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Mutagenicity and Sequence Specificity of Acrolein-DNA Adduct

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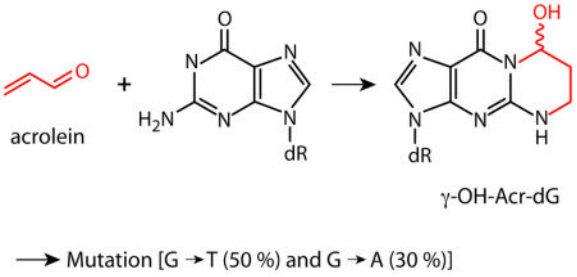
Abstract



Acrolein (Acr) is a major toxicant in cigarette smoke (CS); it can interact with DNA forming two major adduct isomers: α-OH-Acr-dG and γ-OH-Acr-dG. Previously, we found that the Acr-DNA binding pattern in the human p53 gene coincides with the p53 mutational pattern in CS-related lung cancer; hence we proposed that Acr is a major lung cancer etiological agent (1). This hypothesis has been brought into question with recent work that failed to detect Acr-induced mutations in the pSP189 system (2). To resolve this controversy, we determined the level and the type of Acr-dG formation, and the mutagenicity of Acr-dG adducts in the same pSP189 system. We also mapped the Acr-dG adduct distribution at the nucleotide level, and the Acr-dG induced mutational spectrum in this system. We found that 1) γ-OH-Acr-dG is the major adduct formed in Acr-modified DNA based on the LC-ESI-MS/MS analysis; 2) the mutation frequency is proportional to the extent of Acr-modifications and the majority of which are G:C to T:A and G:C to A:T mutations; and 3) sequences with a run of G's are the mutational hotspots. Using the UvrABC nuclease incision method to map the Acr-dG distribution in the *supF* gene sequence, we confirmed that Acr-DNA adducts preferentially form in guanine-rich sequences that are also mutational hotspots. These results reaffirm that Acr-dG adducts are mutagenic, and support our hypothesis that Acr is a major etiological agent for CS and cooking fume-related lung cancer.

Keywords

acrolein; acrolein-DNA adduct; mutation; lung cancer

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Introduction

Acrolein (Acr), a ubiquitous environmental contaminant, is abundant in cigarette smoke (CS) and cooking fumes (3). Acr also can be produced endogenously in cells as a byproduct of lipid peroxidation (LPO) (3,4). Acr is one of the major toxic metabolites of the chemotherapeutic agents, cyclophosphamide and ifosfamide (5,6). Accordingly, it has been proposed that Acr is the major factor for inducing secondary human bladder tumors in patients treated with these agents; indeed, Acr has been demonstrated to induce urinary bladder papillomas in rats through intraperitoneal injection (5–8).

Acr is abundant in ambient air and its content in CS is more than 1000 fold greater than that of polycyclic aromatic hydrocarbons (PAHs) (9,10). It has been shown that Acr is mutagenic to human cells (11) and can cause lung injury, however, the role of Acr in lung cancer has not been established (5,7,12).

Acr can induce α- and γ-hydroxy-1, N^2-propanodeoxyguanosine (α-OH-Acr-dG and γ-OH-Acr-dG) DNA adducts in cultured mammalian cells (13–15). Using an UvrABC incision procedure in combination with ligation-mediated PCR (LMPCR) to fingerprint the Acr-induced DNA damage, we recently found that the distribution of Acr-DNA damage in the p53 gene in normal human bronchial epithelial cells coincides with the p53 mutational spectrum in CS related lung cancer (1). Furthermore, we also found that Acr treatment induces an inhibitory effect on nucleotide excision repair (NER) (1). Hence, we have proposed that Acr is a major etiological agent for CS related lung cancer (1).

While the mutagenicity of α-OH-Acr-dG is well established, the mutagenicity of γ-OH-Acr-dG has been controversial (16–24). For example, using shuttle vector DNA modified with Acr *in vitro*, Kawanishi et al.,(24) have presented evidence to demonstrate that Acr-DNA adducts are mutagenic, inducing mainly G to T transversions in human cells. However, using a shuttle vector containing a site specific γ-OH-Acr-dG, Yang et al. (19) have found that γ-OH-Acr-dG is not mutagenic in human cells. Thus, the mutagenicity of γ-OH-Acr-dG in human cells remains to be clarified.

Recently, Pfeifer's group (2) has reported that they were unable to detect any significant increase of mutations in an Acr-modified pSP189 shuttle vector DNA replicated in human cells, or in the *cII* transgene in Acr-treated mouse fibroblasts. They therefore concluded that Acr-dG adducts are not mutagenic and are not etiological agents for CS related lung cancer. It should be noted however, the results from Pfeifer's group (2) as well as the Kawanishi et al.'s (24) were not based on the determination of either the amount or the type of Acr-dG adducts formed in the Acr-modified plasmid DNA and in the target gene used for mutation detection.

Because of the abundance of Acr in CS and environment, its extreme cytotoxicity, and the similarity of Acr-DNA binding spectrum to the p53 mutational spectrum in CS related lung cancer, the question of whether or not Acr-DNA adducts are mutagenic and carcinogenic is extremely important for risk assessment and for environmental regulation policy making; it therefore merits a careful re-evaluation. In this study, we addressed these issues by determining: 1) the types of Acr-DNA adducts formed in Acr-modified pSP189 shuttle

vectors by the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis, and 2) the *supF* mutations in Acr-modified pSP189 shuttle vectors after replication in cultured human lung cells by DNA sequencing. We also mapped the Acr-dG distribution at the nucleotide level in the *supF* gene in the Acr-modified pSP189 vectors and determined the mutational spectrum in this *supF* gene induced by Acr-modifications. Our results affirm that Acr-DNA adducts are mutagenic and support our hypothesis that Acr is a major etiological agent for lung cancer.

