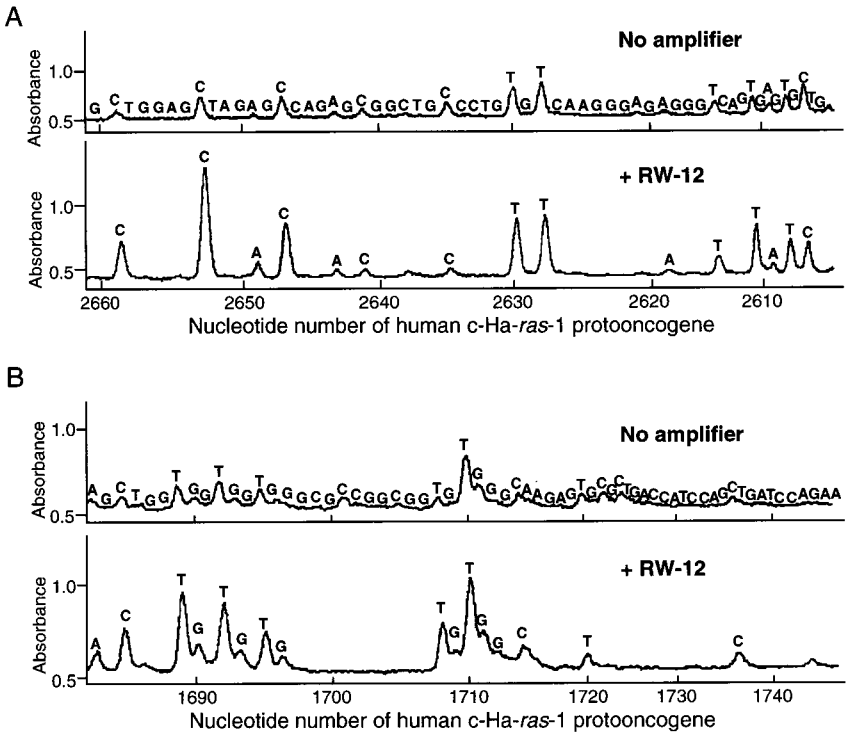

FIGURE 5: Alteration of site specificity of PEM-induced DNA cleavage by amplifiers. (A) The ^{32}P 5′-end-labeled 337 base pair fragment (*Pst*I 2345−*Ava*I* 2681) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 50 μM per base of sonicated calf thymus DNA was incubated with 1 μM PEM, 1 μM $FeSO_4(NH_4)_2SO_4$ in the absence or presence of 20 μM RW-12 at 37 °C for 5 min. (B) The ^{32}P 5′-end-labeled 261 base pair fragment (*Ava*I*1465−*Xba*I 1905) was used. The treated DNA fragments were electrophoresed on an 8% polyacrylamide, 8 M urea gel using a DNA-sequencing system, and the autoradiogram was obtained by exposing X-ray film to the gel. The autoradiogram was scanned with a densitometer (LKB 2222 UltroScan XL). Horizontal axis: the nucleotide number of human c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site (*21*).

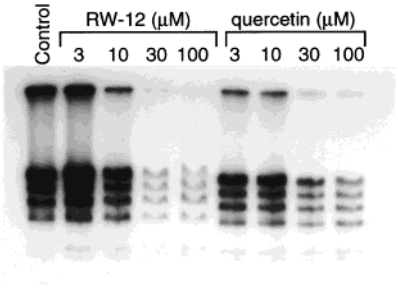

FIGURE 6: Inhibitory effects of RW-12 and quercetin on the activity of human DNA polymerase β. The reaction mixture contained 2 μL of [α-^{32}P]dATP (111 TBq/mmol), 0.5 μg of λDNA/*Hin*dIII, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 2.5 units of β polymerase enzyme, and the indicated concentrations of RW-12 or quercetin in 10 μL of REC Buffer 8. After incubation at 37 °C for 2 h, the radiolabeled DNA product was analyzed as described under Materials and Methods. Control contained neither RW-12 nor quercetin.

by the time course experiment as Figure 2C. In addition, since cancer cells can repair the damage induced by anticancer drugs, it is very interesting to clarify whether amplifiers inhibit the DNA repair. The role of DNA polymerase β in DNA repair strongly suggests the validity of the enzyme as a target for combination chemotherapy (*33*). Activity of human DNA polymerase β was strongly inhibited by RW-12 in vitro, whereas LS-20 exhibited the very weak inhibitory activity. Therefore, the possibility that amplifiers influence human DNA polymerase β has remained. On the basis of these results, it can be concluded that BLM amplifiers bind to DNA and by doing so enhance drug-mediated DNA degradation and may additionally inhibit the DNA repair, ultimately leading to apoptosis.

The observation that unfused tricyclic aromatic cations significantly amplify PEM-mediated cleavage of both isolated and cellular DNA may provide useful information for the design of amplifiers of a great clinical advantage. As a method to approach a new chemotherapy to treat cancer more effectively and to reduce toxic side effects, we have demonstrated the DNA cleavages induced by antitumor drugs were enhanced by DNA binding drugs (*34*, *35*). The concept of synergistic interactions in drug activity at DNA sites may have wide applicability as demonstrated by the BLM amplifiers and in the recent observation of concerted DNA recognition and novel site-specific alkylation by duocarmycin A with distamycin A (*35*−*37*).

