Acrolein induced DNA damage, mutagenicity and effect on DNA repair

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Abstract

Acrolein (Acr) is a ubiquitous environmental contaminant; it also can be generated endogenously by lipid peroxidation. Acr contains a carbonyl group and an olefinic double bond; it can react with many cellular molecules including amino acids, proteins and nucleic acids. In this review article we focus on updating information regarding: 1) Acr induced DNA damage and methods of detection, 2) repair of Acr-DNA damage, 3) mutagenicity of Acr-DNA adducts, 4) sequence specificity and methylation effect on Acr-DNA adduct formation, and 5) the role of Acr in human cancer. We have found that Acr can inhibit DNA repair and induces mutagenic Acr-dG adducts and that the binding spectrum of Acr in the p53 gene in normal human bronchial epithelial cells is similar to the p53 mutational spectrum in lung cancer. Since Acr-DNA adduct has been identified in human lung tissue, and that Acr causes bladder cancer in human and rat models, we conclude that Acr is a major lung and bladder carcinogen, and its carcinogenicity arises via induction of DNA damage and inhibition of DNA repair.

Keywords

Acrolein; DNA Damage; Lung Cancer; Mutation; Repair

Introduction

Acrolein (Acr) is a ubiquitous environmental contaminant. It comes from different sources, including industrial, food, cigarette smoke, incomplete combustion, and cooking [1]. Acr also can be generated endogenously, it is a by-product of lipid peroxidation [2]. Although the levels of Acr in normal cells and in cells under oxidative stress have yet to be established, the interactions of Acr with many cellular molecules including amino acids, proteins and nucleic acids have been extensively studied [3–5]. The most common reaction between Acr and cellular molecules is the Michael addition with and without subsequent

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cyclization [3–6]. It is conceivable that Acr interactions might profoundly affect the functions of these modified molecules. The chemistry and biology of Acr and Acr-DNA adducts have been thoroughly examined in many excellent articles [1–7] This review focuses on updating information regarding: 1) Acr induced DNA damage and methods of detection, 2) repair of Acr-DNA damage, 3) mutagenicity of Acr-DNA adducts, 4) the role of sequence context and cytosine methylation on Acr-DNA adduct formation, and 5) the role of Acr in human cancer. Most of the information was obtained from experimental results that were accomplished by treating cells with exogenous Acr, since little is known about the consequences of elevated endogenous Acr.

Acrolein distribution in human cells

Acr can cross membrane barriers and readily enters human cells [7]. Using anti-hemocyanin-acrolein antibodies Lou et al. have reported that Acr is mainly located in the cytoplasm region of myocytes [8]. Using an anti-Acr-FDP-lysine (N^ε-(3-formyl-3,4-dehydropiperidino)-lysine) antibody specifically for Acr-lysine we found that Acr-lysine is preferentially trapped in the nucleus compared with other regions of the cytoplasm in cultured normal human lung fibroblasts (Fig. 1). If we assume that only the conjugated Acr is recognized by the Acr-lysine antibody then those Acr molecules are not "free form". Whether bonded Acr can further react with nuclear components such as nuclear proteins and DNA, and whether Acr can remain in free form in cytoplasm are unclear.

Acr-DNA interactions in vitro

It has been well established that excyclic amine of guanine residues is a strong Michael addition donor for the olefinic double bond of Acr. At neutral to mild basic pH Acr –DNA interactions generate mainly γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γ-OH-Acr-dG) adducts [6]. However, we found that at high pH both α- and γ-OH-Acr-dG adducts are formed (Fig. 2A). It appears that at neutral and mild pH the N1 of the guanine residue has a higher nucleophilicity than the excyclic amine group. Therefore, it attacks the carbonyl group of the Acr to form primarily γ-OH-Acr-dG adducts. However, at high pH the nucleophilicity of N1 and excyclic amine at guanine residue are reduced and equalized, hence these two moieties have equal probability of attacking the olefinic double bond, as well as the carbonyl group of acrolein, and consequently form both α- and γ-OH-Acr-dG adducts (Fig. 3).

Detection of Acr-dG adducts by the ^{32}P post-labeling-two-dimensional thin layer chromatography (2D-TLC) and high performance liquid chromatography (HPLC)

Two methods have been well established to quantify Acr-DNA adducts: 1) ^{32}P post-labeling label followed by two-dimensional thin layer chromatography (2D-TLC) and high performance liquid chromatography (HPLC) [9], and 2) liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) [10]. The initial step for both methods is to digest DNA to single 3′-mono-phosphate nucleotides by phosphodiesterase. The adducted mono-phosphate nucleotides are separated from normal nucleotide phosphate,

first by solid phase extraction and then followed by LC-ESI-MS/MS. Accurate mass can be identified in the eluent, therefore this method renders a very reliable result [10]. However, this method requires a relatively large amount of genomic DNA. Thus, it is quite a challenging task to apply this method to clinical samples.

For ^{32}P post-labeling analysis, the digested mono-phosphate nucleotides are 5′-end phosphorylated with ^{32}P followed by nuclease P1 (NP1) digestion; adducted nucleotides are relatively resistant to NP1, and the resulting 5′,3′-bisphosphate adducted nucleotides can be separated from normal 5′-^{32}P-mono-phosphate normal nucleotides by 2D-TLC [9]. Although α-OH-Acr-dG from γ-OH-Acr-dG can be well separated in our 2D-TLC system, these two types of adducts co-migrate with crotonaldehyde-adducted deoxyguanosine (Cro-dG) and other unknown DNA adducts, therefore further separation of α- and γ-OH-Acr-dG from Cro-dG and others is necessary (Fig. 2B). We found that the HPLC step can separate Acr-dG, Cro-dG and 4-hydroxy-2-nonenal (HNE)-dG (Fig. 2C). We found that this method is highly reproducible with high recovery (80%). Furthermore, only 5 μg of DNA is needed for adduct analysis using ^{32}P post-labeling 2D-TLC-HPLC method, and this method can simultaneously analyze Acr-dG, Cro-dG, and HNE-dG adducts. Although the LC-ESI-MS/MS method can also have the advantage of separating Acr-dG from others, it requires 50 μg DNA for the same analysis, in addition to requiring the availability of expensive LC-ESI-MS/MS equipment.