Materials and Methods

Shuttle Vector pSP189 DNA Preparation and Acrolein Modifications

The shuttle vector DNA pSP189 was prepared as previously described (25). The DNA was modified with Acr as described prior (1). Briefly, DNA was reacted with different concentrations of Acr in 0.1x TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA) at 37 °C for 16 h, the unreacted Acr was removed by phenol/diethyl ether extractions and the modified DNA was precipitated by ethanol and then dissolved in 1x TE buffer. The amount of Acr-dG formed in the pSP189 DNA was determined by UvrABC nuclease incision and by LC-ESI-MS/MS method as described below.

UvrABC Nuclease Incision on Acr-Modified DNA and Determination of Acr-DNA Adduct Distribution in the *supF* Gene

UvrA, UvrB and UvrC proteins are gene products that can function in concert (termed UvrABC nuclease) in incising bulky DNA damage in *Escherichia coli* cells (26). Methods for the UvrA, UvrB and UvrC protein preparations and the UvrABC nuclease incision conditions were the same as previously described (26). The number of UvrABC incisions on pSP189 supercoiled DNA was calculated based on Poisson distribution equation $P(0) = e^{-n}$, where $P(0)$ represents the fraction of supercoiled DNA and n represents the number of UvrABC incisions (26). Since we have found that UvrABC nuclease can incise Acr-dG quantitatively and specifically (1), we used this incision method to identify and quantify the Acr-dG distribution in the *supF* sequence and in the pSP189 plasmid DNA, respectively. To map the Acr-dG distribution in the *supF* sequence, pSP189 DNA was first digested with restriction enzyme EcoR I, 5′-end-labeled with ^{32}P-γ-ATP and further digested with restriction enzyme BamH I to generate a single 5′-end-^{32}P labeled 131 bp DNA fragment containing *supF* sequence. To generate a single 5′-end-^{32}P labeled 131 bp DNA fragment containing *supF* sequence in the opposite strand, pSP189 DNA was first digested with restriction enzyme BamH I, 5′-end-labeled with ^{32}P-γ-ATP and further digested with restriction enzyme EcoR I. These DNA fragments were modified with Acr and then reacted with UvrABC nuclease. The resultant DNAs were separated by electrophoresis in 8% denaturing polyacrylamide gels in parallel with Maxam and Gilbert sequencing reaction products as previously described (26,27).

Acr-DNA Adduct Analysis by LC-ESI-MS/MS Method

The method for determination of Acr-dG adducts by LC-ESI-MS/MS method was the same as previously described (28). Briefly, DNA (0.1 mg) was dissolved in sodium succinate/$CaCl_2$ (10/5 mM) buffer, pH 7.0, with 50 fmol of [$^{13}C_{10}$,$^{15}N_5$]Acr-dG (α-OH-dG and γ-OH-

dG) as internal standards, heated at 100 °C, 30 min and then quenched in an ice bath. The denatured DNA was digested with micrococcal nuclease (75 U) and phosphodiesterase II (0.5 U) at 37 °C, 6 h, and then alkaline phosphatase (150 U) was added and the mixtures were further incubated at 37 °C overnight. The hydrolysate was further purified using solid-phase extraction (SPE) cartridge, the hydrolysate was loaded first, washed with 1 mL H_2O, 1 mL 5% CH_3OH, and then eluted with 1 mL 70% CH_3OH. The eluant was dried and dissolved in 20 μL H_2O for LC-ESI-MS/MS analysis.

Determination of Acr-DNA Induced Mutational Frequency and Mutational Spectrum in the *supF* Gene

Acr mutagenicity was determined as previously described (29). Briefly, lung fibroblasts CCL-202 cells (American Type Culture Collection (ATCC), Manassas, VA), were grown to 70% confluence in 150 mm tissue-culture dishes and transfected with pSP189 DNA modified by different concentrations of Acr as described previously (1). After transfection, medium containing the transfection mixture was removed and cells were cultured in fresh medium for another 72 h. The transfected plasmids were then rescued from the transfected cells by the alkaline lysis method (1,30). DNA was extracted with phenol and chloroform, precipitated with ethanol, dissolved in TE buffer and then treated with the *Dpn*I restriction enzyme (New England Biolabs, Beverly, MA) to remove the unreplicated plasmids, which bear the bacterial adenine methylation pattern. A known quantity of the replicated plasmids was then electroporated into indicator MBM7070 bacteria, which carry a *lac*Z gene with an amber mutation. The transformed bacteria were plated on LB plates containing ampicillin (50 μg/ml), isopropyl *β*-D-thiogalactoside (IPTG) (190 μg/ml), and 5-bromo-4-chloro-3-indolyl *β*-D-galactoside (X-Gal) (0.8 mg/ml). After overnight incubation at 37 °C, white and light blue mutant colonies were picked from the background of blue wild-type colonies and re-streaked. Plasmids were then extracted and purified using the QIApre-spin plasmid kit (Qiagen, Valencia, CA). The sequences of the *supF* gene of mutant plasmids were determined with the primer 5′-GGC GAC ACG GAA ATG TTG AA-3′.

UV-irradiation Induced Mutation Detection in the *supF* Gene

For comparison purposes, ultraviolet (UV) light (254 nm)-irradiation induced mutations in pSP189 plasmid DNA were also determined. The mutagenicity of the photoproducts induced by UV irradiation in this shuttle system is well established (31–33). The method of detection is the same as described above except that DNA was irradiated with germicidal lamp (>95% emission at 254 nm) for different time periods and the relative amount of DNA lesion formed in the plasmid DNA was determined by the UvrABC incision assay (26).

