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Mechanism of metal-mediated DNA damage induced by a metabolite of carcinogenic acetamide

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Abstract

Acetamide is carcinogenic in rats and mice. To clarify the mechanism of carcinogenesis by acetamide, we investigated DNA damage by an acetamide metabolite, acetohydroxamic acid (AHA), using ^{32}P-5′-end-labeled DNA fragments. AHA treated with amidase induced DNA damage in the presence of Cu(II) and displayed a similar DNA cleavage pattern of hydroxylamine. DNA damage was inhibited by both catalase and bathocuproine, suggesting that H_2O_2 and Cu(I) are involved. Carboxy-PTIO, a specific scavenger of nitric oxide (NO), partially inhibited DNA damage. The amount of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) by amidase-treated AHA was similar to that by hydroxylamine. ESR spectrometry revealed that amidase-treated AHA as well as hydroxylamine generated NO in the presence of Cu(II). From these results, it has been suggested that AHA might be converted into hydroxylamine by amidase. These results suggest that metal-mediated DNA damage mediated by amidase-catalyzed hydroxylamine generation plays an important role in the carcinogenicity of acetamide.

Keywords: DNA damage; Acetamide; Copper; Hydrogen peroxide

1. Introduction

Acetamide, used as a solubilizer, plasticizer and as a stabilizer in the synthesis, is carcinogenic in rats and male mice [1]. Long-term feeding of rats with acetamide is known to result in the development of benign and malignant hepatocellular tumors [2–4]. Recently, it has been reported that chromosome malsegregation is induced by acetamide in Drosophila [5]. Acetamide has been classified by the International Agency for Research on Cancer (IARC) as the agent possibly carcinogenic to humans (group 2B) [1]. Amido derivatives such as hexanamide, adipamide and *p*-tolylurea, have carcinogenic potential [6]. Thioacetamide is hepatocarcinogenic to rats and mice [7]. However, the mechanisms leading to carcinogenesis by acetamide remain to be clarified.

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Acetamide was not mutagenic when tested in *Salmonella typhimurium* [8,9]. In contrast, acetohydroxamic acid (AHA), a putative metabolite of acetamide, displayed genotoxic activity [9]. On the basis of these reports, it was therefore possible that AHA is a proximate carcinogenic metabolite of acetamide. Amidases, which catalyze the hydrolysis of compounds containing amido group, are widely distributed in mammalian organs [10–16]. Therefore, AHA is a possible substrate for amidases to produce ultimate carcinogens. To clarify the mechanism of carcinogenesis induced by acetamide, we examined metal-mediated DNA damage caused by amidase-treated AHA using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene. In addition, we analyzed the formation of 8-oxo-7,8-dihydrodeoxyguanosine (8-oxodG), a characteristic oxidative DNA lesion, caused by AHA in the presence of amidase and metal ions. To clarify nitric oxide (NO) generation from AHA, we performed ESR spin trapping study.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from Boehringer Mannheim GmbH (Germany). T_4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). Diethylenetriamine-*N,N,N′,N″,N‴*-pentaacetic acid (DTPA), carboxy-PTIO, *N*-(dithiocarboxy)sarcosine (DTCS) and bathocuproine disulfonic acid were from Dojin Chemical Co. (Kumamoto, Japan). Calf thymus DNA, calf intestine phosphatase (CIP), superoxide dismutase (SOD, 3000 U/mg from bovine erythrocytes), catalase (45,000 U/mg from bovine liver) and amidase (280 U/mg from *Pseudomonas aeruginosa*, recombinant) were from Sigma Chemical Co. (St. Louis, MO). AHA was from Aldrich Chemical Co. (Milwaukee, IL). Nuclease P_1 (400 U/mg) was from Yamasa Shoyu Co. (Chiba, Japan). *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) was obtained from Trevigen Inc. (Gaithersburg, MD, USA).

2.2. Preparation of ^{32}P-5′-end-labeled DNA fragments

Exon-containing DNA fragments were obtained from the human *p53* tumor suppressor gene [17]. The 5′-end-labeled 443 bp (*Apa*I 14179-*Eco*RI* 14621) and 211 bp fragments (*Hin*d III* 13972-*Apa*I 14182) were obtained as described previously [18]. Exon-containing DNA fragments were also obtained from the human *p16* tumor suppressor gene [19]. The 490 bp fragment was further digested with *Mro*I to obtain singly labeled 328 bp fragment (*Eco*RI* 5841-*Mro*I 6168) as described previously [20]. DNA fragments were also obtained from the human c-Ha-*ras*-1 protooncogene [21]. DNA fragments were prepared from the plasmid pbcNI, which carries a 6.6 kb *Bam* HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene. Singly labeled 341 bp (*Xba*I 1906-*Ava*I* 2246) and 337 bp fragments (*Pst*I 2345-*Ava*I* 2681) were prepared as described previously [22]. Nucleotide numbering for the human c-Ha-*ras*-1 protooncogene starts with the *Bam*HI site [21].

2.3. Detection of DNA damage caused by AHA treated with amidase in the presence of metal ions

Standard reaction mixtures (in a 1.5 ml Eppendorf microtube) contained AHA, ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (20 μM/base) and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Where indicated, AHA was pretreated with 0.5 U of amidase in 10 mM sodium phosphate buffer for 1 h at 37 °C prior to incubation. Following a 0.5 h incubation at 37 °C, the DNA fragments were heated for 20 min at 90 °C in 1 M piperidine and electrophoresed followed by autoradiography as described previously [22]. Subsequently, DNA was treated with 1 M piperidine for 20 min at 90 °C or 10 U of Fpg protein in the reaction buffer (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA and 0.1 mg/ml BSA) for 2 h at 37 °C. After ethanol precipitation, the DNA fragments were electrophoresed and the autoradiogram was obtained by exposing X-ray film to the gel as described previously [23]. 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 [24] using a

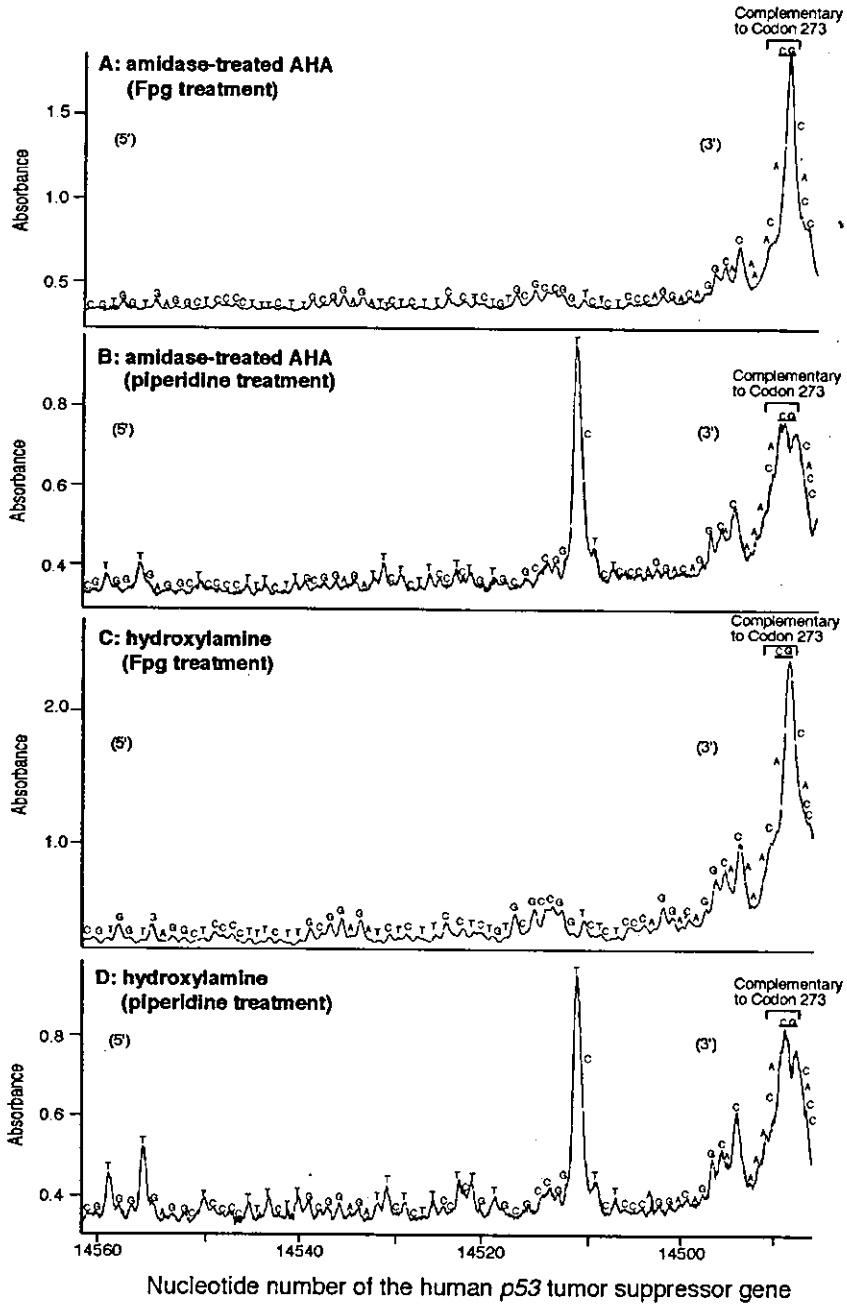

Fig. 1. Site specificity of DNA cleavage induced by amidase-treated AHA or hydroxylamine in the presence of Cu(II). Reaction mixtures contained either the ^{32}P-5′-end-labeled 443 bp fragment (*Apa*I 14179-*Eco*RI* 14621), 20 μM/base of calf thymus DNA, 50 μM amidase-treated AHA (A and B) or hydroxylamine (C and D) and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were incubated for 0.5 h at 37 °C. Following Fpg (A and C) or piperidine treatment (B and D), the DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel. Autoradiograms were visualized by exposing an X-ray film to the gel. The relative quantities of oligonucleotides were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech). Underlined bases represent double-base lesions detected by the treatment with piperidine and Fpg protein.

DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

2.4. Analysis of 8-oxodG formation in calf thymus DNA by AHA treated with amidase in the presence of metal ions

Calf thymus DNA fragments (100 μM/base) were incubated for 0.5 h at 37 °C with AHA or hydroxylamine and 20 μM $CuCl_2$ in 200 μl of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. In a certain experiment, AHA was pretreated with amidase for 1 h at 37 °C. Following ethanol precipitation, the DNA fragments were digested into the nucleosides with nuclease P_1 and calf intestine phosphate, and then analyzed by HPLC-ECD, as described previously [25,26].

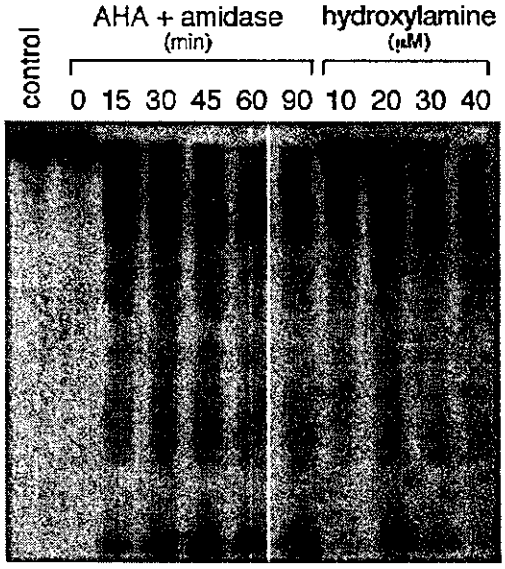

Fig. 2. DNA cleavage induced by amidase-treated AHA or hydroxylamine in the presence of Cu(II). Reaction mixtures contained the ^{32}P-5′-end-labeled 328 bp DNA fragment, 20 μM/base of calf thymus DNA, 50 μM AHA treated with 0.5 U of amidase or indicated concentration of hydroxylamine and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were incubated for indicated time (amidase-treated AHA) or 90 min (hydroxylamine) at 37 °C. DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel.

2.5. Electron spin resonance spin-trapping studies

$Fe(DTCS)_3$ was used as a spin-trapping agent. Reaction mixtures contained either 1 mM hydroxylamine or amidase-treated AHA, 20 μM $CuCl_2$ and $Fe(DTCS)_3$ solution ([Fe] = 10 mM, [DTCS] = 30 mM) in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The $Fe(DTCS)_3$ solution was produced by aerobically dissolving DTCS and $FeCl_3$ in H_2O [20]. Where indicated, AHA was treated for 1 h with amidase

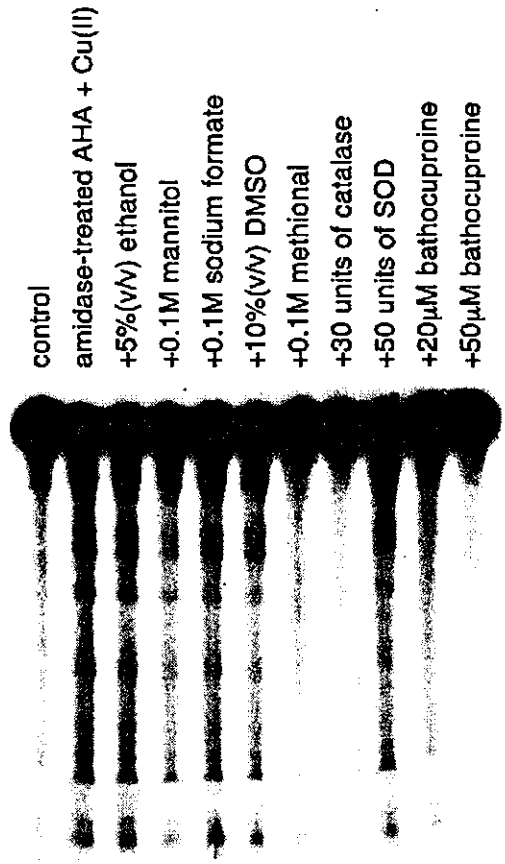

Fig. 3. Effects of scavengers and bathocuproine on DNA damage induced by AHA treated with amidase in the presence of Cu(II). Reaction mixtures contained the ^{32}P-5′-end-labeled 211 bp DNA fragment, 20 μM/base of calf thymus DNA, 50 μM AHA treated with 0.5 U of amidase and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were incubated for 0.5 h at 37° C. DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel.

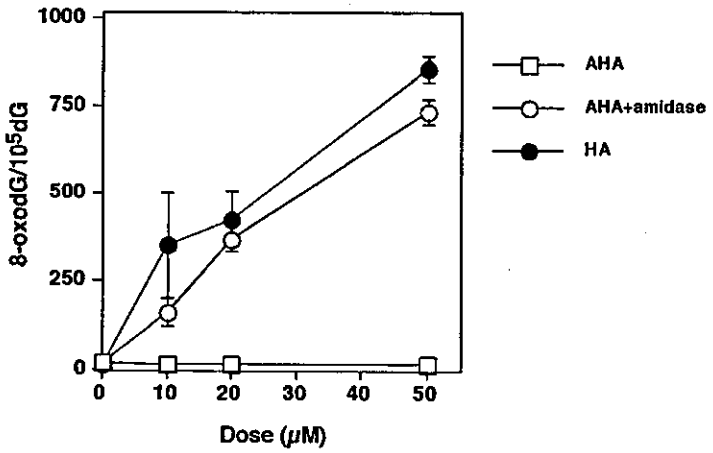

Fig. 4. Formation of 8-oxodG by AHA, amidase-treated AHA and hydroxylamine (HA) in the presence of Cu(II). Reaction mixtures contained calf thymus DNA (100 μM/base), the indicated concentrations of AHA, amidase-treated AHA or hydroxylamine plus 20 μM $CuCl_2$ in 400 μl of 4 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. In a certain experiment, AHA was pretreated with amidase for 1 h at 37 °C. Following an incubation for 0.5 h at 37 °C, 0.2 mM DTPA was added to stop the reaction and then the DNA was precipitated in ethanol. DNA was subjected to enzymatic digestion and analyzed by an HPLC-ECD. Data represent the means ± S.D. of three independent experiments.

at 37 °C. Reaction mixtures were taken up capillary tubes. ESR spectra were measured at room temperature (25 °C), using a JES-TE-100 (JEOL, Tokyo, Japan) spectrometer with 100 kHz field modulation. Spectra were recorded at room temperature with a microwave power of 16 mW, a modulation amplitude of 0.05 mT, a receiver gain of 500, a time constant of 1 s and a sweep time of 4 min. Magnetic fields were calculated using the splitting of Mn(II) in MgO (ΔH_{3-4} = 8.69 mT).

3. Results

3.1. Damage to ^{32}P-labeled DNA fragments by AHA treated with amidase

Fig. 1 showed the site specificity of DNA damage by amidase-treated AHA and hydroxylamine in the presence of Cu(II). The DNA was frequently damaged at T and C with a piperidine treatment (Fig. 1B). With Fpg treatment, the DNA cleavage occurred mainly at G residues (Fig. 1A). Especially, Fpg treatment induced cleavage sites mainly at G residue of the 5′-ACG-3′ sequence complementary to codon 273 of the *p53* gene, whereas piperidine treatment induced cleavage at C of 5′-ACG-3′ (Fig. 1A and B). Therefore, the treated AHA can cause double base lesions at the 5′-CG-3′ sequence at high frequency. Hydroxylamine displayed DNA cleavage pattern similar to that induced by amidase-treated AHA (Fig. 1C and D). AHA alone did not cause DNA damage (data not shown). The treated AHA did not induce DNA damage in the presence of other metal ions, including Co(II), Ni(II), Mn(II), Mn(III), Fe(II), Fe(III) or Fe(III)EDTA (data not shown).

Fig. 2 shows the time course of DNA damage induced by amidase-treated AHA in comparison with dose-dependent DNA damage induced by hydroxylamine. Amidase-treated AHA (50 μM) induced DNA damage to a similar extent to DNA damage induced by 40 μM hydroxylamine for 90 min. This result suggests that approximately 80% of AHA was converted to hydroxylamine.