Analysis of Acr-dG formation at the DNA sequence level

We have found that *Escherichia coli* nucleotide excision repair enzymes, UvrA, UvrB and UvrC working in concert (collectively, known as UvrABC nuclease), can incise Acr-dG specifically and quantitatively [9, 11]. Using UvrABC incision with and without ligation-mediated PCR, we have developed methods to map the distribution of Acr-dG in the p53 gene and the *supF* gene at the nucleotide level in the defined DNA fragments and in genomic DNA isolated from human cells [9, 11]. We have found that 1) Acr-DNA adducts form exclusively at guanine residues in DNA, 2) sequences of a run of guanine residues, -GA- and –AG- are the preferential sites for Acr-DNA adduct formation, and 3) the C5 cytosine methylation at –CpG- sequence greatly enhances Acr-dG adduction at this site (Fig. 4). Our results indicate that Acr-dG formation is affected by DNA sequence context and cytosine methylation [9, 11].

Acrolein-induced cytotoxicity and mutations in mammalian cells

Acr has been shown to be both cytotoxic and mutagenic to Chinese hamster ovary (CHO) cells [12–15]. However, its genotoxic and cytotoxic effects in human cells are less straightforward [16, 17]. It has also been demonstrated that Acr induces a concentration-dependent cell killing and mutation increase in nucleotide excision repair (NER) deficient xeroderma pigmentosum complement group A (XPA) cells [18]. These results indicate that Acr-induced DNA damage is not only mutagenic but also cytotoxic. However, although Acr induces a concentration-dependent cell killing in NER proficient human fibroblasts and the D37 for these cells is 4–5 times higher than for XPA cells, intriguingly, Acr does not induce a significant number of mutations in NER proficient human cells even at the level that it

induces 90% of cell killing [18]. Kim et al. [19] also failed to detect Acr-induced mutation in the *cII* transgene in transgenic mouse fibroblasts treated with the Acr (<100 μM) at the concentrations that causes a substantial amount of cell killing and DNA damage detected by an extremely sensitive terminal transferase-dependent PCR method. Together, these results suggest that either Acr-induced DNA damage is efficiently repaired in normal human fibroblasts and mouse ES cells or that the path that Acr induces cell death in normal human and mouse ES cells is different from the path for XPA cells.

Since Acr can readily interact with many proteins and numerous cellular components it is conceivable that multiple pathways can lead to cell death by Acr and that DNA damage is one of them. For example, it has been found that Acr can induce apoptosis through interaction with mitochondria [20–26]. To further test this possibility, we determined the Acr-induced cytotoxicity and DNA damage in mitochondria depleted rho zero cells and their parental human lung epithelial cells. We have found that rho zero cells are much more resistant to Acr-induced cell killing [27] (Fig. 5). These results indicate that mitochondria indeed mediate Acr-induced cell death. These results also indicate that unlike many DNA damaging agents that induce cytotoxicity through their genotoxicity, the relationship between Acr-induced cytotoxicity and genotoxicity is not equal and is cell type dependent.

Mutagenicity of DNA adducts: α-OH-Acr-dG versus γ-OH-Acr-dG

While the relationship between Acr-induced cytotoxicity and genotoxicity is not straightforward, the mutagenicity of Acr-induced DNA adducts is no less confusing. It is well established that modifications of deoxyguanosine mono-phosphate result in generating two stereoisomeric α-OH-Acr-dG adducts and two stereoisomeric γ-OH-Acr-dG Acr adducts (Fig. 3). Although the γ-OH-Acr-dG adduct is the major DNA adduct detected in cultured human cells that have been treated with Acr, both α-OH-Acr-dG and γ-OH-Acr-dG are detected in human lung tissues [10]. Therefore, it is essential to assess the mutagenicity of these two types of DNA adducts in order to understand the carcinogenicity of Acr. While the mutagenicity of Acr-induced DNA adducts in CHO and human cells has been firmly established a question remains: which isomeric Acr-dG is mutagenic? Using a construct that contains a site-specific α-OH-Acr-dG, it has been shown that both G to T and G to A mutations are induced at the adducted guanine position in both mammalian and *Escherichia coli* cells [28]. Hence, it is generally believed that α-OH-Acr-dG adducts are mutagenic. The mutagenicity of γ-OH-Acr-dG is controversial. Using a construct containing a site-specific γ-OH-Acr-dG, both Moriya's and Marnett's laboratories have shown that no significant mutations were observed at the adducted guanine site [29, 30]. On the other hand, using a *supF* containing shuttle vector randomly modified with Acr that generates mainly γ-OH-Acr-dG adducts (97%), both Yagi and Tang's laboratories have found that γ-OH-Acr-dG adducts are mutagenic and induce G to T and G to A mutations at the adducted guanines [11, 31]. Using a site-specific construct containing a γ-OH-Acr-dG adduct Lloyd's laboratory has also found G to T and G to A mutations at the adducted guanine position in Cos cells [32–34]. These results indicate that both sequence context and host cells plays a role in determining the Acr-dG adduct mutagenicity. Caution must be exercised in generalizing results, particularly those results obtained from a site-directed Acr-dG containing constructs, because site specific Acr-DNA adduct induced mutations represent

only a "snapshot" of Acr-DNA adduct mutagenicity at that particular sequence context and may not represent the mutagenicity of the same adduct at other sequences.