Results

The Major Type of Adduct Formed in Acr-Modified DNA Is γ-OH-Acr-dG

Although Acr can interact with DNA to form covalent DNA adducts, this process is slow at neutral pH and ambient temperature. In order to obtain reproducible Acr-DNA modifications, we incubated DNA with different concentrations of Acr in 0.1x TE buffer (pH 8.0) at 37 °C for 16 h. To identify and quantify Acr-DNA adducts formed under these conditions, the modified and mock-modified DNAs were first heat denatured, then digested

with micrococcal nucleases, phosphodiesterases and alkaline phosphatases, and the adducted nucleosides in the hydrolysates were further purified by using an SPE cartridge for LC-ESI-MS/MS analysis. It should be noted that Zhang et al. (28) has shown that this method is able to achieve >90 % recovery for both α-OH-Acr-dG and γ-OH-Acr-dG adducts. The results in Table 1 and Figure 2 show that 95% of Acr-dG adducts formed under in vitro modification conditions are γ-OH-Acr-dG and 5% are α-OH-Acr-dG. The levels of both γ-OH-Acr-dG and α-OH-Acr-dG adduct formation are proportional to the Acr concentrations used for DNA modifications.

In earlier published work, we demonstrated that the *E. coli* NER enzyme UvrABC nuclease is able to incise Acr-modified DNAs quantitatively and specifically (1). To determine the amount of Acr-dG adduct formed in the Acr-modified supercoiled pSP189 shuttle vector DNAs were reacted with UvrABC nuclease and the resultant DNAs separated electrophoretically on 1% agarose gels. The number of UvrABC incisions was calculated based on Poisson equation $P(0) = e^{-n}$ assuming the distribution of Acr-dG adducts in the plasmid DNA is random (26). The results in Figure 3A show that the number of UvrABC incisions on Acr-modified pSP189 DNA is proportional to the amount of Acr used for DNA modifications. The number of UvrABC incisions is slightly lower than the amount of Acr-dG adduct per molecule of pSP189 plasmid DNA calculated based on LC-ESI-MS/MS analysis (Figure 3B). These results indicate that the LC-ESI-MS/MS method is more sensitive than the UvrABC incision method for quantifying Acr-dG adducts. Since 95% Acr-dG adducts in the pSP189 DNA are γ-OH-Acr-dG, and more than 50% of these Acr-dG adducts can be detected by UvrABC incision method, these results indicate that γ-OH-Acr-dG adduct is a substrate for the UvrABC nuclease.

Determination of the Mutagenicity of the Acr-dG DNA Adducts

Having establishing that γ-OH-Acr-dG is the major DNA adduct formed in the Acr modified pSP189 shuttle vectors, we then determined the mutagenicity of these DNA adducts. Cultured primary human lung fibroblasts (CCL-202) were transfected with a supercoiled form of pSP189 DNAs modified with different concentrations of Acr; the transfected cells were then incubated for 72 h to allow the transfected shuttle vector to replicate. The replicated DNAs were subsequently isolated by Hirt's method (30) and the nonreplicated pSP189 DNAs were removed by restriction enzyme *Dpn*I digestion. The mutagenicity of Acr-dG adducts was determined by transforming a known quantity of the replicated pSP189 DNA recovered from transfected human cells into *E. coli* MB7070 indicator cells and the transformed cells were plated onto growth medium containing ampicillin, IPTG and X-Gal. Only the cells carrying pSP189 will form colonies in this growth medium. MB7070 cells carry an amber codon in the *lac Z* gene; if the cells are transformed with pSP189 shuttle vectors then *supF* will be able to suppress the nonsense mutation and the phenotype of the transformed cells will be Lac Z^+. On the other hand, if the cells are transformed with a mutated *supF* gene that is no longer able to suppress the nonsense mutation, then the transformed cells will remain phenotypically LacZ^-. A total of ~10,000 colonies that resulted from transfection of the pSP189 plasmid DNA modified with each concentration of Acr were counted and the white colonies were picked and confirmed by re-streak onto indicator plates. The mutation frequency was calculated as the ratio of white colonies (which

carry mutated *supF*) to total colonies including blue colonies (which carry functional *supF*). Results in Figure 4 and Table 2 show that Acr-dG adducts induce mutations and that the mutation level is proportional to the amount of Acr used for DNA modifications; the increase of mutations in pSP189 plasmid DNA modified with 0.1, 0.5, 1 and 2.5 mM Acr is 3.5, 6, 13, and 23 fold, respectively.

The relative mutagenicity of Acr-dG was compared with UV-induced DNA lesions [cyclobutane pyrimidine dimer (CPD) and <6-4>photoproduct], a well-known mutagenic DNA damage. Plasmid pSP189 was irradiated with different doses of UV and the mutations induced were determined by the same method as for Acr modifications. The number of Acr-dG adducts and UV-induced DNA lesions in the pSP189 DNA was determined by UvrABC incision. Results in Figure 4B show that the mutagenicity of Acr-dG adducts, defined as mutant/DNA adduct, is significantly greater than that of UV-induced DNA lesion, defined as mutant/UV-induced DNA lesion (18.3×10^{-4} mutant/Acr-dG vs. 8.7×10^{-4} mutant/UV-induced DNA lesion).

Comparison of the Acr-dG Adduct Distribution and Acr-dG Induced Mutation Distribution in the *supF* gene

The mutations induced by Acr-dG adduct in the pSP189 vectors were sequenced and the types of mutation induced by Acr-dG and the distribution of mutations in the *supF* gene are presented in Figure 5 and Table 2. From these results, we conclude: 1) base substitutions at G:C positions are the major type of mutations induced by Acr-modifications indicating Acr induces mainly targeted mutations; 2) Acr-dG adducts induce mainly G:C to T:A and G:C to A:T mutations; and 3) 4 out 5 mutation hotspots induced by Acr occur in sequence with runs of 2–5 G's.