3.2. Effects of scavengers and a metal chelator on DNA damage induced by AHA treated with amidase

Fig. 3 shows the effects of scavengers and a metal chelator on Cu(II)-mediated DNA damage induced by amidase-treated AHA. Catalase and bathocuproine, a Cu(I)-specific chelator, both inhibited DNA damage, suggesting the involvement of H_2O_2 and Cu(I) (Fig. 3). Free hydroxyl radical (•OH) scavengers, such as ethanol, mannitol, sodium formate and DMSO, demonstrated little or no inhibitory effect on the DNA damage. Methional inhibited the DNA damage. Methional

is capable of scavenging both •OH and species with weaker reactivity [28]. Similar results were obtained for DNA damage caused by hydroxylamine (data not shown). Carboxy-PTIO, a specific scavenger of NO, partially inhibited DNA damage induced by amidase-treaed AHA and hydroxylamine (data not shown).

3.3. Formation of 8-oxodG in calf thymus DNA by AHA treated with amidase

Using an HPLC-ECD, we measured the quantity of 8-oxodG, an indicator of oxidative base damage [25,26], in calf thymus DNA following treatment with variable concentrations of AHA treated with amidase in the presence of Cu(II). The level of 8-oxodG significantly increased with increasing concentrations of AHA (Fig. 4). The amount of 8-oxodG was similar to that by hydroxylamine. AHA alone without amidase treatment did not increase 8-oxodG formation.

3.4. Production of NO from AHA treated with amidase and hydroxylamine

Using $Fe(DTCS)_3$ as a spin-trapping agent, we detected NO generated from amidase-treated AHA and hydroxylamine in the presence of Cu(II) by ESR spin trapping spectroscopy. The ESR spectrum of the NO-bound iron complex [29] generated from amidase-treated AHA had distinct triplet signals with a_N = 1.27 mT and g_{iso} = 2.041, reasonably assigned to the $Fe(DTCS)_2(NO)$ [27] (Fig. 5A). The NO-Fe-DTCS complex [28] generated from hydroxylamine displayed a spectrum similar to that generated from amidase-treated AHA (a_N = 1.27 mT and g_{iso} = 2.040) (Fig. 5B). No signal was observed with non-treated AHA (Fig. 5C).

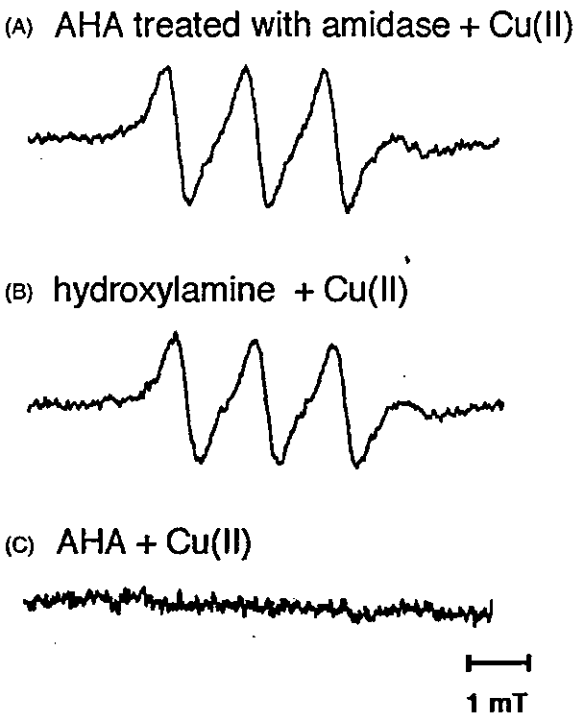

Fig. 5. ESR spectra of $Fe(DTCS)_2(NO)$ complex, derived from hydroxylamine or amidase-treated AHA in the presence of Cu(II). Reaction mixtures contained 20 μM $CuCl_2$, $Fe(DTCS)_3$ solution ([Fe] = 10 mM, [DTCS] = 30 mM) and either 1 mM AHA (A and C) or 1 mM hydroxylamine (B) in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Where indicated, AHA was pretreated with 2.5 U of amidase for 1 h at 37 °C (A). Immediately after the addition of $CuCl_2$ and $Fe(DTCS)_3$ solution, the reaction mixtures were taken up capillary tubes and spectra were then measured at room temperature.

4. Discussion

In this study, AHA caused oxidative DNA damage in the presence of Cu(II), when it was treated by amidase, which is widely distributed in mammalian organs [10–16]. The treated AHA can cause double-base lesions at the CG sequence at high frequency in 5′-ACG-3′ sequence complementary to codon 273, a well-known hot spot [30,31] of the *p53* gene. Recently, it has been reported that reactive oxygen species induce double base lesions, consisting of guanine and an adjacent pyrimidine base [32–34]. It is reported that clustered damage including double-base lesions, which can be demonstrated in living cells, is poorly repaired [35]. Therefore, double-base lesions caused by reactive oxygen species generated from amidase-treated AHA appear to play an important role in acetamide-induced carcinogenesis.

The inhibitory effects of catalase and bathocuproine on the DNA damage suggest that both H_2O_2 and Cu(I) participate in DNA damage. Typical •OH scavengers demonstrated little or no inhibitory effect, whereas methional inhibited DNA damage. This result suggests the involvement of reactive species with a similar reactivity to •OH [28]. It is considered that reactive

species such as the Cu(I)-hydroperoxo complex formed from H_2O_2 and Cu(I) are involved in DNA damage by amidase-treated AHA. However, •OH may participate in DNA damage through the formation of the DNA-Cu(I)-hydroperoxo complex, which releases •OH to attack the adjacent DNA constituents prior to being scavenged by •OH scavengers [36].

Hydroxylamine displayed DNA cleavage pattern similar to that induced by amidase-treated AHA. The amount of 8-oxodG by amidase-treated AHA was also similar to that by hydroxylamine. Furthermore, ESR spectrometry revealed that amidase-treated AHA as well as hydroxylamine generated NO in the presence of Cu(II). Amidases have been detected in human liver [13,15]. It has been demonstrated that human liver microsome preparations are capable of catalyzing hydrolysis of compounds containing amido group, and the hydrolysis was inhibited by an amidase inhibitor [14]. These references and our results support an idea that a large part of AHA can undergo amidase-catalyzed hydrolysis to generate hydroxylamine as an ultimate hepatocarcinogen in humans. Hydroxylamine derivatives are capable of form DNA adduct formation [37] in addition to oxidative DNA damage. Thus, there remains the possibility that DNA adduct formation by hydroxylamine is involved in acetamide-induced carcinogenesis.

On the basis of these results and reports, we propose a possible mechanism whereby amidase-treated AHA induces Cu(II)-mediated DNA damage (Fig. 6). Amidase catalyzed AHA to hydroxylamine and acetic acid. Acetic acid has been reported to be an important metabolite of AHA in mice [38]. Hydroxylamine is then autoxidized by Cu(II) into the hydronitroxide radical, leading to the production of HNO and NO [39]. Cu(II) is reduced to Cu(I) during the autoxidation, and O_2^- is concomitantly generated, followed by dismutation to H_2O_2. H_2O_2 interacts with Cu(I) to form the Cu(I)-hydroperoxo complex, capable of inducing DNA damage. In addition, amidase-treated AHA generated NO in the presence of Cu(II). The generation of NO is confirmed by the finding that carboxy-PTIO, a specific scavenger of NO, showed an inhibitory effect on DNA damage induced by amidase-treated AHA and hydroxylamine. We have previously demonstrated that depurination of DNA is mediated by peroxynitrite generated from NO plus O_2^- during Cu(II)-mediated autoxidation of hydroxylamine generated from esterase-treated urethane metabolite [40]. Therefore, NO may also be concerned with DNA damage by amidase-treated AHA. Copper exists in the mammalian cell nucleus, and may contribute to high-order chromatin structures [41]. Copper, which occurs in the mammalian cell nucleus, is believed to play a central role in the formation

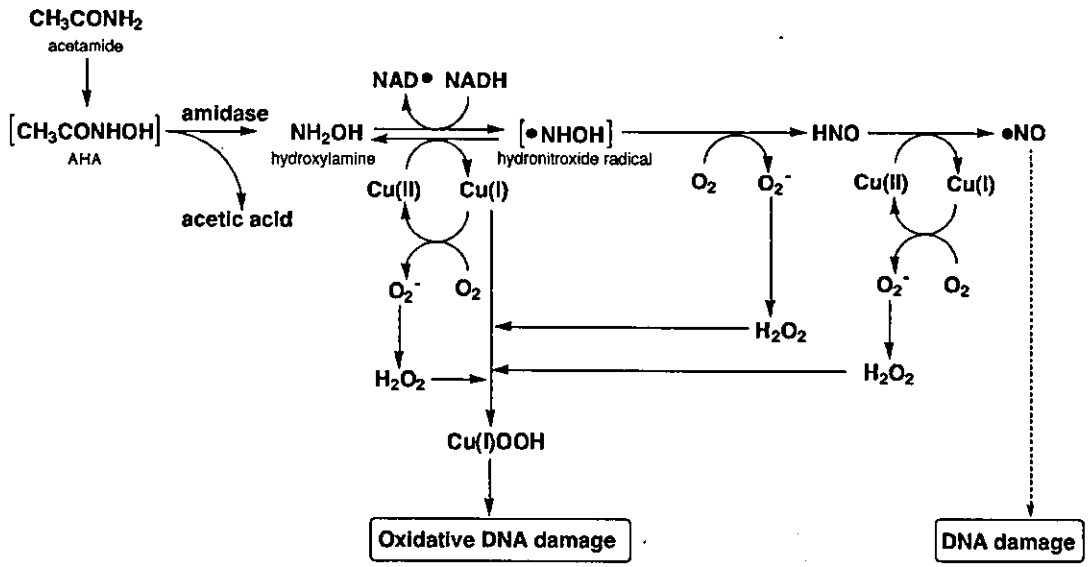

Fig. 6. Possible mechanisms whereby acetamide metabolites induce DNA damage in the presence of Cu(II).

of reactive oxygen species and produce DNA damage leading to carcinogenesis [42–45]. These studies support the finding that amidase-treated AHA caused DNA damage by generating ROS and NO through the interaction with copper.