Since we have found that Acr-DNA modifications under various pH conditions produce different ratios of Acr-dG stereoisomers (Fig. 2), we modified *supF* containing shuttle vectors under pH 8 and 9.8 conditions to produce plasmid DNA with either mainly γ-OH-Acr-dG (97%) or plasmid DNA with 50% γ-OH-Acr-dG adducts and 50% α-OH-Acr-dG adducts. We have found that the latter adduct ratio induced significantly less mutations/DNA adducts than the former in both normal human bronchial epithelial (NHBE) cells and in normal human lung fibroblasts (NHLF) (CCL202) (Table 1). Intriguingly, we also found that the γ-OH-Acr-dG adduct is more mutagenic in NHBE cells than in NHLF. In contrast, the α-OH-Acr-dG adduct is more mutagenic in NHLF than in NHBE cells. These results suggest that α-OH-Acr-dG adduct could be either repaired efficiently and/or are less mutagenic than the γ-OH-Acr-dG adduct.

Acr-DNA and Acr-protein-DNA crosslinks

It has been found that γ-OH-Acr-dG adduct can undergo further chemical reactions forming interstrand or intrastrand DNA cross-links at 5′-CpG-3′ sequences. Acr can also cause DNA-protein conjugates. The chemistry and biological processing of these cross-links were extensive reviewed by Minko et al. and Stone et al. [5, 35]. Interestingly, Minko et al. [36] have found that while mutations are rarely generated during processing of these interstrand or intrastrand DNA cross-links, the reduced γ-OH-Acr-dG-peptide conjugates can be more mutagenic than the corresponding mono-adduct and its potential to cause mutations is dependent on the peptide attachment. Current data suggest that γOH-Acr-dG-peptide conjugates are formed as intermediates in the process of repairing γ-OH-Acr-dG [37]. These results suggest that the mutagenicity of γ-OH-Acr-dG can be derived from adduct itself and its peptide conjugates.

Repairability of α-OH-Acr-dG adducts versus γ-OH-Acr-dG adducts

To determine the repairability of the two stereoisomeric Acr-dG adducts, supercoiled plasmid DNA was modified with Acr under pH 8 and pH 9.8 conditions that produce different percentages of α-OH-Acr-dG adducts versus γOH-Acr-dG adducts; these DNAs were then used as substrates for *in vitro* DNA damage dependent repair synthesis which was carried out using cell lysates isolated from human lung fibroblasts (CCL202) and XPA cells (Fig. 6). We have found that 1) DNA modified with Acr under pH 9.8 conditions produces lower total Acr-dG adducts but significantly higher α-OH-Acr-dG/γ-OH-Acr-dG ratios than DNA modified with Acr under pH 8.0 conditions, and 2) DNA modified with Acr under pH 9.8 conditions induces more repair synthesis than DNA modified with Acr under pH 8.0 conditions. These results indicate that α-OH-Acr-dG adducts induce more repair synthesis than γ-OH-Acr-dG adducts suggesting that α-OH-Acr-dG adducts are better substrates for excision repair. We also found that lysates from XPA cells are deficient in carrying out repair synthesis for both substrates, indicating that NER is the major mechanism for Acr-dG adduct repair (Fig. 6).

Acrolein effect on DNA repair

Because Acr contains a carbonyl group and an olefinic double bond, once it enters cells Acr can interact with numerous cellular components such as proteins and DNA through Michael additions. Two systems have been used to measure Acr effect on DNA repair: host cell reactivation (HCR) of UV-modified vector containing luciferase gene and *in vitro* DNA damage dependent repair synthesis. In the former, luciferase will not be expressed until the UV damage in the target gene is repaired. In the latter, the level of repair synthesis, which can be measured by incorporation of ^{32}P labeled nucleotides, is directly proportional to the DNA repair capacity in the cell lysates [9]. We have found that cells treated with Acr have lower HCR for UV-irradiated luciferase, indicating that the Acr treatment causes an inhibitory effect on DNA repair (Fig. 7A) [9]. This interpretation was further confirmed by results showing that cell lysates isolated from Acr-treated cells have lower capacity to carry out repair synthesis using UV-damaged templates (Fig. 7B & D) [9]. The reduction of repair capacity in cells treated with Acr apparently comes from Acr-repair protein interactions based on results which show that the addition of Acr directly to the cell free cell lysates can also effectively inhibit cell lysates from carrying out repair synthesis (Fig. 7C & D) [9].

In the process of mapping Acr-DNA adduct repair kinetics in the p53 gene in cells treated with Acr, we found that Acr-dG adducts are not significantly repaired in the p53 gene, as well as in genome overall, after 8 h incubation in NHLF; similar results were found in XPA cells (Fig. 8A & B). Since we have found that Acr-dG adducts are substrates for NER and that Acr cause an inhibitory effect on DNA repair for UV-induced DNA damage, together, these results suggest that Acr treatment causes two detrimental effects: DNA damage and reduction of repair capacity for Acr-induced DNA adducts.

Conclusions

Acr is not only a ubiquitous environmental contaminant, but also can be generated endogenously under oxidative stress. Ample evidence indicates that Acr interacts with many cellular components, resulting in deleterious effects. We have shown that through the interaction with DNA Acr induces two major stereoisomeric DNA adducts: α–OH-Acr-dG and γ–OH-Acr-dG. These two types of DNA adducts are also found in human lung tissue and furthermore, the amount of Acr-dG is significantly higher than benzo(a)pyrene diol epoxide (BPDE)-dG adducts [38, 39] The mechanisms that lead to the production of these two types of adducts is unclear. Nonetheless, both types of Acr-dG are mutagenic in different assay systems [11, 31–33]. Acr also has also been found to cause an inhibitory effect on DNA repair [9]. It seems that this effect is through its direct interaction with DNA repair proteins [9].

