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Cadmium Inhibits BPDE Alkylation of DNA in the Major Groove but Not in the Minor Groove

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Cadmium, a constituent of tobacco, has the potential to act in synergy with other carcinogens in tobacco smoke. Working on the hypothesis that cadmium interactions with DNA enhances the mutagenic lesions induced by tobacco carcinogens, we investigated the site and sequence selectivity of DNA binding by cadmium using DNA reactive chemical probes. Our results show that this divalent cation binds to N^7 guanines with a great preference for those occurring in runs of G's. Further, cadmium considerably diminishes N^7 guanine alkylation by the tobacco carcinogen metabolite BPDE; however, the biologically relevant guanine alkylation in the minor groove by BPDE was not affected. The relevance of our findings to cadmium's role in the tobacco carcinogenesis is discussed.

Cadmium (Cd^{2+}) is a widely distributed metallic pollutant and a known carcinogen (1). Human exposure to Cd^{2+} is increasing through foods grown in contaminated soils and manufacturing processes (2). The most important source of Cd^{2+} exposure of non-smokers is through the diet consumption of Cd^{2+} contaminated vegetables. The problem of food contamination with Cd^{2+} is likely to remain important since fertilisers and pesticides are increasingly contaminated.

The concentration of Cd^{2+} is elevated in smokers' tissues compared to non-smokers and may play a role in the pathology and carcinogenesis (3). Tobacco contains from 0.01 to 0.35 μg of Cd^{2+} per cigarette of which about 10-20% is inhaled in the mainstream phase (4). Cd^{2+} has been detected in both neoplastic and adjacent non-neoplastic lung tissues (5,6). Cadmium concentrations in tumours and adjacent non-involved tissues were higher than in lung tissue from non-smokers (7). In addition to Cd^{2+} smoke contains polycyclic aromatic hydrocarbons (PAH), nitroso compounds and aldehydes that may contribute to lung cancer. PAH, aromatic amines, nitro aromatic amines, nitrosamines, hydrazines, aflatoxins, and halogenated hydrocarbons can be activated by P-450 to form compounds capable of covalent binding to DNA. Alkylation of DNA is believed to be the first step in the initiation of chemically induced carcinogenesis. Modified bases in specific codons of a proto-oncogene lead to point mutations, which in turn lead to protein products with deleterious effects. *Ras* genes, for instance, are activated when mutation occurs in codons 12 or 61 depending on the nature of the alkylating agent. Methylnitrosourea modifies guanine in codon 12 leading to G-A transition while activated dimethylbenzanthracene (DMBA) alkylates adenine in codon 61 leading to A-T transversion. Smoke-derived DNA adducts have been detected in human lung tissues from cigarette smokers (8). Benzo(a)pyrene, a common PAH found in cigarette smoke, when activated to its ultimate metabolite form, (±)*trans*-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), reacts with the exocyclic amino group of guanine in DNA (9). It also alkylates the N^7 sites in guanines but it is the former which is considered to be the mutagenic event (10).

Increasing exposure to Cd^{2+} is of concern because of its association with renal toxicity and because it is a known carcinogen. However, it is more likely that in the near future that the exposure levels will not be sufficient to induce over kidney damage in the population but it may pose a significant threat as a carcinogen or co-carcinogen. Based on limited epidemiological data and a few animal studies Cd^{2+} has been classified as a carcinogen, though the carcinogenic mechanism has not been delineated. Mandel and Ryser (11) demonstrated that cadmium chloride is a weak mutagen in *S. typhimurium*, but had a strong co-mutagenic effect with the methylating agents methylnitrosourea (MNU) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). The co-mutagenic effect of Cd^{2+} with MNU was confirmed by Takahashi *et al.*, (12) using *E. Coli*. These results suggest that Cd^{2+} augments the formation of

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DNA lesions, the initiating events in the chemical carcinogenesis.

Working on our hypothesis that selective binding of Cd^{2+} to DNA modulates the reactivity of DNA towards electrophilic carcinogens we report here our findings on the interaction of Cd^{2+} with DNA and its effect on the reactivity of BPDE. Like other known heavy metal divalent cations such as Pt^{2+} and Pd^{2+}, it is known that Cd^{2+} binds to N^7 of guanine in the major groove of DNA (13). Here we present evidence using chemical footprinting technique that Cd^{2+} binds to DNA in a sequence selective manner in the major groove but it does not bind in the minor groove. We further show using chemical footprinting and fluorescence techniques that Cd^{2+} inhibits major groove alkylation by BPDE but not its biologically important minor groove alkylation.