In this study, we have demonstrated that amidase-treated AHA can cause oxidative DNA damage, probably double-base lesions at the 5′-CG-3′ sequence. We have shown that G residue in 5′-CG-3′ sequence was oxidized to 8-oxodG, which can cause the misreplication of DNA (G:C → T:A transversion) that might lead to mutation or cancer [46–48]. It is considered that oxidation of DNA by hydroxylamine appears to play an important role in carcinogenesis induced by acetamide and compounds containing amido group, hexanamide, adipamide thioacetamide and *p*-tolylurea. AHA is a putative metabolite of acetamide and displays genotoxic activity [9]. *N*-fluoren-2-yl-acetamide (2-acetylaminofluorene) is known to be metabolized to *N*-fluoren-2-yl-acetohydroxamic acid (*N*-hydroxy-2-acetylaminofluorene) [49]. Therefore, acetamide and its derivatives could be metabolized to corresponding *N*-hydroxamic acids. *N*-hydroxamic acids, the derivatives of hydroxylamine, are proximate carcinogenic metabolites of several hepatocarcinogenic aromatic amines [50]. In conclusion, these compounds may participate in their carcinogenicity through similar mechanisms.

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Amplification of Anticancer Drug-Induced DNA Damage and Apoptosis by DNA-Binding Compounds

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Abstract: A number of anticancer drugs exert their effect by causing DNA damage and subsequent apoptosis induction. Most anticancer drugs are known to cause severe side effects. Nontoxic amplification of DNA-cleaving activity of anticancer drugs would enable to reduce drug dose and side effects, leading to development of effective chemotherapy. As a method to approach new cancer chemotherapy, we have investigated the enhancing effects of DNA-binding ligands ("amplifiers"), especially minor groove binders and intercalators, on anticancer drug-induced apoptosis and DNA cleavage, using human cultured cells and ^{32}P-labeled DNA fragments obtained from the human genes. We have demonstrated as follows: a) DNA-binding molecules (unfused aromatic cations, distamycin A and synthtic triamides) induced amplification of bleomycin-induced DNA cleavage and apoptosis; b) a minor-groove binder distamycin A enhanced duocarmycin A-induced DNA cleavage; c) actinomycin D altered the site specificity of neocarzinostatin-induced DNA cleavage and distamycin A enhanced C1027-induced apoptosis. The mechanism of amplification of DNA cleavage can be explained by assuming that binding of amplifier changes the DNA conformation to allow anticancer drug to interact more appropriately with the specific sequences, resulting in enhancement of anticancer effect. The study on amplifiers of anticancer agents shows a novel approach to the potentially effective anticancer therapy.

Key Words: Amplifier, anticancer drug, DNA damage, apoptosis, DNA-binding compound, minor-groove binder, bleomycin, enediyne.

1. INTRODUCTION

DNA is the molecular target for a number of anticancer drugs [1]. Such anticancer drugs exert their effects by inducing apoptosis following DNA damage [2-6] Most anticancer drugs are known to cause severe side effects. Nontoxic amplification of DNA-cleaving activities of anticancer drugs would enable to reduce drug dose and side effects, leading to development of effective chemotherapy. It is very attractive idea that DNA-binding ligands, which recognize specific base sequences, could amplify the activity of anticancer drugs, when they appropriately interact with DNA microstructure [7, 8]. We discovered that distamycin A enhanced duocarmycin A-induced alkylation at G•C rich sequences through cooperative recognition [9]. Since then, as a method to approach new cancer chemotherapy, we have investigated the enhancing effects of DNA-binding molecules ("amplifiers"), including minor groove binders and intercalators, on anticancer drug-induced DNA cleavage and apoptosis. The effects of DNA-binding ligands on anticancer drug-induced DNA cleavage are summarized in Table 1. The chemical structures of anticancer drugs and DNA-binding compounds used in our studies are shown in Fig. (1).

2. EFFECTS OF DNA BINDING LIGANDS ON BLEOMYCIN-INDUCED DNA CLEAVAGE AND APOPTOSIS

Bleomycins have been used for the treatment of tumors of head and neck, lungs and testes. Bleomycins are known to cause severe side effects, such as pulmonary fibrosis [10-12]. It was suggested that toxic O_2-derived species participate in the bleomycin lung toxicity [13]. Bleomycins are activated in the presence of Fe(II) and O_2 through the formation of the bleomycin-iron-oxygen complex, which induces DNA cleavage preferably at 5'-GC-3' and 5'-GT-3' sequences (damaged bases are underlined) by abstracting hydrogen from deoxyribose [14]. Bleomycins recognize the guanine base using the metal-binding domain and the HO_2^- group is positioned close to the thymine or cytosine residues [14]. The bithiazole group intercalates into between base pairs [15]. The carbohydrate moiety of bleomycin is also important for DNA recognition [16]. The bleomycin-mediated DNA degradation was amplified by polyamines through the binding to the major groove [17]. The addition of actinomycin caused alterations in the sequence specificity of bleomycin-induced DNA cleavage [16]. We have investigated the effects of various DNA-binding molecules (unfused aromatic cations, distamycin A and synthtic triamides) on bleomycin (pepleomycin)-induced DNA cleavage and cytotoxicity. Pepleomycin, which has a phenyl ring attached to the bithiazole moiety, is a semi-synthetic bleomycin with cytostatic activity and less pulmonary toxicity than the natural bleomycin mixture.

2.1. Unfused Aromatic Cations (RW-12, LS-20, 1S-5Me)

We have investigated whether pepleomycin-mediated DNA cleavage, cytotoxicity and apoptosis are amplifed by unfused aromatic cations (RW-12, LS-20 and 1S-5Me). These aromatic cations bind to DNA by a nonstandard intercalation mode [18]. RW-12 enhanced most effectively pepleomycin-induced cytotoxicity and apoptosis in human myelogenous leukemic cell line HL-60 [7] (Fig. (2)). The

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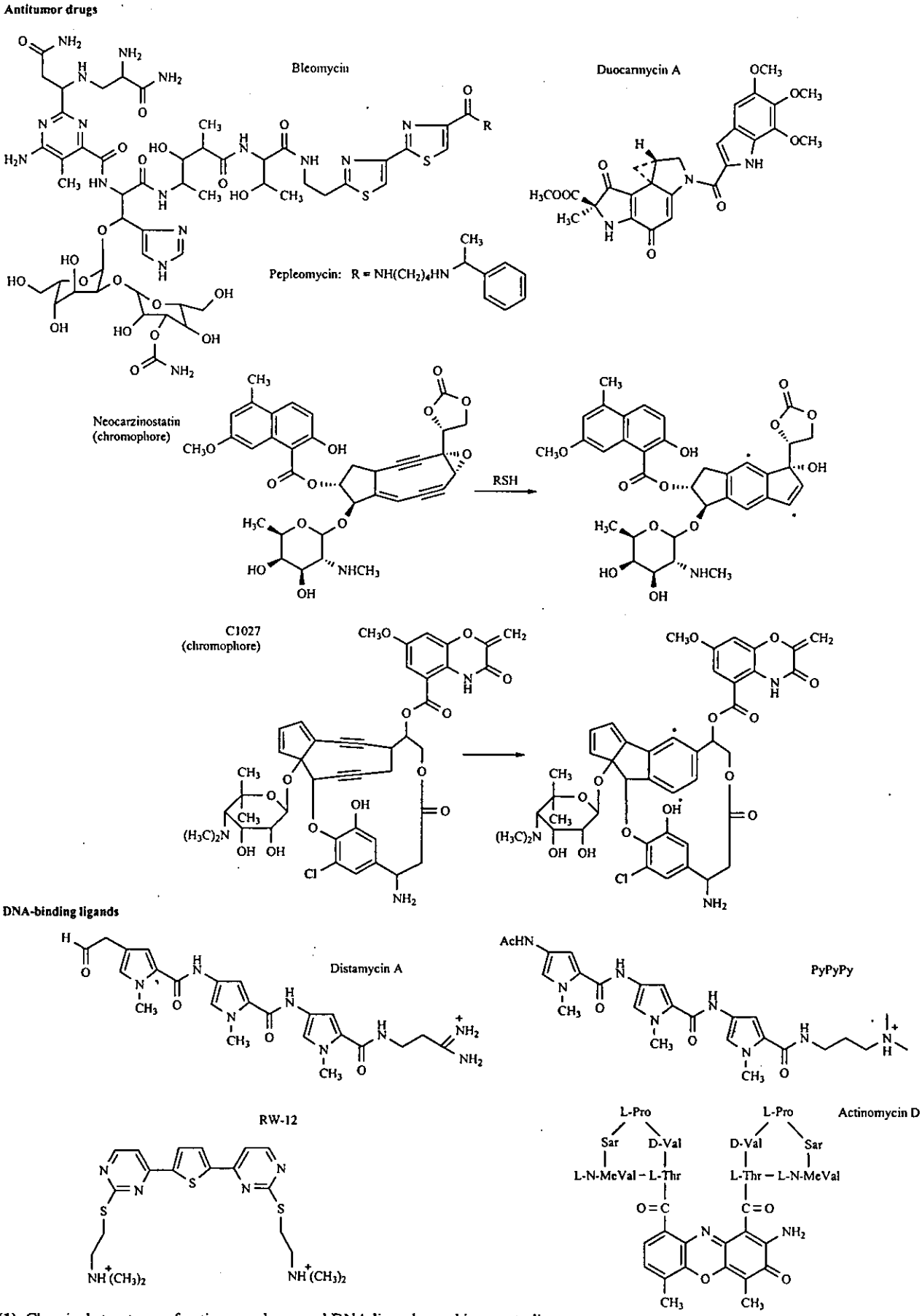

Fig. (1). Chemical structures of anticancer drugs and DNA ligands used in our studies.