Intraperitoneal injection of Acr causes bladder cancer in rat models [3, 40, 41]. Metabolically produced Acr, by antitumor drugs such as cyclphosphamide and ifosfamide, can cause bladder cancer in humans [3, 40, 41]. We have found that the distribution of the Acr-DNA adduct in the p53 gene coincides with the p53 mutational pattern in cigarette smoke-related lung cancer [9]. Furthermore, the Acr-dG formed in the p53 gene, as well as in genome overall, is poorly repaired (Fig. 8). Therefore, based on the findings that: Acr-

induces DNA damage that is mutagenic, Acr causes an inhibitory effect on DNA repair, and Acr causes bladder carcinogenesis, we conclude that Acr is a carcinogen for human lung and bladder cancers. This conclusion raises an intriguing question: If Acr has such detrimental effects then why to date there is no evidence to imply Acr in cancers other than lung and bladder cancers in human? The answer probably will be found by understanding how Acr is metabolized in human body. Except for occupational and/or accidental exposure, which can occur anywhere, in the body, inhalation is the major entry route for exogenous Acr to the body. It is conceivable that bronchial epithelial cells are the first targets to be exposed to inhaled Acr. Once entering the blood stream, Acr is most likely to interact with amino acids and serum proteins through Schiff base formation. Those protein or amino acid-associated Acr is no longer "active" in causing detrimental effects until they reach the renal system, which under normal physiological conditions will free Acr from proteins and amino acids. The freed Acr is excreted in urine, accumulated in the bladder, and consequently causes detrimental effects to urothelial cells, including initiating bladder carcinogenesis.

In summary, Acr is a ubiquitous environmental contaminant; it is abundant in cigarette smoke and in cooking fumes. Acr is very reactive with various cellular components including amino acids, proteins and nucleic acids; it causes an inhibitory effect on DNA repair and induces mutagenic Acr-dG adducts. Acr causes bladder cancer in human and rat models. The binding spectrum of Acr in the p53 gene in normal human bronchial epithelial cells is similar to the p53 mutational spectrum in lung cancer. Therefore, we conclude that Acr is a major lung and bladder carcinogen, and its carcinogenicity arises via induction of DNA damage and inhibition of DNA repair.

Acknowledgments

Abbreviations

Acr	acrolein
2D-TLC	two dimensional thin layer chromatography
HPLC	high performance liquid chromatography
α- and γ-OH-Acr-dG	α- and γ-hydroxy-1,N^2-propano-2′-deoxyguanosine
NP1	nuclease P1
Cro-dG	crotonaldehyde adducted propano-dG
HNE	4-hydroxy-2-nonenal
HNE-dG	HNE adducted propano-dG
CHO	Chinese hamster ovary
NER	nucleotide excision repair
XPA	xeroderma pigmentosum complement group A
D37	dose yielding 37% survival

| NHBE | normal human bronchial epithelial |
NHLF	normal human lung fibroblasts
HCR	host cell reactivation
BPDE	benzo(a)pyrene diol epoxide
LC-ESI-MS/MS	liquid chromatography electrospray ionization tandem mass spectrometry