Two possible mechanisms may account for the mutation hotspots in the *supF* gene: one, Acr-dG adducts are preferentially formed at these hotspot sequences, and two, that error-prone translesion synthesis occurs more often at these hotspots. Previously we have shown that UvrABC nuclease is able to incise Acr-dG DNA adducts specifically and quantitatively (1). Hence, to test the first possibility, we determined the Acr-dG formation in the *supF* gene sequence using UvrABC incision method. Acr-modified pSP189 DNAs were first cut with restriction enzyme EcoRI, followed by 5′-^{32}P labeling of the *supF* gene nontranscribed strand, and then digested with a second restriction enzyme (BamH I). Alternatively, the sample was cut with BamH I first, 5′-^{32}P labeled end of the transcribed strand of the *supF* gene, and then digested with the second restriction enzyme (EcoR I). In both cases, the resulting fragments were then incubated with UvrABC nucleases and subsequently separated by electrophoresis in a sequencing gel in parallel with Maxam and Gilbert sequencing reaction products (27). The results in Figure 6 show that almost all UvrABC incision bands can be attributed to G residues indicating that Acr-DNA adducts formed mainly at G residues; the results are consistent with the results shown in Figure 2 that Acr-modifications produce Acr-dG adducts. The results in Figure 6 also show that the intensities of UvrABC incision bands are great for sequence runs of G's. Since UvrABC is able to incise Acr-dG adducts quantitatively, these results indicate that Acr-dG adducts are preferentially formed in sequence runs of G's.

In order to determine the role of the sequence context and extent of Acr-dG formation in mutation induction, the relative Acr-dG formation at both strands of the *supF* gene was plotted against the mutational spectrum in this gene (Figure 7). The results show that mutational hotspots occurred at these preferential sites for Acr-dG formation, leading us to conclude that preferential Acr-dG formation contributes to Acr-induced mutational hotspots in the *supF* sequence. However, while the formations of Acr-dG at positions 4 and 35 are modest, mutation frequencies at these sites are relatively high, suggesting that either Acr-dG formed at these sites are relatively resistant to repair mechanism and/or adducts formed at these sites allow high frequency of translesion synthesis with a compromised fidelity.

Discussion

In this study we address three major questions regarding Acr mutagenicity: first and foremost, are the Acr-dG DNA adducts mutagenic? Second, which isomer of Acr-dG is the major adduct formed in Acr-modified DNA? Third, what role does DNA sequence context play in determining Acr-dG adduct formation and mutations?

Based on LC-ESI-MS/MS analysis, we conclude that γ-OH-Acr-dG is the major adduct isomer formed when Acr reacts with DNA at 37 °C in TE buffer at pH 8.0. Our results are thus consistent with those reported by Chung et al (13) for Acr-modified calf thymus DNA.

We found that Acr-modifications induce mutations in the *supF* gene, and that the level of mutations is proportional to the extent of Acr-DNA adduct formation in the pSP189 plasmid DNA. Furthermore, we found that the mutagenicity of the Acr-dG is higher than the mutagenicityof the UV-induced DNA lesion. Our results are consistent with prior observations by Kawanishi et al (24) that Acr-dG adducts are mutagenic. Since more than 95% of Acr-induced DNA adducts in the pSP189 plasmids are γ-OH-Acr-dG adducts, our results indicate that these isomers are mutagenic.

Finally, our results show that all base substitution mutations in the *supF* sequence induced by Acr modification are located at guanine residues, and that the Acr-induced mutational spectrum is distinctly different from the spontaneous mutational spectrum. These results strongly suggest that Acr modification induced mutations are due to Acr-dG adduct formation. Using the UvrABC incision method to map the Acr-DNA adduct distribution in the *supF* sequence we found that most, if not all, Acr-DNA adducts form at deoxyguanosine positions. Furthermore, we found that Acr-dG adducts preferentially form at runs of G sites in the *supF* sequences; these sites are also the mutational hotspots induced by Acr modifications.

Taken in sum, these results not only strengthen the conclusion that Acr-dG adduct is mutagenic but also suggest that Acr-induced mutations result from translesional synthesis. However, we also found that a relatively low level of Acr-dG formation at sequences, such as positions 4 and 35, nevertheless resulted in a relatively high level of mutations. We hypothesize that Acr-dG adducts formed at these sequences allow more erroneous translesional synthesis and/or are less susceptible to DNA repair mechanisms. It is worth noting that 50% of Acr modification induced mutations are G to T transversions and 30%

are G to A transitions; this mutational signature is similar to that found in the p53 gene in the CS related lung cancer (1,34).

It is not clear why Pfeifer laboratory (2) was unable to detect a significant increase of mutation in pSP189 shuttle vectors modified with up to 1 mM Acr in human cells, except to note that these authors did not use direct adduct analysis method to quantify the Acr-dG formation in the pSP189 plasmid DNA used for transfection. However, based on the terminal transferase dependent (TD)-LMPCR signals presented by these authors, the extent of Acr-DNA modifications appears to be 10–20 times less than the number of UVB-DNA modifications that induced 4 to 20 fold increase in mutations compared to unmodified DNA in repair proficient and deficient human fibroblasts, respectively. If this indeed is the case, then this low level of Acr-dG in pSP189 would not be expected to induce mutations significantly above background, even though using the same detection system, our results show that the mutagenicity of the Acr-dG adduct is higher than UV-induced DNA lesions. Hence, it is likely that the extent of Acr-dG formations in the pSP189 DNA used by Pfeifer laboratory (2) was too low to generate significant increase of mutations.

Recently Zhang et al (28) have reported that almost equal amounts of α- and γ-OH-Acr-dG are found in human lung tissues, and that the amount of these adducts is comparable to the total DNA adduct burden detected in cigarette smokers with similar cigarette smoke history. Gupta laboratory (35) recently reported that the majority of diagonal radioactive zones (DRZs) detected in cigarette smokers using ^{32}P post-labeling and 2 dimensional thin layer chromatograph (2D-TLC) method are not PAHs and aromatic amines. Interestingly, these workers also found that DNA modified with acetyl aldehydes and formaldehydes produced the same DRZs as DNA from lung tissues of cigarette smokers. It should be noted that the amount of Acr in CS is more than 1000 fold higher than PAHs (7,8). Among aldehydes found in CS, Acr is one of the most active and is readily taken up by cells (3,36).