MATERIALS AND METHODS

Methylnitrosourea (MNU) and (±)*trans*-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) were obtained from Sigma and Midwest Research Institute (USA) respectively. Fotemustine (FM) was a gift from Servier Labs Australia and bromoethyl phenol (BEP) was a gift from Dr. David Young, Griffith University, Brisbane. The structures of the probes and BPDE are shown in Figure 1. The plasmid pBR322, the restriction enzymes *ECo*R1 and *Bam*H1 and the Klenow fragment were purchased from Promega. The [α-^{32}P] dATP was obtained from Bresatec Ltd.

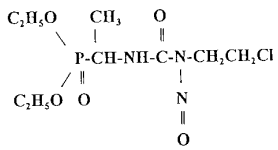
Labeling and isolation of DNA fragment. A 375 base pair *BamHI/EcoRI* fragment of pBR322 was 3′-end-labeled at the *EcoRI* site using [α-^{32}P] dATP with Klenow fragment and was isolated on a 4% non-denaturing polyacrylamide gel (14).

Alkylation of labeled DNA with chemical probes (BPDE, MNU, FM & BEP). The labeled DNA (approx. 15,000 cpm) was incubated in presence and absence of cadmium chloride (final conc. 0.5-10 μM) with different concentrations of chemical probes (BPDE-0.4 μM; MNU-500 μM; FM-125 μM and BEP-125 μM) in TEA buffer (10mM, pH 6.7) for 1 hr at 37° C. Incubation was stopped by ethanol precipitation. The BEP-treated DNA pellet was redissolved in 100 μl of TE buffer (pH 7.4) and incubated at 60° C for 1 hr to induce quantitative depurination at alkylated sites. Higher temperatures were avoided as it causes significant level of depurination in the metal containing DNA control samples. The DNA was then precipitated and all reaction samples were then dissolved in 100 μl of 1M piperidine and subjected to heat treatment (90° C for 10 min). In the case of BEP the two heat treatments lead to depurination and subsequent strand breaks at all purines alkylated at N^7 of guanines and N^3 of adenines. With the other probes, which alkylate guanines but not adenines, the piperidine treatment alone leads to strand breaks at all alkylated N^7 guanine sites. Prior to loading on a 6% denaturing polyacrylamide gel, samples were dissolved in formamide dye and denatured at 90° C for 2 min. Sequencing lanes corresponding to G, Pu and T were loaded to aid sequence identification. The gels were run on a Bio-Rad Sequi-Gen apparatus at 70W (gel temp.50° C) until xylene cyanol had migrated to 26 cm. The gels were then dried and exposed to X-ray film (Kodak 5-XAR) overnight prior to development.

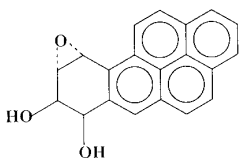
Fluorescence assay. BPDE covalently binds to DNA at N^7 guanine in the major groove and at exocyclic amino group of guanine in the minor groove. The N^7 adduct is piperidine sensitive and can be detected on a sequencing gel as described above. To detect the minor groove adduct which is resistant to chemical treatments used in the sequencing protocols, a fluorescence method is used. BPDE and its

STRUCTURES OF CHEMICAL PROBES

Fotemustine (FM)



Benzo(a)pyrene diol epoxide (BPDE)



Bromoethyl phenol (BEP)

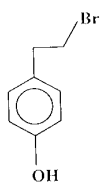


FIG. 1. Structures of FM, BPDE and BEP.

DNA adducts are non-fluorescent but its hydrolyzed product, a tetrol, is highly fluorescent (15). The minor groove adduct can be separated from the major groove adducts by heat treatment at neutral pH which will release all major groove adducts as depurinated products. The precipitated DNA will carry only minor groove adducts which when treated in 0.1N HCl will hydrolyze to a fluorescent tetrol (16).