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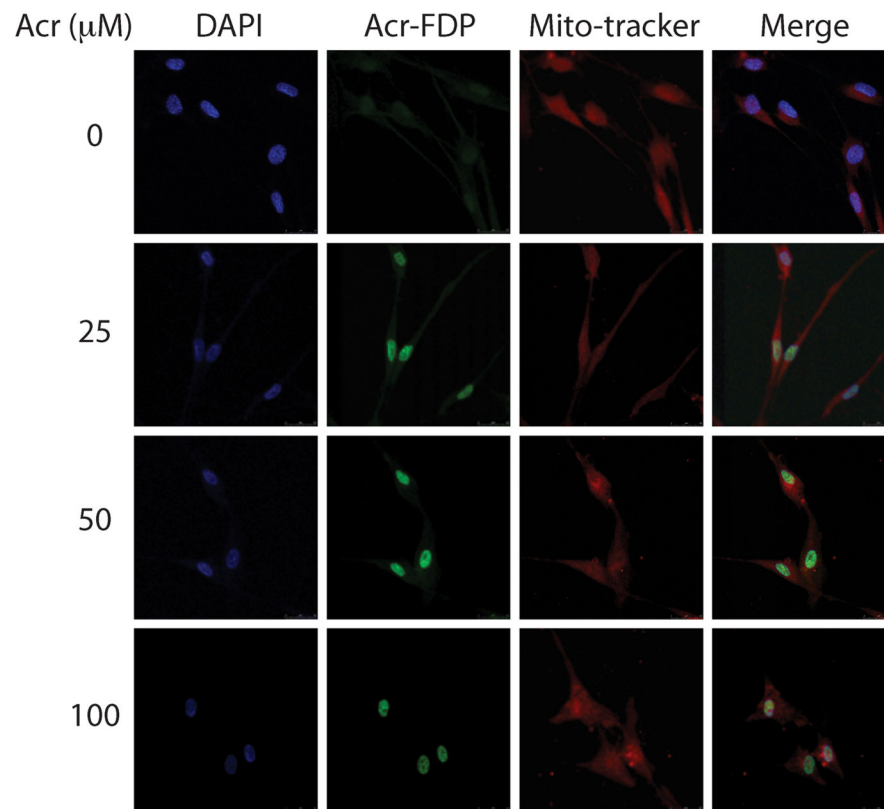
Figure 1.
Acrolein distribution in normal human lung fibroblasts (CCL202). Cultured CCL202 cells were treated with Acr (0–100 μM, 1 h), fixed, stained with anit-Acr-FDP-lysine (N^ε-(3-formyl-3,4-dehydropiperidino)-lysine) antibody followed by goat anti-mouse FITC-conjugated secondary antibody, and then examined by microscopy. The method is the same as previously described [8]. Note: DAPI stained