Table 1. Effects of DNA Binding Ligands on DNA Cleavage Induced by Anticancer Drugs

DNA binding ligands	*Anticancer drugs*			
	Bleomycin (Pepleomycin)	Duocarmycin	Neocarzinostatin	C1027
Distamycin A	+ [19]	+ [9]	-	+ [28]
PyPyPy	+ [20]	+ [22]		
RW-12	+ [7]		-	
Actinomycin D	+ [16]		+ [27]	-
Chromomycin A_3	+ [19]		-	±
Mithramycin A	+		-	

+: enhanced anticancer drug-induced DNA cleavage at specific sequences

-: no effect on DNA cleavage

[ ]: reference number

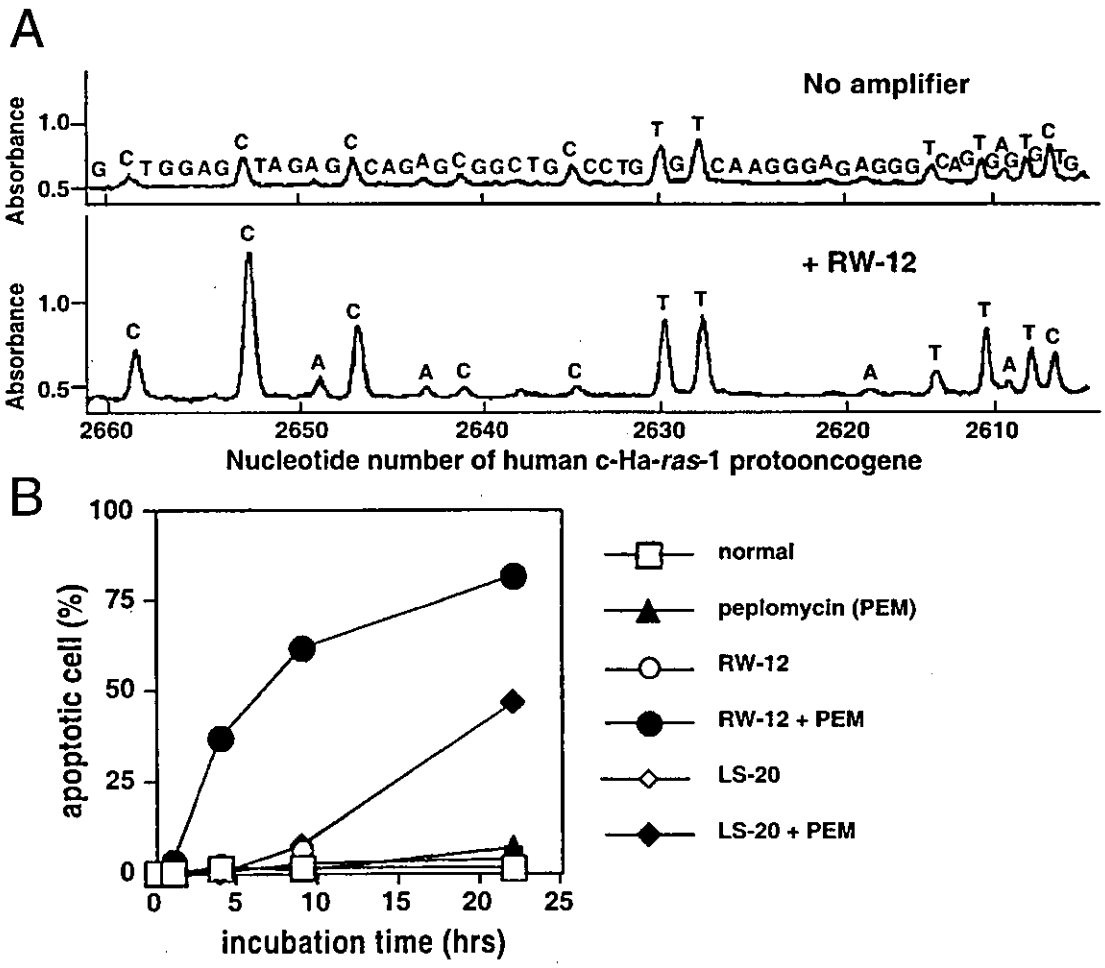

Fig. (2). Amplification of peplomycin-induced DNA cleavage and cytotoxicity by unfused aromatic cations. (A) Alteration of site specificity of peplomycin-induced DNA cleavage by amplifiers. The ^{32}P 5' end-labeled DNA fragment in 10 mM sodium phosphate buffer at pH 7.9 containing 50 μM/base of sonicated calf thymus DNA was incubated with 1 μM pepleomycin, 1 μM $FeSO_4(NH_4)_2SO_4$ in the absence or presence of 20 μM RW-12 at 37 °C for 5 min. The treated DNA fragments were electrophoresed and analyzed as reported previously [7]. (B) Apoptosis in HL-60 cells treated with pepleomycin 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 cells showing condensed chromatin in the acridine orange staining was determined.

order of the enhancing effect of these compounds on pepleomycin-mediated cytotoxicity was RW-12 > LS-20 > 1S-5Me. These compounds induced amplification of pepleomycin-induced cleavage of DNA fragments obtained from human cancer-relevant genes in the same order. Pepleomycin plus Fe(II) caused DNA cleavage at the 5'-GC-3' and 5'-GT-3' sequences (damaged bases are underlined). When DNA was incubated with RW-12 prior to addition of pepleomycin, 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'. Wilson *et al* [18] have reported that the order of the DNA binding constants of these compounds is RW-12 > LS-20 > 1S-5Me. These results suggest that these aromatic cations bind to DNA and then enhance pepleomycin-mediated DNA cleavage, ultimately leading to apoptosis.

2.2. Distamycin A

We examined the effects of a minor groove binder, distamycin A, which binds to A•T rich sequences, on bleomycin-induced DNA cleavage. The addition of distamycin A enhanced bleomycin-induced DNA cleavage at G•C rich sequences such as 5'-GGGGC-3' [19]. The cleavage in this sequence in the presence of distamycin A was about 100-fold greater than that in the absence of distamycin A. Hoechst 33258, 4', 6-diamidino-2-phenylindole (DAPI) and berenil did not cause extensive enhancement of DNA cleavage. These results suggest that the distamycin-induced conformational changes of DNA through interactions other than the DNA minor groove binding in A•T-rich sequences are specifically suitable for the bleomycin action.

2.3. Synthetic Triamides

We also investigated the amplification of pepleomycin-induced DNA cleavage by synthetic triamides containing *N*-methylpyrrole (Py) and/or *N*-methylimidazole (Im), PyPyPy, PyPyIm, PyImPy, and PyImIm, which are minor groove binders. The addition of PyPyPy dramatically enhanced the DNA cleavage at cytosine residues 3' to consecutive guanines (5'-GGC-3', 5'-GGGC-3' and 5'-GGGGC-3'), and to a lesser extent, at thymines in the 5'-GGGGT-3' sequence and guanines in the 5'-GG-3' sequence, whereas the other triamides did not. DNase I footprinting revealed that PyPyPy bound to the sites adjacent to the sites where DNA cleavage was enhanced by PyPyPy [20]. These findings suggest that binding of distamycin A or PyPyPy to the DNA minor groove changes the DNA conformation to allow pepleomycin to cleave DNA more efficiently at GC-rich sequences, particularly at cytosines at the 3'-side of polyguanines.