REFERENCES

1. Gutteridge, J. M., West, M., Eneff, K., and Floyd, R. A. (1990) *Free Radical Res. Commun. 10*, 159−165.
2. Takeshita, M., Grollman, A. P., Ohtsubo, E., and Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983−5987.
3. Bearden, J., Jr., and Haidle, C. W. (1975) *Biochem. Biophys. Res. Commun. 65*, 371−377.
4. Allen, T. E., Brown, D. J., Cowden, W. B., Griff, G. W., Hart, N. K., Lamberton, J. A., and Lane, A. (1984) *J. Antibiot. 37*, 376−383.
5. Strekowski, L. (1992) in *Advances in Detailed Reaction Mechanisms* (Coxon, J. M., Ed.) pp 61−109, JAI Press, Greenwich, CT.
6. Wilson, W. D., Tanious, F. A., Watson, R. A., Barton, H. J., Strekowska, A., Harden, D. B., and Strekowski, L. (1989) *Biochemistry 28*, 1984−1992.

7. Wilson, W. D., Strekowski, L., Tanious, F. A., Watson, R. A., Mokrosz, J. L., Strekowska, A., Webster, G. D., and Neidle, S. (1988) *J. Am. Chem. Soc. 110*, 8292−8299.
8. Strekowski, L., Mokrosz, J. L., Tanious, F. A., Watson, R. A., Harden, D., Mokrosz, M., Edward, W. D., and Wilson, W. D. (1988) *J. Med. Chem. 31*, 1231−1240.
9. Strekowski, L., Wilson, W. D., Mokrosz, J. L., Strekowska, A., Koziol, A. E., and Palenik, G. J. (1988) *Anti-Cancer Drug Des. 2*, 387−398.
10. Strekowski, L., Strekowska, A., Watson, R. A., Tanious, F. A., Nguyen, L. T., and Wilson, W. D. (1987) *J. Med. Chem. 30*, 415−1420.
11. Strekowski, L., Chandrasekaran, S., Wang, Y.-H., Edward, W. D., and Wilson, W. D. (1986) *J. Med. Chem. 29*, 1311−1315.
12. Tada-Oikawa, S., Oikawa, S., Kawanishi, M., Yamada, M., and Kawanishi, S. (1999) *FEBS Lett. 442*, 65−69.
13. Guchelaar, H. J., Vermes, A., Vermes, I., and Haanen, C. (1997) *Pharm. World Sci. 19*, 119−125.
14. Gorczyca, W., Gong, J., Ardelt, B., Traganos, F., and Darzynkiewicz, Z. (1993) *Cancer Res. 53*, 3186−3192.
15. Kaufmann, S. H. (1989) *Cancer Res. 49*, 5870−5878.
16. Hickman, J. A. (1992) *Cancer Metastasis Rev. 11*, 121−139.
17. Hiraku, Y., and Kawanishi, S. (1996) *Cancer Res. 56*, 5172−5178.
18. Ito, K., Yamamoto, K., and Kawanishi, S. (1992) *Biochemistry 31*, 11606−11613.
19. Yamamoto, K., and Kawanishi, S. (1991) *J. Biol. Chem. 266*, 1509−1515.
20. Kawanishi, S., and Yamamoto, K. (1991) *Biochemistry 30*, 3069−3075.
21. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Goeddel, D. V. (1983) *Nature 302*, 3337.
22. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol. 65*, 499−560.
23. Mirabelli, C. K., Ting, A., Huang, C.-H., Mong, S., and Crooke, S. T. (1982) *Cancer Res. 42*, 2779−2785.
24. D'Andrea, A. D., and Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608−3612.
25. Ono, K., Nakane, H., Fukushima, M., Chermann, J. C., and Barre-Sinoussi, F. (1990) *Eur. J. Biochem. 190*, 469−476.
26. Kuwahara, J., and Sugiura, Y. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2459−2463.
27. Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer, New York.
28. Norden, B., and Tjerneld, F. (1977) *Chem. Phys. Lett. 50*, 508−512.
29. Williams, L. D., and Goldberg, I. H. (1988) *Biochemistry 27*, 3004−3011.
30. Suh, D., and Povirk, L. F. (1997) *Biochemistry 36*, 4248−4257.
31. Sucheck, S. J., Ellena, J. F., and Hecht, S. M. (1988) *J. Am. Chem. Soc. 120*, 7450−7460.
32. Stubbe, J., Kozarich, J. W., Wu, W., and Vanderwall, D. E. (1996) *Acc. Chem. Res. 29*, 322−330.
33. Starck, S. R., Deng, J. Z., and Hecht, S. M. (2000) *Biochemistry 39*, 2413−2419.
34. Hiraku, Y., and Kawanishi, S. (1997) *Biochem. Biophys. Res. Commun. 239*, 134−138.
35. Yamamoto, K., and Kawanishi, S. (1992) *Biochem. Biophys. Res. Commun. 183*, 292−299.
36. Sugiyama, H., Lian, C., Isomura, M., Saito, I., and Wang, A. H. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14405−14410.
37. Yamamoto, K., Sugiyama, H., and Kawanishi, S. (1993) *Biochemistry 32*, 1059−1066.

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