The calf thymus DNA (approx. 35μg in 1ml) was incubated in the presence and in the absence of cadmium chloride (5μl of 5 mM) with BPDE (5μl of 1mM) for 1 hr at 37°C. At the end of incubation time the reaction products were precipitated with ammonium acetate and ethanol. The supernatant (sup **I**) contained tetrol from unreacted BPDE and was saved for fluorescence measurements. The precipitate (DNA pellet) was then redissolved in TEA buffer and heated at 90°C for 10 min. The reaction products were brought to room temperature before precipitation with ammonium acetate and ethanol. This supernatant (sup **II**) contained depurinated adducts and intercalated tetrol from the precipitated DNA. This time the DNA pellet was redissolved in 0.1N HCl and heated at 90°C for 1 hr. Followed by neutralization with NaOH to pH 7-7.5 before precipitation. The supernatant (sup **III**) contained mainly tetrol released from N^2 adducts. All three supernatant solutions (containing 67% ethanol and roughly 2.5 mM ammonium acetate) were directly used for fluorescence studies without further manipulation. The emission spectra of each su-

pernatant fraction were obtained using Perkin Elmer Luminescence Spectrometer LS50B at a fixed excitation wavelength of 350nm. This wavelength corresponds to a near isosbestic point in the absorbance spectra of BPDE run with and without DNA and/or Cd^{2+}. Therefore the changes in the intensity of the fluorescence peaks observed at this wavelength corresponds to true ground state quenching of fluorescence and not is an artifact arising from absorbance shift in the presence of DNA or Cd^{2+}. It should be noted that the fluorescence spectrum of tetrol in the supernatant solution produces a broad signal unlike the well-resolved peaks normally observed in an aqueous solution.

RESULTS

Figure 2 shows the autoradiogram of a chemical footprinting assay. Fotemustine (FM) is a chloroethylnitrosourea that generates piperidine-sensitive N^7 alkyl guanines with a preference for G's occurring in runs of guanines (bases 80-81, 121-122 and 129-131 (17). Lanes 8 and 9 show the alkylation pattern produced by FM with and without Cd^{2+}. It is evident that Cd^{2+} inhibits bands corresponding to GG sequences more intensely than isolated G's (bases 70, 74 and 78.). On the other hand, the divalent cation Mg^{2+} has much less effect even at 5 times the concentration of Cd^{2+} (lane 10). Lanes 1 to 4 shows the piperidine-induced strand cleavage pattern obtained from BPDE-modified DNA in the absence and in the presence of various concentrations of Cd^{2+}. BPDE in the absence of the metal alkylates all guanines with a preference for runs of guanines (lane 4). It is well known that the majority of BPDE alkylation occurs at the exocyclic amino group of guanine in the minor groove (18) but this lesion is resistant to piperidine treatment (10). However, the diffuse appearance of the bands is an indication that the piperidine-induced DNA strands carry minor groove alkylated guanines leading to lower mobility bands. In the presence of Cd^{2+} the N^7 guanine alkylation is strongly inhibited even at metal concentrations as low as 0.4 μM (lane 1-3).

The second chemical probe BEP used in this study alkylates N3 of adenine in the minor groove and N^7 of guanine in the major groove (17,19). Figure 3 is an autoradiogram of BEP alkylation pattern in the absence and in the presence of Cd^{2+} or Mg^{2+}. Lanes 3 and 4 show the alkylation pattern of BEP with (lane 3) and without (lane 4) Cd^{2+}. Guanine bands are strongly inhibited (bases 80-81, 121-123, 129-131) whereas the adenines are only marginally affected (bases 46-48, 57, 62, 66, 87 and 95). Even among guanines, isolated guanines are not quenched as strongly as the runs of guanines. Again Mg^{2+} has only marginal effect on the pattern (lane 5).