3. EFFECT OF DISTAMYCIN A ON DUOCARMYCIN A-INDUCED DNA CLEAVAGE

Duocarmycin A is an anticancer antibiotic containing a reactive cyclopropane ring. The cyclopropyl moiety alkylates *N*3 of adenine at the 3' end of sequences of three or more consecutive A or T in double-stranded DNA [14]. The recognition of the A•T rich sequences by duocarmycin A is thought to be through binding to the minor groove. We examined the effects of minor groove binders with affinity to A•T rich sequences, distamycin A, berenil, Hoechst 33258 and DAPI, on DNA cleavage by duocarmycin A. The treatment of DNA with duocarmycin A plus distamycin A caused alkylation of guanine residues at the 5' end of G•C rich sequences, which was not alkylated by duocarmycin A alone. Guanine alkylation by duocarmycin A was not observed with berenil, Hoechst 33258 or DAPI. HPLC product analysis showed that duocarmycin A plus distamycin A produced a duocarmycin A-guanine adduct in double helical DNA octamer $d(CCCCGGGG)_2$. These results suggest that duocarmycin A and distamycin A cooperatively recognize G•C rich sequences of DNA, resulting in enhanced alkylation at these sequences [9]. Our hypothesis has been confirmed by the NMR spectroscopy data showing that duocarmycin A, distamycin A and double-stranded oligonucleotide form the ternary alkylated complex [21]. It has been reported that the site specificity of duocarmycin-induced DNA alkylation is also altered by addition of pyrrole-imidazole triamides (PyPy Py, PyPyIm, ImPyPy, PyImIm, PyImPy and ImImIm) [22].

4. EFFECTS OF DNA BINDING LIGANDS ON ENEDIYNE-INDUCED DNA CLEAVAGE AND APOPTOSIS

Neocarzinostatin (NCS) and C1027 are enediyne anticancer antibiotics containing apoprotein and chromophore. The enediyne moiety of the chromophore of NCS undergoes rearrangement to form a highly reactive benzenoid diradical species in the presence of thiols, such as glutathione, whereas in the case of C1027, the enediyne ring is spontaneously converted to the corresponding diradical (Fig. (**1**)). These diradicals, positioned in the DNA minor groove, abstract hydrogen atoms from the deoxyribose, resulting in double-strand breaks with a two-nucleotide 3'-stagger of the cleaved residues [23-25].

4.1. Effect of Actinomycin D on NCS-Induced DNA Cleavage

We examined the effect of actinomycin D (ActD) on NCS-induced DNA cleavage. ActD is an anticancer antibiotic, which is known to inhibit mRNA synthesis. The phenoxazone ring of ActD intercalates particularly at the 5'-GC-3' sequence, and each pentapeptide is located in the minor groove of the DNA helix to form a hydrogen bond with a deoxyguanosine residue to stabilize the DNA-ActD complex [14, 26]. The intercalation of ActD unwinds the DNA helix and widens the minor groove [26]. In our study, NCS plus glutathione caused DNA cleavage at thymine and adenine residues. The addition of ActD enhanced the double stranded DNA cleavage particularly at the 5'-TCT-3'/3'-AGA-5', 5'-TGT-3'/3'-ACA-5' and 5'-ACT-3'/3'-TGA-5' sequences [27]. The mechanism of ActD-mediated amplification of DNA cleavage can be explained by assuming that binding of ActD to DNA changes the DNA conformation to allow NCS to bind to DNA at the specific sequences.

4.2. Effect of Distamycin A on C1027-Induced DNA Cleavage and Apoptosis

In addition, we examined the effects of a minor groove binder, distamycin A, on DNA cleavage and apoptosis induced by C1027. C1027 alone induced DNA cleavage particularly at the 5'-TTTT-3'/3'-AAAA-5' sequence. The

addition of distamycin A enhanced the double-strand DNA cleavage at the 5'-CCT-3'/3'-GGA-5' and 5'-CCA-3'/3'-GGT-5' sequences. Distamycin A enhanced C1027-induced cytotoxicity and DNA ladder formation, a characteristic of apoptosis, in human promyelocytic HL-60 leukemia cells [28]. These results suggest that distamycin A forms a heterodimer with C1027 to bind to DNA at GC-rich regions, resulting in amplification of DNA cleavage and apoptosis. Therefore, amplification of DNA cleavage at GC-rich regions may result in enhancement of apoptosis.

CONCLUSION

We examined the effects of various DNA-binding ligands, particularly minor groove binders and intercalators, on anticancer drug-induced DNA cleavage and apoptosis. The mechanism of DNA-binding compound-mediated amplification of anticancer drug-induced DNA cleavage can be explained by assuming that binding of amplifier to DNA changes the DNA conformation to allow anticancer drug to bind to DNA at the specific sequences. The observation that these compounds significantly amplify DNA cleavage and apoptosis may provide useful information for the design of amplifiers of a great clinical advantage. Recently, it has been reported that DNA-binding molecules conjugated with alkylating agents, such as an imidazole-pyrrole diamide-cyclopropylpyrroloindole conjugate, induce highly sequence-selective alkylation of double-stranded DNA [29, 30]. The present study on amplifiers of anticancer agents showed a novel approach to the potentially effective anticancer therapy.

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Original Contribution

OXIDATIVE DNA DAMAGE INDUCED BY A HYDROPEROXIDE DERIVATIVE OF CYCLOPHOSPHAMIDE

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Abstract—Interstrand DNA cross-linking has been considered to be the primary action mechanism of cyclophosphamide (CP) and its hydroperoxide derivative, 4-hydroperoxycyclophosphamide (4-HC). To clarify the mechanism of anti-tumor effects by 4-HC, we investigated DNA damage in a human leukemia cell line, HL-60, and its H_2O_2-resistant clone HP100. Apoptosis DNA ladder formation was detected in HL-60 cells treated with 4-HC, whereas it was not observed in HP100 cells. 4-HC significantly increased 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation, a marker of oxidative DNA damage, in HL-60 cells. On the other hand, CP did not significantly induce 8-oxodG formation and apoptosis in HL-60 cells under the same conditions as did 4-HC. Using ^{32}P-labeled DNA fragments from the human *p53* tumor suppressor gene, 4-HC was found to cause Cu(II)-mediated oxidative DNA damage, but CP did not. Catalase inhibited 4-HC-induced DNA damage, including 8-oxodG formation, suggesting the involvement of H_2O_2. The generation of H_2O_2 during 4-HC degradation was ascertained by procedures using scopoletin and potassium iodide. We conclude that, in addition to DNA cross-linking, oxidative DNA damage through H_2O_2 generation may participate in the anti-tumor effects of 4-HC.

Keywords—4-Hydroperoxycyclophosphamide, Cyclophosphamide, Hydrogen peroxide, Oxidative DNA damage, Apoptosis, Free radicals

INTRODUCTION

Cyclophosphamide (CP) is a widely used antineoplastic drug for treating malignant lymphoma, multiple myeloma, leukemia, and other malignant diseases. 4-Hydroperoxycyclophosphamide (4-HC) is a hydroperoxide derivative of cyclophosphamide. In autologous hematopoietic stem cell transplantation, 4-HC has been used to decrease numbers of infused tumor cells as pharmacological purging since 1980 [1].

CP is metabolized to 4-hydroxy-CP in the liver by cytochrome P450 2B1 isozyme. 4-HC is readily converted into 4-hydroxy-CP without metabolic activation [2]. 4-Hydroxy-CP is unstable, so it spontaneously breaks down to the reactive intermediates phosphoramide mustard (PAM) and acrolein. PAM is converted to aziridinium ion, which then alkylates at the N7 position of guanine residue in DNA. This reaction with another guanine in the other strand makes the interstrand DNA cross-link. Interstrand DNA cross-links are found after exposure of cultured tumor cells to PAM [3,4]. The interstrand DNA cross-linking is considered to be the primary action mechanism of CP and 4-HC [5,6]. However, 4-HC-induced cytotoxicity was linked to not only DNA cross-links but also DNA fragmentation [7,8]. Millar et al. suggested that the formation of cross-links was not very important for the toxicity of 4-HC [9]. 4-HC was about 250- and 2-fold more effective in induction of DNA single-strand breaks and alkali labile sites than CP and PAM, respectively [10]. Furthermore, 4-HC inhibited tumor

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growth more effectively than CP [11]. On the basis of the fact that 4-HC is a hydroperoxide derivative, we expected that oxidative DNA damage by 4-HC may also play a role in anti-tumor effects, in addition to DNA cross-linking.

In this study, we examined 4-HC-induced apoptosis using a human leukemia cell line, HL-60, and its hydrogen peroxide (H_2O_2)-resistant clone HP100. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is one of the most widely studied lesions and has attracted attention due to its mutagenic property, although many types of oxidatively modified bases of DNA have been identified [12,13]. We measured 8-oxodG formation in HL-60 as a marker for oxidative DNA damage by high-pressure liquid chromatography coupled with an electrochemical detector (HPLC-ECD). Furthermore, we compared the abilities of CP and 4-HC to cause DNA damage, using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and c-Ha-*ras*-1 proto-oncogene. We also measured 8-oxodG formation in calf thymus DNA in the presence of Cu(II) and NADH.