We previously found that the Acr-dG distribution in the p53 gene coincides with the p53 mutational spectrum in CS related lung cancer, and that Acr can have an inhibitory effect on DNA nucleotide excision repair (1). Since the results presented here further confirm that Acr-dG adducts are mutagenic and induce G to T and G to A mutations that are similar to the mutational signature found in the p53 gene in the CS related lung cancer, together these results support the hypothesis that Acr is a major etiological agent for CS related lung cancer and cooking fume related lung cancer. It is possible that the susceptibility to Acr-induced adduct formation, and the repair capacity for Acr-dG adducts may play a major role in determining an individual’s susceptibility to CS-induced lung carcinogenesis.

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Abbreviations

CS	cigarette smoke

Acr	acrolein
dG	deoxyguanosine
α-OH-Acr-dG	α-OH-propanodeoxyguanosine
γ-OH-Acr-dG	γ-OH-propanodeoxyguanosine
LC-ESI-MS/MS	liquid chromatography-electrospray ionization-tandem mass spectrometry
LPO	lipid peroxidation
PAHs	polycyclic aromatic hydrocarbons
LMPCR	ligation-mediated polymerase chain reactions
TD-LMPCR	terminal transferase-dependent LMPCR
NER	nucleotide excision repair
TE	tris-EDTA
IPTG	isopropyl β-D-thiogalactoside
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactoside
CPD	cyclobutane pyrimidine dimer
DRZ	diagonal radioactive zones
TLC	thin layer chromatography

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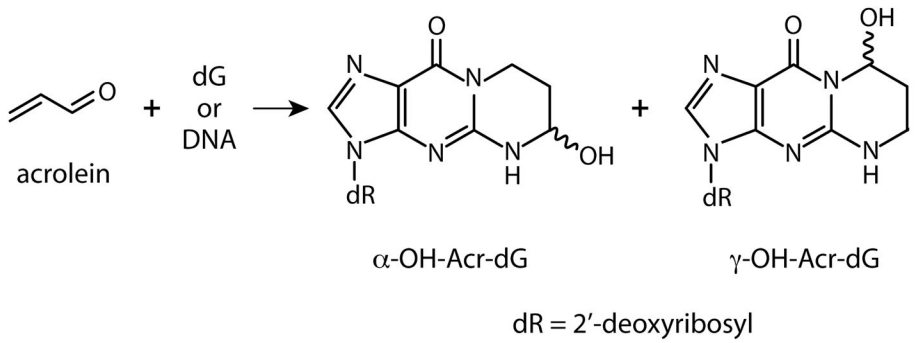

Figure 1.
Structures of α-OH-Acr-dG and γ-OH-Acr-dG adducts resulting from reaction of Acr with deoxyguanosine (dG) residues.

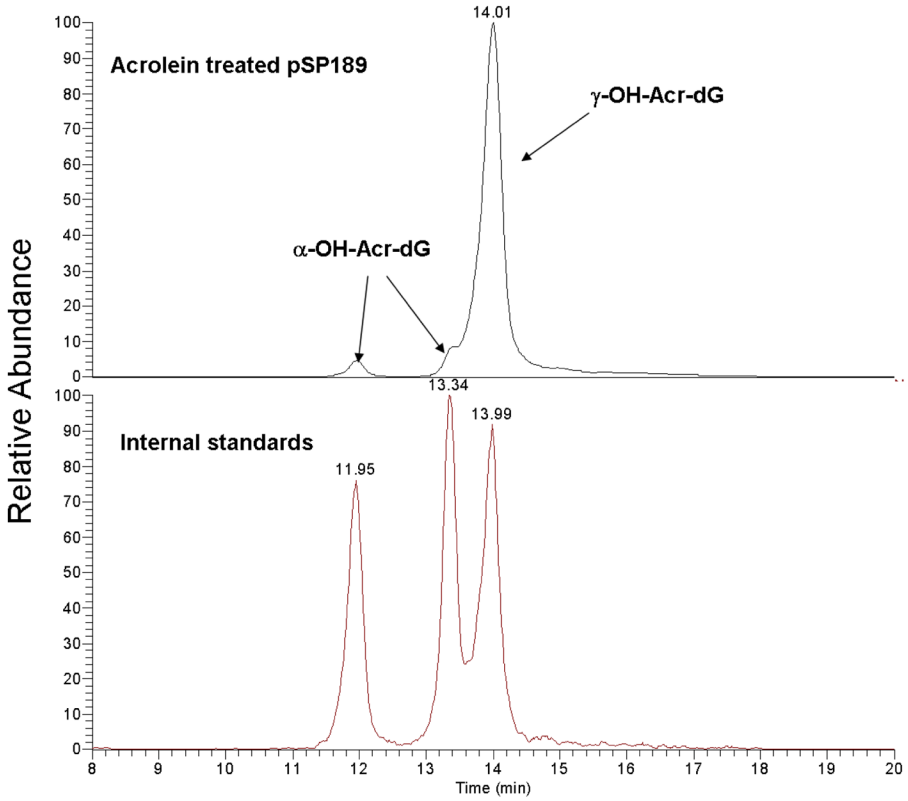

Figure 2.

Typical chromatograms obtained from LC-ESI-MS/MS analysis of Acr-modified pSP189 DNA. Double-stranded pSP189 DNA modified with different concentrations of Acr were denatured, digested with micrococcal nuclease, phosphodiesterase II, and alkaline phosphatase; the Acr-dG adducts were purified by SPE, and analyzed. Acr-dG adducts (upper trace) and standards of purified α-OH-Acr-dG and γ-OH-Acr-dG adducts (lower trace) was shown in. Note: two (+/−) stereoisomers of α-OH-Acr-dG were well separated while two (+/−) stereoisomers of γ-OH-Acr-dG cochromatographed at the same position (28).