In order to detect the effect of Cd^{2+} on the minor groove alkylation by BPDE, we utilized the fact that when treated in acid the minor groove adduct releases the hydrolysis product of BPDE, a tetrol with a high fluorescent quantum yield (16). In this assay the BPDE-modified calf thymus DNA is precipitated to remove unreacted BPDE as tetrol in the supernatant **I.** This is followed by heat treatment and precipitation of

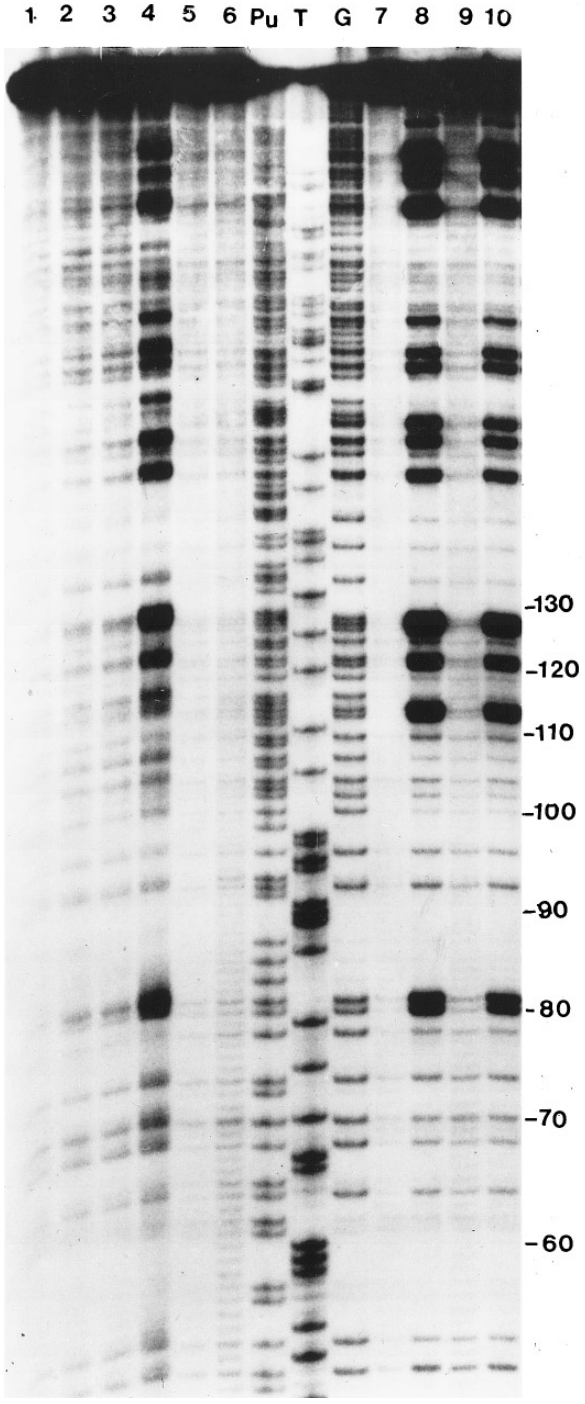


FIG. 2. Autoradiogram of alkylation patterns of BPDE and FM with and without Cd^{2+} or Mg^{2+}. Lanes 1-3, BPDE (0.4μM) + Cd^{2+} (5.0, 1.0, 0.5μM); lane 4, BPDE (0.4μM); lane 5,Cd^{2+} (5.0μM); lane 6, control; lane 7, Mg^{2+}(5.0μM); lane 8, FM (125μM); lane 9, FM + Cd^{2+} (5.0μM); lane 10, FM + Mg^{2+}(5.0μM). Pu, T, and G are sequencing lanes.