DNA, Acr-FDP stained Acr-lysine adducts and Mito-tracker stained mitochondria.
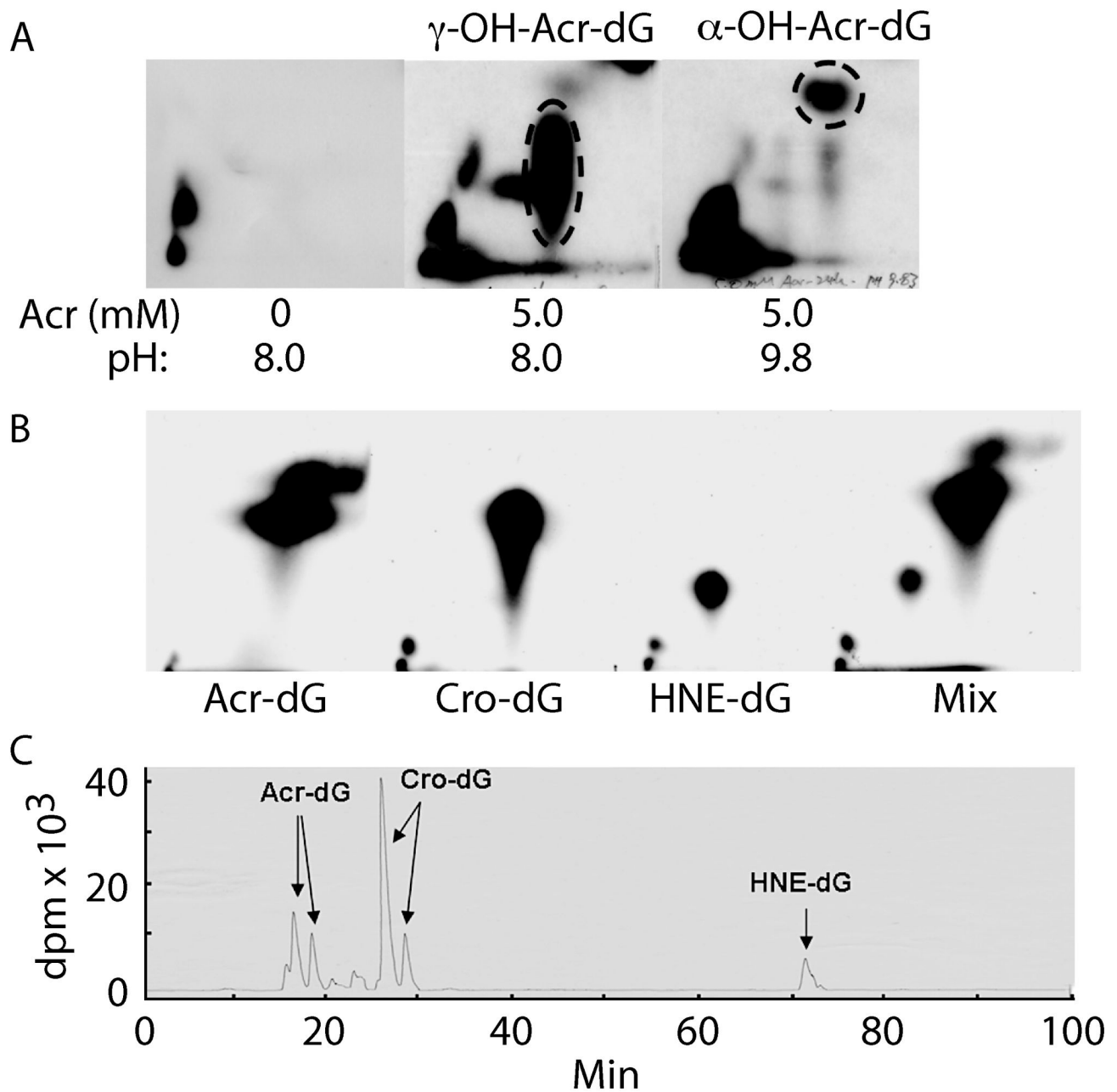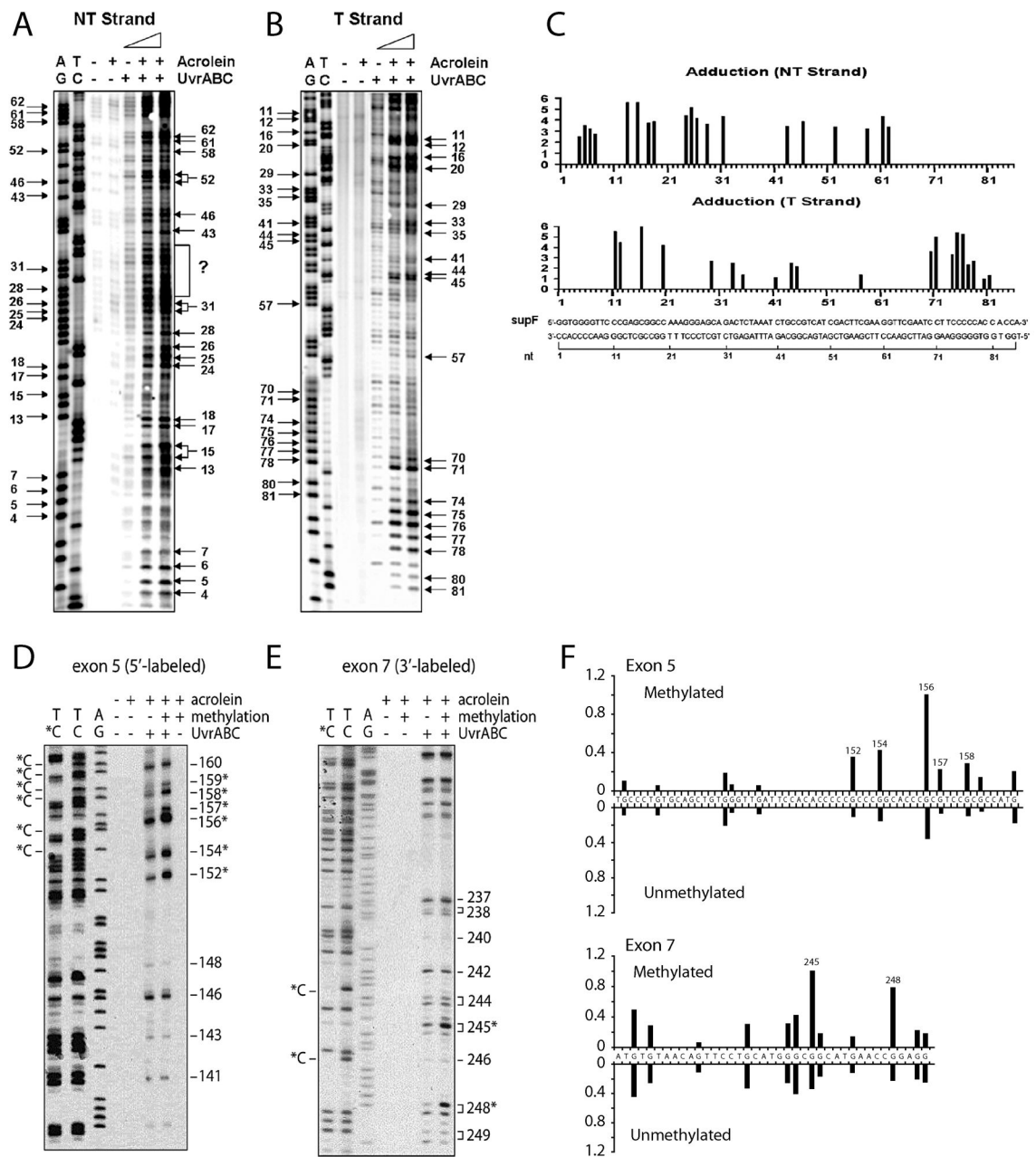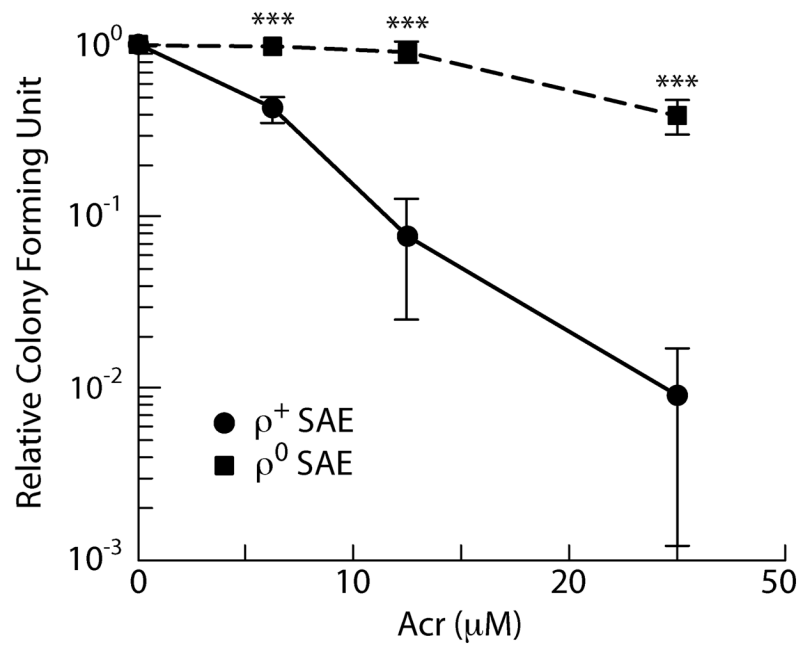
Figure 2.
Analysis of acrolein induced DNA adduct under pH 8 and pH 9.8 reaction conditions by ^{32}P post-labeling, 2 dimensional thin layer chromatography (2D-TLC) (A) followed by high performance liquid chromatography (HPLC). The DNA adducts in the indicated spots that contain Acr-dG