MATERIALS AND METHODS

Materials

Restriction enzymes (*Sma*I, *Eco*RI, *Apa*I, and *Sty*I) and proteinase K were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Restriction enzymes (*Hin*dIII, *Ava*I, and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). [γ-^{32}P]ATP (222 TBq/mmol) was obtained from New England Nuclear (Boston, MA, USA). 4-HC was a kind gift from Shionogi Co. (Osaka, Japan). Cyclophosphamide monohydrate was from ICN Biomedicals, Inc. (Aurora, OH, USA). β-Nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH) was purchased from Kohjin Co. (Tokyo, Japan). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were obtained from Dojin Chemicals Co. (Kumamoto, Japan). Superoxide dismutase (SOD; 3000 U/mg from bovine erythrocytes), catalase (45,000 U/mg from bovine liver), bacterial alkaline phosphatase, and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lysis buffer for DNA extraction (Model 340A) was purchased from Applied Biosystems (Foster City, CA, USA). Copper(II) chloride dihydrate, ethanol, and scopoletin were from Nacalai Tesque, Inc. (Kyoto, Japan). Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). Horseradish peroxidase was from Toyobo Co. (Osaka, Japan). Bacterial formamidopyrimidine-DNA glycosylase (Fpg protein) was from Trevigen, Inc. (Gaithersburg, MD, USA).

Detection of apoptotic cells and DNA ladder formation induced by 4-HC

Human leukemia HL-60 cells and their H_2O_2-resistant clone HP100 cells [14] were grown in RPMI 1640 supplemented with 6% fetal bovine serum at 37°C under 5% CO_2 in a humidified atmosphere. HL-60 cells and HP100 (1×10^6 cells/ml) were incubated with 4-HC in 2 ml of RPMI 1640 supplemented with 6% fetal bovine serum for indicated times at 37°C. Apoptotic cells were determined by fluorescence microscopy after staining with 0.02 mg/ml acridine orange. For the detection of DNA ladder formation, the medium was removed and the cells were washed twice with PBS. The cells were suspended in 1 ml of cytoplasm extraction buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $MgCl_2$ in 0.5% Triton X) and centrifuged. The pellet containing nuclei was treated with lysis buffer (10 mM Tris, pH 7.5, 400 mM NaCl, and 1 mM EDTA in 1% Triton X) for 10 min and centrifuged at 4°C. Precipitation containing nuclear membrane and large intact DNA was discarded. The supernatant containing mainly low-molecular-weight DNA due to apoptotic fragmentation was treated with 0.2 mg/ml RNase overnight at room temperature and subsequently with 0.1 mg/ml proteinase K for 2 h at 37°C. The DNA was extracted with phenol/chloroform and then with water-saturated ether and precipitated with ethanol overnight at −20°C. The pellet was dissolved in 20 μl of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). The low-molecular-weight DNA was electrophoresed on 1.4% agarose gel containing 0.375 μg/ml ethidium bromide in 0.5× TBE buffer.

Measurement of 8-oxodG in cultured cells

HL-60 cells (1×10^6 cells/ml) were incubated with 4-HC for 2 h at 37°C and immediately washed three times with cold PBS. DNA was extracted by using 500 μl lysis buffer, 0.05 mg/ml RNase A, and 0.5 mg/ml proteinase K at 60°C for 1 h. DNA was digested to component nucleosides with nuclease P_1 and bacterial alkaline phosphatase and analyzed by HPLC-ECD using a previously described method [15,16].

Preparation of ^{32}P-5′-end-labeled DNA fragments

Exon-containing DNA fragments obtained from the human *p53* tumor suppressor gene [17] were prepared as described previously [18]. A 5′-end-labeled 650 bp fragment (*Hin*d III*13972–*Eco*RI*14621) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase (*, ^{32}P label). The 650 bp fragment was further digested with *Apa*I to obtain a singly labeled 443 bp fragment (*Apa*I 14179–*Eco*RI*14621) and a 211 bp

fragment (*Hin*dIII*13972–*Apa*I 14182). A DNA fragment was also obtained from the human c-Ha-*ras*-1 proto-oncogene [19]. The fragment was prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene. A singly labeled 341 bp fragment (*Xba*I 1906–*Ava*I*2246) and a 337 bp fragment (*Pst*I 2345–*Ava*I*2681) were obtained according to the method described previously [20]. Nucleotide numbering starts with the *Bam*HI site [19].

Detection of DNA damage by 4-HC in the presence of Cu(II) and NADH

A standard reaction mixture (in a 1.5 ml Eppendorf microtube) contained 4-HC, Cu(II), ^{32}P-5′-end-labeled DNA fragments, and calf thymus DNA (5 μM per base) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37°C for 1 h, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min where indicated and treated as described previously [20]. For Fpg treatment, the DNA fragments were incubated with 6 units (13.6 μg protein) of Fpg protein in 20 μl of reaction buffer (10 mM Hepes–KOH (pH 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/ml BSA) at 37°C for 2 h. The preferred cleavage sites were determined by direct comparison of the labeled, cleaved oligonucleotides with a standard 5′-end-labeled Maxam–Gilbert sequencing reaction [21] (LKB 2010 Macrophor; LKB Pharmacia Biotechnology, Inc., Uppsala, Sweden). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL; LKB Pharmacia Biotechnology, Inc.).

Analysis of 8-oxodG formation in calf thymus DNA by 4-HC in the presence of Cu(II) and NADH

Native or denatured DNA fragments (100 μM per base) from calf thymus were incubated with 4-HC and Cu(II) at 37°C for the indicated duration. After ethanol precipitation, DNA was digested to its component nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by HPLC-ECD as described previously [15].

Detection of H_2O_2 generation during the degradation of 4-HC

The amounts of H_2O_2 were determined by measuring the extinction of scopoletin fluorescence during its oxidation by horseradish peroxidase [22]. Reactions were performed in cuvettes containing 10 μM scopoletin and 10 μM 4-HC with or without catalase in 10 mM phosphate buffer (pH 7.4) at 37°C. The reaction was initiated by the addition of 1 μM horseradish peroxidase. Fluorescence was measured with a spectrofluorometer (RF-5300PC; Shimadzu, Kyoto, Japan) with sample excitation at 365 nm and emission at 450 nm. The amounts of peroxides, which include 4-HC and H_2O_2, were also measured using potassium iodide [23]. Reaction mixtures contained 4-HC with or without catalase in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. Cuvettes containing sample, acetic acid, and chloroform were deaerated by N_2, and then 50% potassium iodide solution was added. Hydroperoxides have the oxidative capacity to convert iodide to iodine, which can be measured photometrically at 365 nm with a UV–visible spectrophotometer (UV-2500PC; Shimadzu). After the sample was allowed to stand for 30 min in the dark at ambient temperature, absorbance was measured at 365 nm with a spectrophotometer. In both methods, 30 units of catalase was added to detect H_2O_2 generation and calibration curves were obtained using H_2O_2 of known concentrations.

RESULTS

DNA ladder formation and apoptotic changes in cultured cells treated with 4-HC

The electrophoresis of DNA isolated from apoptotic cells reveals characteristic fragmentation by endonuclease, i.e., "DNA ladder" [24]. Figure 1 shows the DNA ladder formation in HL-60 cells treated with 4-HC at 37°C for 4 h. When HL-60 cells were treated with 50 μM CP at 37°C for 4 h, the ladder formation was not observed. Apparent fragmentation was detected in HL-60 cells treated with 4-HC, whereas the ladder formation was not observed in HP100 cells (Fig. 2). Apoptotic

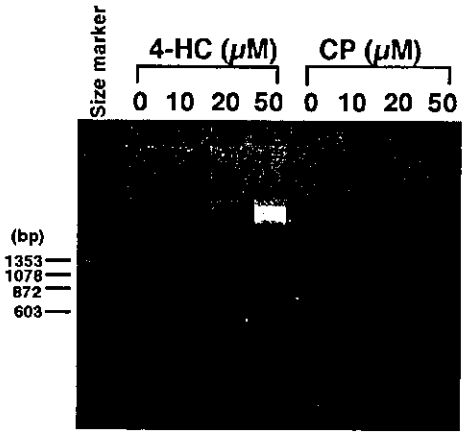

Fig. 1. DNA ladder formation in HL-60 cells induced by 4-HC. HL-60 cells were incubated with the indicated concentrations of 4-HC or CP at 37°C for 4 h. After low-molecular-weight DNA was refined, the DNA was electrophoresed on an agarose gel containing ethidium bromide. The first lane shows the DNA size markers (*Hae*III-digested øX174 DNA).

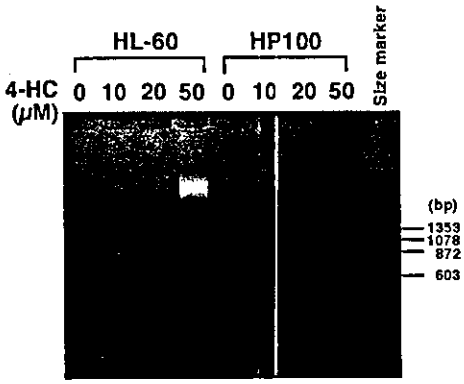

Fig. 2. DNA ladder formation in HL-60 cells and HP100 cells induced by 4-HC. HL-60 cells and HP100 cells were incubated with 4-HC at 37°C for 4 h. After low-molecular-weight DNA was refined, DNA ladder formation was analyzed as described in the legend to Fig. 1. The last lane shows the DNA size markers (*Hae*III-digested øX174 DNA).

cells were observed with acridine orange staining by fluorescence microscopy. 4-HC increased apoptotic cells, identified by chromatin condensation, nuclear fragmentation, and cytoplasmic budding, in HL-60 cells (data not shown). Apparent apoptotic changes were not observed in HP100 cells treated with 4-HC (data not shown).