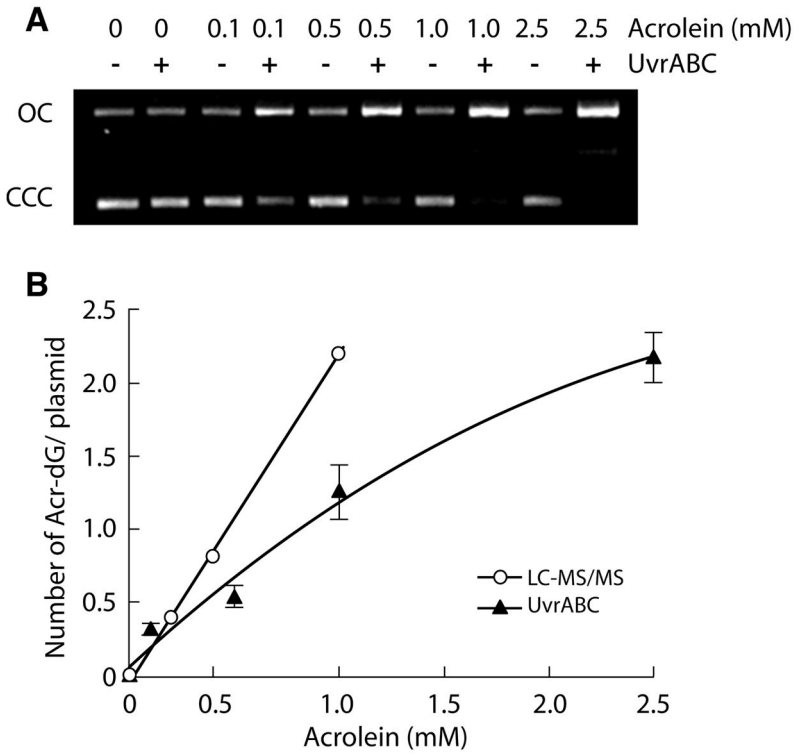

Figure 3.

UvrABC incisions on Acr-modified supercoiled pSP189 DNA. The same Acr-modified supercoiled pSP189 DNAs described in Figure 2 were reacted with UvrABC nuclease and the resultant DNAs were separated by electrophoresis in a 1 % agarose and stained with ethidium bromide. A) Photograph of a typical gel. Symbols: OC, open circle and CCC, closed covalent circle. B) Quantitative determination of Acr-dG formation in supercoiled pSP189 DNA. The number of Acr-dG adducts in the pSP189 plasmid DNA modified with different concentrations of Acr was calculated from the number of UvrABC nuclease incisions based on Poisson distribution equation (26) (—▲—). The numbers represent the average of two independent experiment results. For comparison the number of Acr-dG adduct per plasmid was also calculated from results of LC-ESI-MS/MS analysis (—○—).

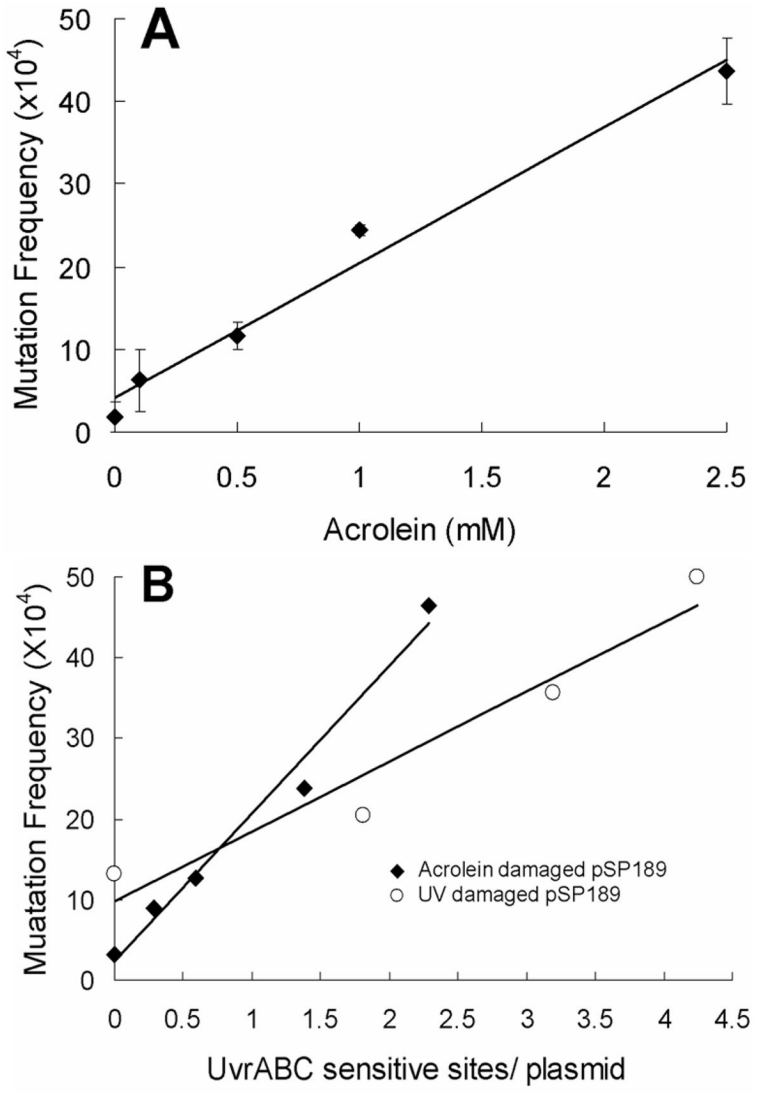

Figure 4.

The effect of Acr-modifications and UV-irradiation on the mutation induction in the *supF* gene. A) The same Acr-modified supercoiled pSP189 DNAs as described in Figures 2 and 3 were used for transfection in human lung fibroblasts CCL-202. The methods of transfection and the *supF* gene mutation detection are described in **Materials and Methods**. The mutant plasmids were purified and the mutations in *supF* gene in these plasmids were confirmed by DNA sequencing. Mutation frequency was calculated based the ratio of the number of white colonies over the number of total colonies. Results represent three independent experiments. In B) mutagenicity of Acr-dG was compared with mutagenicity of UV-irradiation induced DNA lesions. The amount of Acr-dG formed in the pSP189 DNA was calculated based on the UvrABC incision assay (26). For detection of UV-induced mutations, pSP189 DNA was irradiated with different fluences of UV and the mutation detection was the same as for Acr-modification-induced mutation detection. The amount of DNA lesions produced by UV-irradiation was also based on the UvrABC incision assay (26).