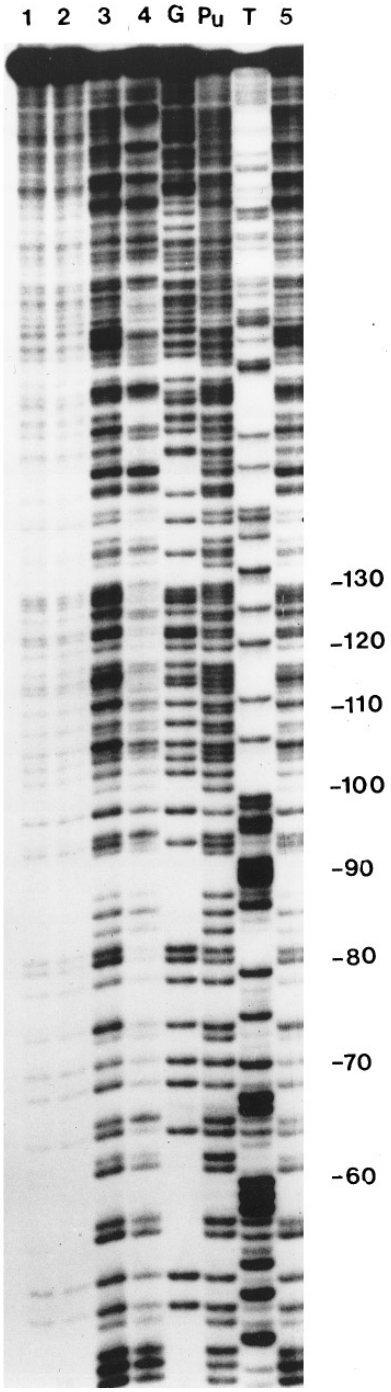


FIG. 3. Autoradiogram of alkylation pattern of BEP with and without 5.0μM of Cd^{2+} or Mg^{2+}. Lane 1, control; lane 2, Cd^{2+}; lane 3, BEP (125μM); lane 4, BEP + Cd^{2+}; lane 5, BEP + Mg^{2+}. G, Pu and T are sequencing lanes.

the dissolved DNA sample in neutral buffer to release quantitatively all N^7 guanine adducts plus DNA-intercalated tetrol from the first precipitation step in to the supernatant **II.** The last step involves acid hydrolysis to release minor groove adduct as tetrol in to supernatant **III.** Figure 4 shows the fluorescence spectra of tetrol in the supernatants from DNA samples treated with BPDE with (solid lines) and without Cd^{2+} (dashed lines). It should be noted that the fluorescence spectrum of tetrol in the supernatant solution produces a broad signal unlike the well-resolved peaks observed in an aqueous solution. The spectra labeled **I** are from supernatant **I** which contains mainly hydrolyzed BPDE catalyzed by acidic DNA. The intensity is greatest in this supernatant as only a small fraction of BPDE actually covalently binds to DNA. The intensities of the peaks are more or less the same with and without Cd^{2+}. The small increase (2%) in the Cd^{2+} sample is presumably from the hydrolysis of the fraction of BPDE prevented from reaction in the major groove. Curves labelled **II** show the fluorescence from supernatant **II** with and without Cd^{2+}. The small fluorescence observed here is likely to be from the intercalated tetrol that precipitated with DNA from the first step. The depurinated adduct, like BPDE, has very low fluorescence quantum yield and hence does not contribute to the intensity of the peaks (15). Curves labelled **III** show the fluorescence from the tetrol released from the acid-treated minor groove adducts. There is a marginal and statistically insignificant increase in the fluorescence intensity in the presence of Cd^{2+}. This increase is unrelated to that observed in **I** since in the former case it is from unreacted BPDE while in the latter it is from

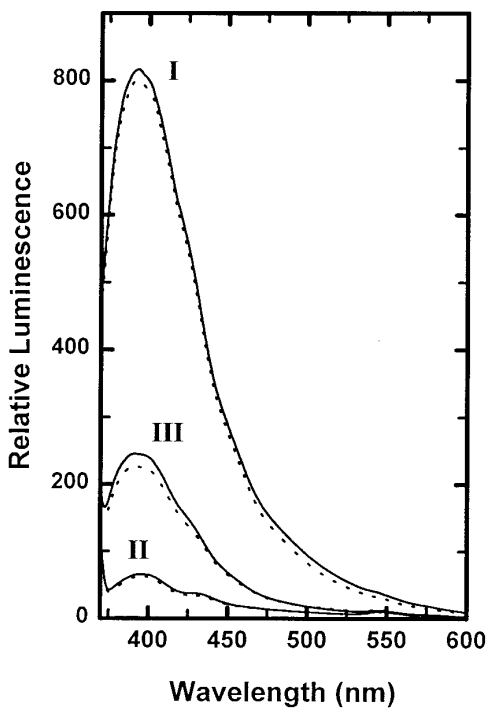


FIG. 4. Fluorescence spectra of BP tetrol from DNA-BPDE reaction products with Cd^{2+} (solid lines) or without Cd^{2+} (dashed lines). **I, II** and **III** correspond to the three supernatant fractions explained in the text.

the hydrolysis of the adducts. There was no detectable fluorescence in the redissolved final DNA pellet (data not shown).