Induction of 8-oxodG formation in human cultured cells treated with 4-HC and CP

Figure 3 shows 8-oxodG formation in HL-60 cells. 4-HC at the concentration of 50 μM induced a significant increase in 8-oxodG formation compared with negative

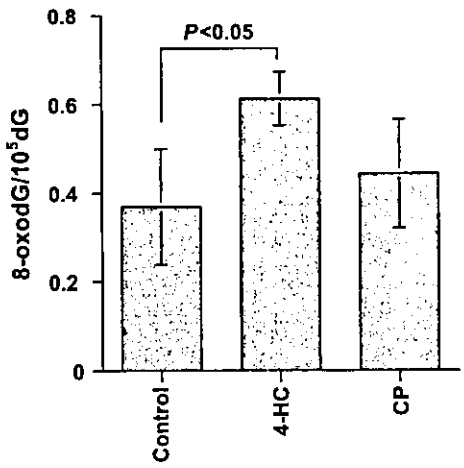

Fig. 3. Comparison of 8-oxodG formation in HL-60 cells treated with 4-HC and CP. Cells were incubated with 4-HC or CP at 37°C for 2 h. After the incubation, the cells were treated for DNA extraction. DNA was digested to nucleosides enzymatically and 8-oxodG content was analyzed by HPLC-ECD, as described under Materials and Methods. Results are expressed as means and SD of values obtained from four independent experiments. Significant differences were analyzed by *t* test compared with control.

control ($p < .05$). On the other hand, CP-induced 8-oxodG formation is not significantly different from negative control.

Damage to ^{32}P-labeled DNA fragments by 4-HC in the presence of NADH and Cu(II)

Figure 4 shows an autoradiogram of DNA fragments treated with CP and 4-HC in the presence of Cu(II) and NADH. The intensity of Cu(II)-mediated DNA damage by 4-HC increased with increasing concentrations of 4-HC. Addition of NADH induced approximately five times stronger DNA damage. CP caused no DNA damage even in the presence of Cu(II) and NADH. 4-HC alone did not cause DNA damage (data not shown). 4-HC induced DNA fragmentation without piperidine or Fpg treatment, indicating strand breakage. Piperidine increased fragmentation of 4-HC-damaged DNA, suggesting existence of base modifications and/or abasic sites. Fpg treatment also increased the number of strand breaks. This result can be explained on the basis of the reports that the Fpg-sensitive lesions were 8-oxo-7,8-dihydroguanine and other oxidized bases [25,26] as well as imidazole-ring-opened alkylated purines [27].

Effects of scavengers and chelators on DNA damage induced by 4-HC in the presence of Cu(II)

Figure 5 shows the effects of scavengers and metal chelators on DNA damage induced by 4-HC in the

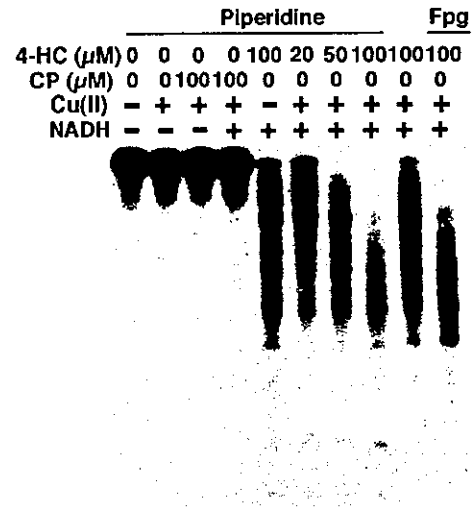

Fig. 4. Autoradiogram of ^{32}P-labeled DNA fragments incubated with 4-HC and CP in the presence and absence of NADH and Cu(II). The reaction mixture contained ^{32}P-5'-end-labeled 443 bp DNA fragments, 5 μM per base calf thymus DNA, indicated concentrations of 4-HC, 200 μM NADH, and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37°C for 1 h, followed by no, piperidine, or Fpg treatment, where indicated. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing a radiographic film to the gel.

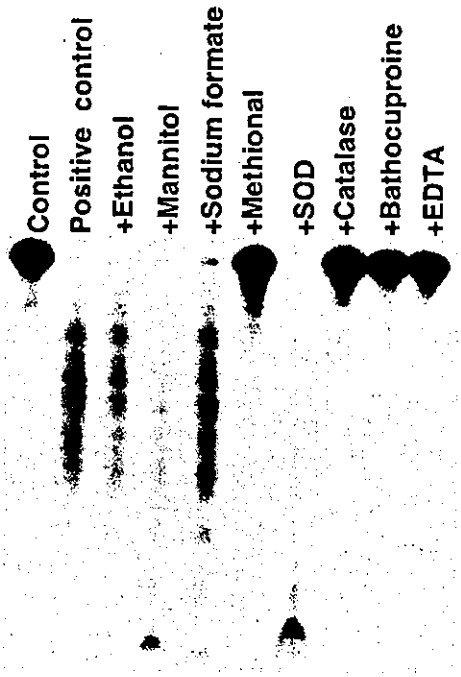

Fig. 5. Effects of scavengers, bathocuproine, and EDTA on DNA damage induced by 4-HC in the presence of Cu(II) and NADH. The reaction mixture contained ^{32}P-5′-end-labeled 443 bp DNA fragments, 5 μM per base calf thymus DNA, 200 μM 4-HC, 200 μM NADH, and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37°C for 1 h, followed by piperidine treatment. The DNA fragments were analyzed as described in the legend to Fig. 4. The concentrations of scavengers, bathocuproine, and EDTA were as follows: 5% (v/v) ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.1 M methional, 30 units of SOD, 30 units of catalase, 50 μM bathocuproine, 50 μM EDTA.

presence of Cu(II). Catalase, bathocuproine, and EDTA inhibited DNA damage, indicating the involvement of H_2O_2, Cu(I), and Cu(II). Methional reduced the amount of DNA damage, although typical hydroxyl radical ($^{\bullet}OH$) scavengers, such as ethanol, mannitol, and sodium formate, did not. SOD slightly enhanced DNA damage.

Site specificity of DNA cleavage by 4-HC in the presence of NADH and Cu(II)

An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA cleavage in the human *p53* tumor suppressor gene. 4-HC together with Cu(II)/NADH induced piperidine-labile sites preferentially at cytosine and thymine residues (Fig. 6A). When Fpg treatment was used instead of piperidine, DNA cleavage occurred more frequently at guanine residues (Fig. 6B). These results concerning piperidine and Fpg treatment led us to the idea that 4-HC induced potential double-base damage at C and G of the 5′-ACG-3′ sequence, the complementary sequence to codon 273, a known hot spot in exon 8 of the *p53* gene [28,29]. The existence of NADH did not affect the cleavage pattern. A similar pattern was also observed in the c-Ha-*ras*-1 proto-oncogene (data not shown).

Formation of 8-oxodG in calf thymus DNA by 4-HC in the presence of Cu(II) and NADH

Cu(II)-mediated 8-oxodG formation in calf thymus DNA treated with 4-HC in the presence and absence of NADH was addressed using HPLC-ECD. The amount of 8-oxodG increased with increasing concentrations of 4-HC in the presence of Cu(II). The formation of 8-oxodG was increased by DNA denaturation (data not shown). When NADH was added, the amount of 8-oxodG formation represented a 2- to 3-fold increase (Fig. 7A). When catalase was added, the formation of 8-oxodG was remarkably inhibited (Fig. 7B).

H_2O_2 generation during the degradation of 4-HC

H_2O_2 was not detected before incubation by the method using scopoletin. After the incubation for 1 h, H_2O_2 was generated which was 65.6% of 4-HC (Fig. 8A). By the iodometric procedure, H_2O_2 was detected as 31.9 and 86.9% of 4-HC before and after incubation, respectively. Concomitantly, peroxide decreased 50.8 to 5.4%, suggesting that the incubation caused the degradation of 4-HC and the formation of H_2O_2 (Fig. 8B). The difference in the H_2O_2 amount between scopoletin and iodometric procedure is explained by the additional reaction time of the latter method.

DISCUSSION

The present study has demonstrated that 4-HC has the ability to cause oxidative damage, including 8-oxodG formation, to cellular and isolated DNA. Formation of 8-oxodG, a marker of oxidative DNA damage, in HL-60 cells was increased by 4-HC, but not increased by CP. Furthermore, apparent DNA ladder formation was detected in HL-60 cells treated with 4-HC, but not CP, under the condition used. On the other hand, 4-HC did not induce the ladder formation in HP100 cells, of which catalase activity of HP100 cells is 18 times higher than that of parent HL-60 cells [14]. We also confirmed that 4-HC-induced 8-oxodG formation preceded DNA ladder formation. Our previous studies have shown that reactive oxygen species induce 8-oxodG formation, followed by the loss of mitochondrial membrane potential ($\Delta\Psi_m$) and subsequent activation of caspase-3, resulting in apoptosis [30–32]. These findings indicate the possibility that oxidative DNA damage through the generation of H_2O_2 induces apoptosis. CP was shown to induce apoptosis in many cell types with higher concentrations and longer incubation times [33,34]. 4-HC induced apoptosis more efficiently than CP under our experimental design (4 h incubation, 50 μM). This may