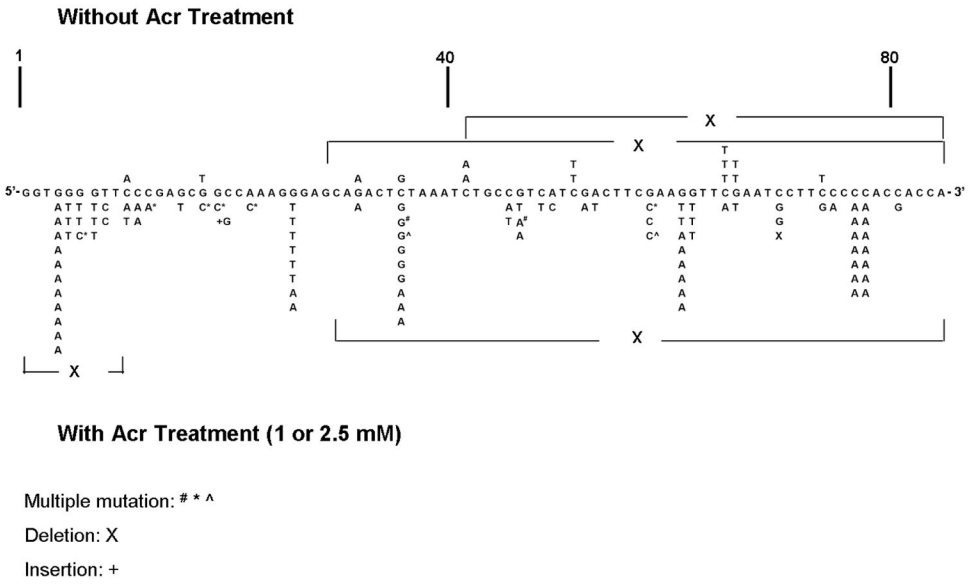

Figure 5.

Acr-modification induced mutational spectrum in the *supF* gene in pSP189 plasmids replicated in human lung fibroblasts CCL-202. Mutations induced by Acr-modifications are shown in the lower panel and spontaneous mutations are shown in the upper panel. Methods for transfection and *supF* gene mutation detection are described in **Materials and Methods**. The mutant plasmids were purified (90 mutant plasmids resulted from plasmid modified with 1 and 2.5 mM Acr and 18 mutants plasmids resulted from unmodified plasmids) and the *supF* gene in these plasmids was sequenced. The symbols denoted are as follows: ^{X}, single base deletions; $^{\#\ \wedge}$, double mutations (two mutations occurring in the same plasmid); *, multiple mutation (more than two mutations occurring in the same plasmid); +, single base insertion; —X— fragment deletion.

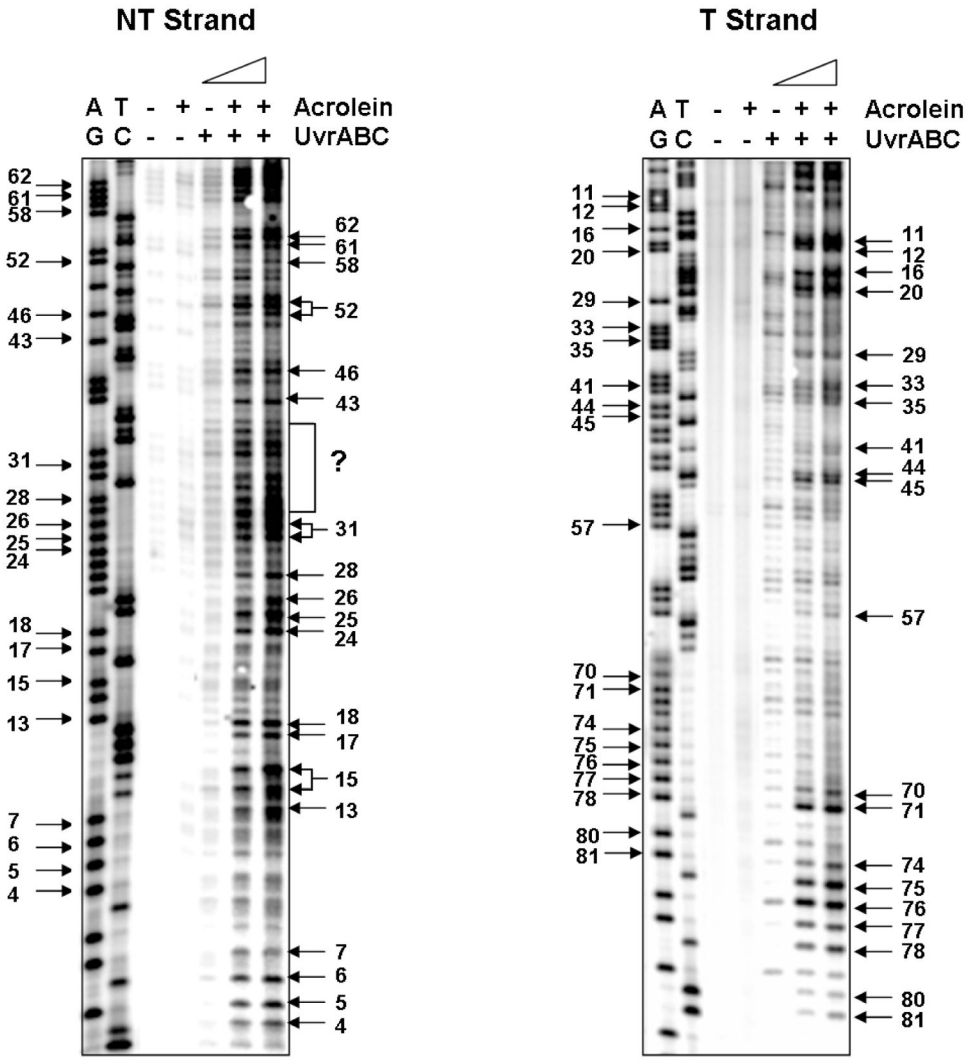

Figure 6.