were extracted and further separated by HPLC. Acr-dG, and Cro-dG co-migrate in 2D-TLC (B). Acr-dG, Cro-dG and HNE-dG can be well separated by HPLC (C). The methods for Acr-DNA modification and adduct analysis are the same as previously described [9, 11].
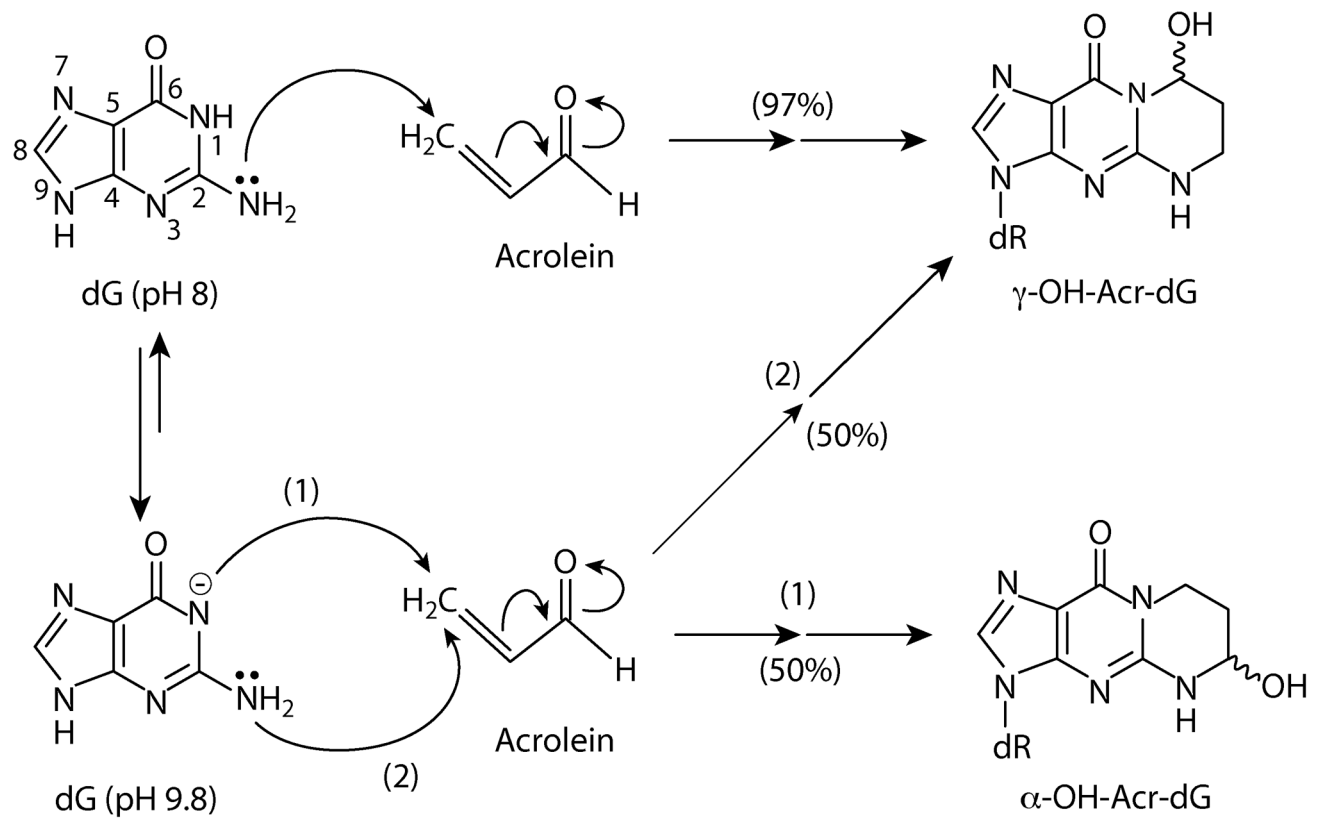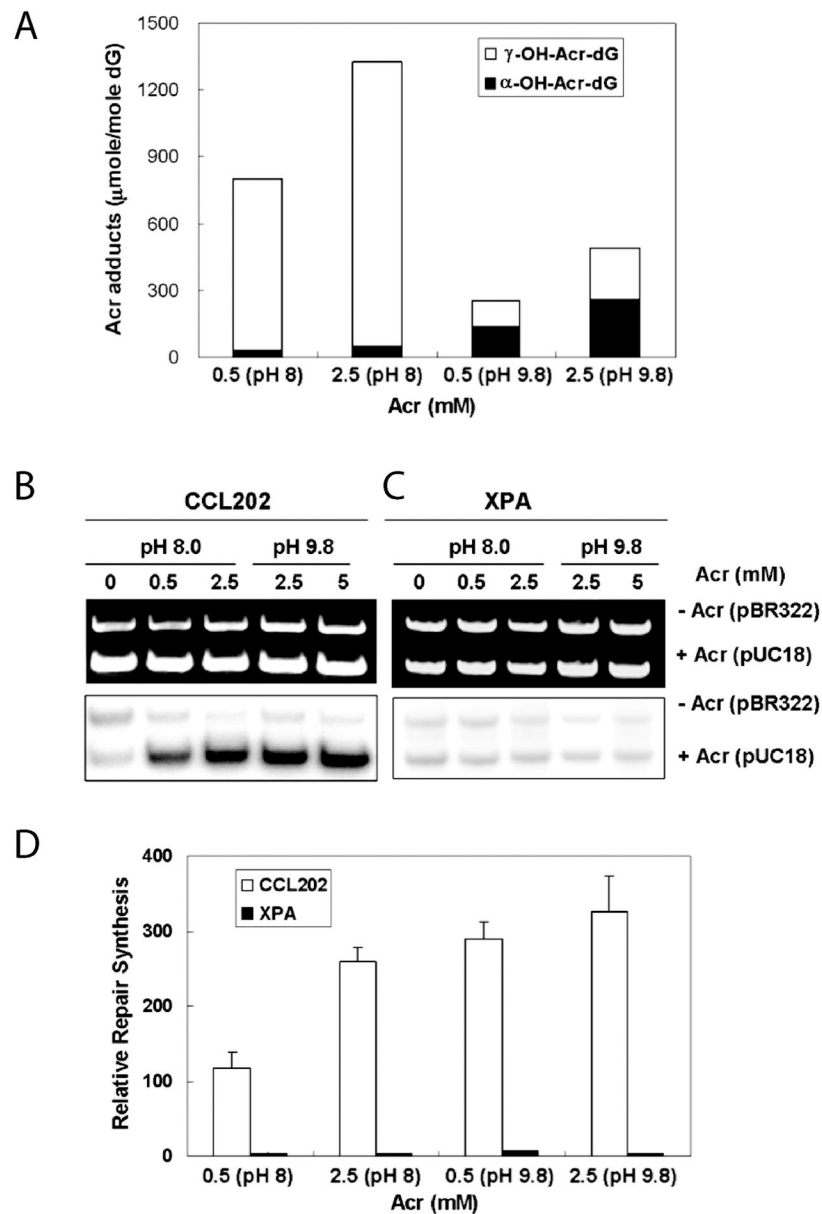
Figure 3.
Hypothetical acrolein – deoxyguanosine reactions under pH 8 and pH 9.8 conditions that lead to formation of α-OH-Acr-dG and γ-OH-Acr-dG adducts.