DISCUSSION

The extent of covalent DNA damage in lung and larynx parallel cancer risk and mortality, which are known to increase with the number of cigarettes smoked per day and the duration of smoking (20,21). BP when activated to its ultimate metabolite form, BPDE, reacts with the exocyclic amino group of guanine in DNA. It also alkylates the N^7 sites in guanines but it is the former that is considered to be the mutagenic event (10). Van Schooten *et al.* (8) have demonstrated the presence of BPDE-DNA adducts in the human lung cancer tissues using ^{32}P-postlabelling technique. In addition, there have been numerous studies in the last ten years demonstrating the presence of DNA adducts of tobacco related carcinogens in various tissue samples obtained from smokers and ex-smokers (22,23).

In this study we investigated the interactions of another tobacco carcinogen, the transition metal Cd^{2+} with DNA *in vitro* and its effect on the binding of BPDE to DNA. We wanted to investigate whether Cd^{2+} enhances the reactivity of DNA to other carcinogens via its direct interaction with DNA for the following reasons: It is known that Cd^{2+} i) binds to DNA bases, specifically to N^7 of guanine (13), ii) is an established co-mutagen (11) and iii) levels in smokers' lung tissues are higher than in non-smokers' tissues (7).

Both FM and BPDE alkylate N^7 of guanines in the major groove, which is sensitive to piperidine cleavage. In a normal sequencing gel assay these chemicals produce characteristic band pattern which correspond to guanines with hot spots occurring in sequences containing runs of guanines (Fig. 2, lanes 4 and 8). Guanines in these sequences possess higher nucleophilicity than those in other sequences (24) and hence their higher reactivity towards electrophilic reagents. The presence of Cd^{2+} alters the alkylation patterns of both chemicals considerably. The alkylation of G's in contiguous guanines are dramatically inhibited while those in other sequences are either weakly affected or not at all. In contrast, the Mg^{2+} at the same concentration has very little effect on the alkylation pattern of these compounds. These results clearly demonstrate that Cd^{2+} preferentially binds to N^7 of guanine occurring in runs of guanines. Similar results have been obtained for major groove binding anthracycline antibiotics using FM as a probe (17).

In order to determine if Cd^{2+} can bind in the minor groove as well, we used BEP, which has been successfully used as a minor groove probe in our earlier studies (17). This chemical alkylates N3 of adenines in the minor groove and to a small extent N^7 of guanines in the major groove (Fig. 3, lane 3). As reported by White et al., (19) it preferentially alkylates A's occurring in runs of A's and those having runs of T's at the 5′ end. The presence of Cd^{2+} strongly inhibited guanine alkylation specially those occurring in runs of guanines, but not adenine alkylation (lane 4). Mg^{2+} again has only a slight effect on the alkylation pattern. Thus it is clear from these two gel experiments that Cd^{2+} is a major groove binder and that it inhibits other molecules from interacting with DNA at the site of its binding.

In addition to major groove alkylation, BPDE also alkylates exocyclic amino group of guanine in the minor groove but this lesion is not susceptible to chemical cleavage. The factors governing alkylation site preferences of electrophiles such as BPDE is not very well understood. Pearson's theory of electrophile-nucleophile interactions based on Hard and Soft Acid-Base (HSAB) comes closest in explaining these (see review, 25). In the case of BPDE, the minor groove alkylation is considered to be the key lesion leading to mutation and subsequent initiation of cancer (10). Thus any process that may enhance this lesion will act as an effective co-carcinogen. The fact that over 90% of BPDE alkylation occurs in the minor groove (26) makes it hard to detect any marginal increase in alkylation at this site as result of inhibition of alkylation at the other site. However it is important to note that in cells there are DNA repair enzymes such as glycosylases which are induced by N^7 guanine alkylation (27) and have the ability to repair DNA lesions both at N^7 and N^2 guanines (review, 28). Thus inhibition of the N^7 guanine alkylation could lead to suppression of repair of the minor groove alkylation. Based on our findings about the binding preferences of Cd^{2+} we tentatively propose that Cd^{2+} has the potential to enhance mutagenic responses of BPDE through modulation of the reactivity of cellular DNA. Our future studies will use animals to monitor the effect of Cd^{2+} on DNA damage and tumor formation in BP treated animals.

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