The Acr-dG adduct distribution detected by UvrABC incision method in the *supF* gene sequence. The *supF* DNA fragments were prepared from pSP189 plasmid DNA modified with different concentrations of Acr, single 5′-end labeled with ^{32}P at the nontranscribed stand (NT strand) or at the transcribed strand (T strand), reacted with UvrABC, and the resultant DNAs were separated by 8 % denatured polyacrylamide gel electrophoresis as described in **Materials and Methods**. A) represents a typical radiogram resulting from single 5′-end labeled with ^{32}P at the transcribed strand, and B) represents a typical radiogram resulting from single 5′-end labeled with ^{32}P at the nontranscribed strand. Symbols: GA and TC represent Maxam and Gilbert sequencing reaction products, and +/− represents reaction with Acr or UvrABC nuclease. Guanine residue numbers in the sequence are labeled at the left and the corresponding UvrABC incision bands are labeled at the right. Note: we were unable to assign the UvrABC incision bands within the (?) area to corresponding guanine residues in the *supF* sequence.

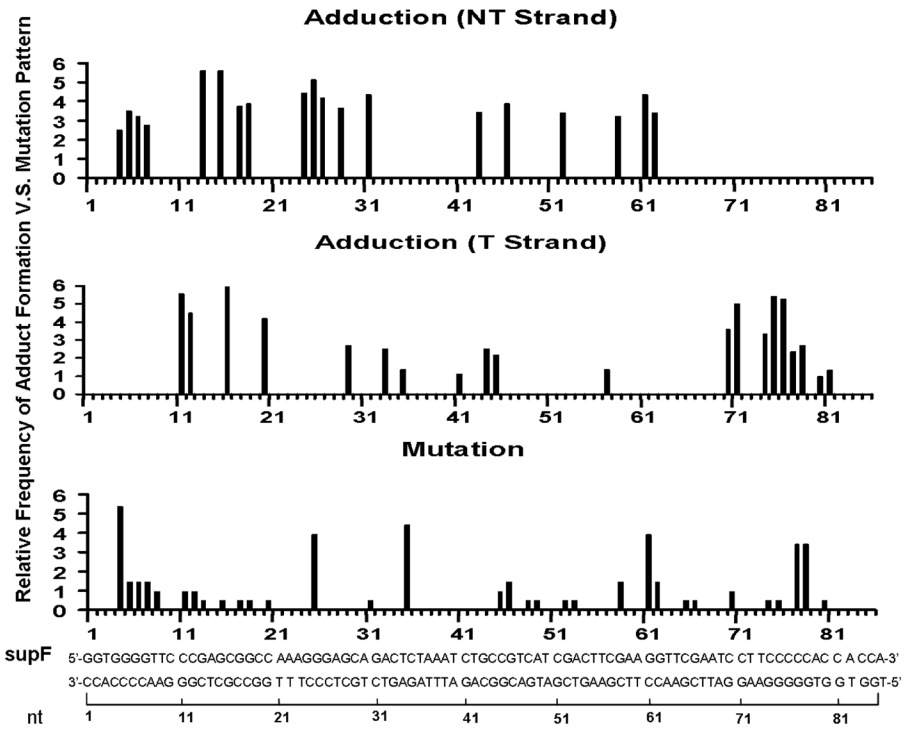

Figure 7.

Comparison of the Acr-dG adduct distribution detected by UvrABC incision method with the relative mutation frequency induced by Acr modification in the *supF* gene sequence. The relative Acr-dG adduct formation at different sequences of the nontranscribed strands (NT strands) and the transcribed strands (T strands) of the *supF* gene were determined by the UvrABC incision method as shown in Figure 6. The Acr-modification induced relative mutation frequency at different sequences of the *supF* gene was calculated from Figure 5.

Table 1

LC-MS/MS Analysis of Acrolein-Treated pSP189.

	Acr-dG concentration (μ mol/mol dG)		
Acrolein concentration (mM)	**α-OH-Acr-dG**	**γ-OH-Acr-dG**	**Total**
0	0.1	0.5	0.6
0.2	7.6	148	155
0.4	15.8	307	323
1.0	41.6	848	890

Table 2

Types of Mutations in the *supF* Gene in Acrolein-Treated pSP189 Plasmids Replicated in Human Normal Lung Fibroblasts CCL-202.

	CCL-202	
	Control	**Treated***
Single base substition		
G•C to T•A	6 (43%)	44 (53%)
G•C to A•T	7 (50%)	25 (30%)
G•C to C•G	1 (7%)	10 (12%)
A•T to T•A	0 (0%)	1 (1%)
A•T to C•G	0 (0%)	1 (1%)
A•T to G•C	0 (0%)	2 (2%)
Total	14	83
Base substition**		
G•C to T•A	6 (43%)	45 (48%)
G•C to A•T	7 (50%)	27 (29%)
G•C to C•G	1 (7%)	17 (18%)
A•T to T•A	0 (0%)	1 (1%)
A•T to C•G	0 (0%)	1 (1%)
A•T to G•C	0 (0%)	2 (2%)
Total	14	93
Single base substition	14 (78%)	83 (92%)
Single base deletion	0 (0%)	1 (1%)
Fragment deletion	4 (22%)	2 (2%)
Multiple muation***	0 (0%)	3 (3%)
Single base insertion	0 (0%)	1 (1%)
Total	18	90

* Plasmids were treated with 1 or 2.5 mM Acr.

** Including single base substitution, and multiple mutations.

*** Two or more than two mutations occur in the same plasmid.