Figure 4.
Sequence specificity and cytosine methylation effect on Acr-dG adduct formation. A) and B) are ^{32}P 5′-single end labeled *supF* DNA fragments. D) and E) are ^{32}P 5′-single end labeled exon 5 of the p53 gene and ^{32}P 3′-single end labeled exon 7 of the p53 gene fragments, respectively (with and without methylated at –CpG sequences by CpG methylase). Labeled DNA were modified with Acr and then reacted with UvrABC. The resultant DNAs were separated by denaturing DNA sequencing gel electrophoresis. C) and F) are quantitations. AG and TC represent Maxam and Gilbert sequencing reaction products; the positions of the methylated cytosines, which are resistant to Maxam Gilbert reactions, are indicated by C* [9, 11].

Figure 5.
Acrolein-induced cell killing in human small airway epithelial (SAE) cells and their mitochondria depleted derivatives ($\rho°$). Exponentially growing SAE and cells $\rho°$ cells were treated with different concentrations of acrolein for 1 h and the cell survival was determined by colony forming ability. The method for generating $\rho°$ cells is the same as described by Huang [27].

Figure 6.
α- and γ-OH-Acr-dG induced DNA repair synthesis carried out by cell lysates isolated from normal human lung fibroblasts (CCL202) and xeroderma pigmentosum group A (XPA) cells. Supercoiled plasmid DNA pUC18 was modified with Acr under pH 8 and pH 9.8 conditions which produce different ratios of α-OH-Acr-dG : γ-OH-Acr-dG adducts (3 : 97 for pH 8 and 50 : 50 for pH 9.8). These Acr-modified pUC18 and pBR322 were used as substrates for *in vitro* repair synthesis in the presence of ^{32}P-dATP, the same manner as previously described [9]. (A) Acr-dG adduct analysis and (B–D) *in vitro* repair synthesis carried out by cell lysates of CCL202 and XPA cells. Note: DNA modified with Acr under pH 9.8 conditions produces lower total Acr-dG adducts but higher α-OH-Acr-dG/γ-OH-Acr-dG ratios than DNA modified with Acr under pH 8.0 conditions, and DNA modified with
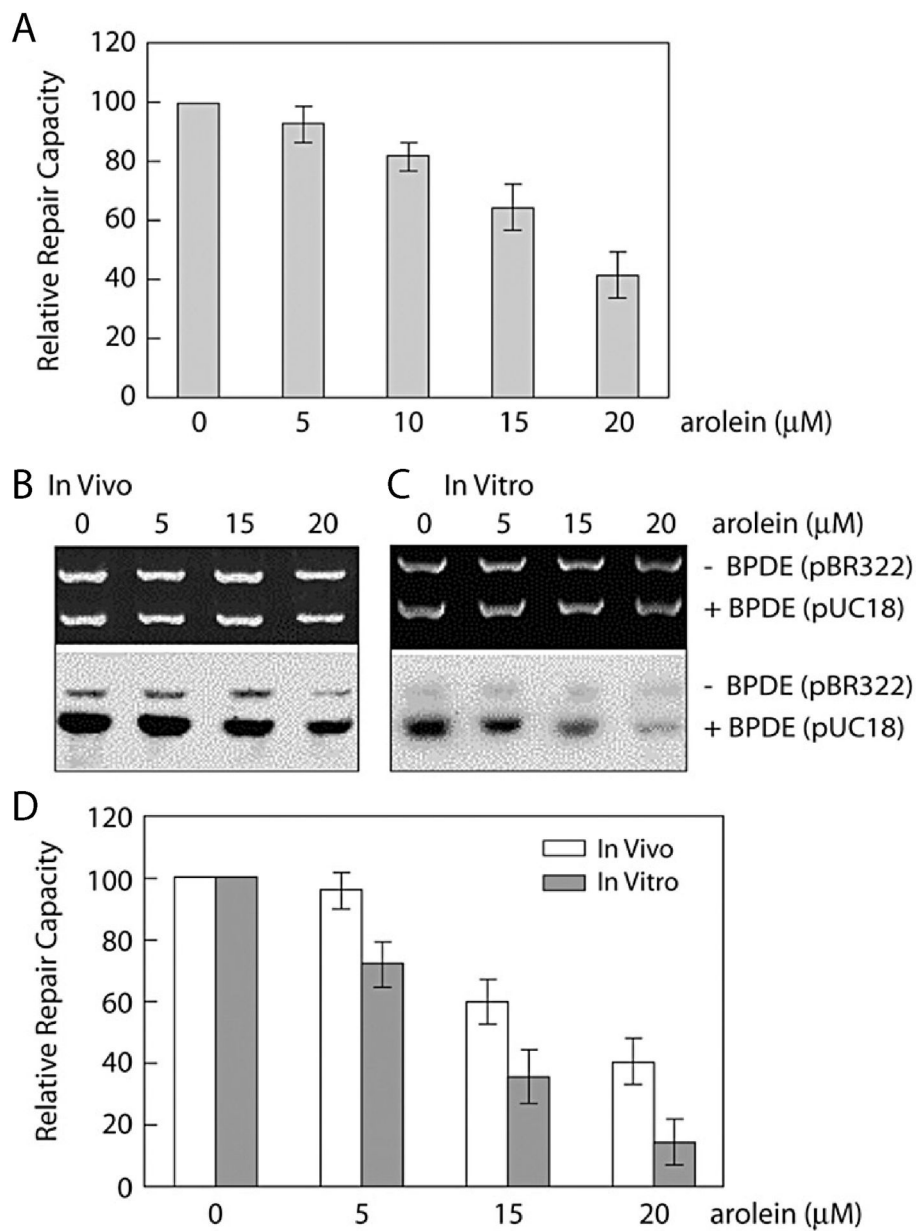
Acr under pH 9.8 conditions induces more repair synthesis than DNA modified with Acr under pH 8.0 conditions. These results indicate that α-OH-Acr-dG adducts are better substrates for repair synthesis.

Figure 7.
Inhibitory effect on DNA repair capacity by Acr. (A) Host cell reactivation (HCR) assay.
UV-irradiated luciferase reporter plasmids and unmodified β-galactosidase plasmids were
transfected into normal human lung fibroblasts (CCL202) (NHLF) with Acr treatments, and
the luciferase and β-galactosidase activities were measured post-transfection. (B–D) *In vitro*
DNA repair synthesis assay. UV-irradiated pUCC18 and unmodified pBR322 plasmids were
used as DNA substrates for *in vitro* DNA repair synthesis assay. In (B) NHLF were treated
with Acr first and the cell extracts were used for repair assay. In (C) Acr was added directly
into cell extracts prepared from untreated NHLF. In the upper panel are ethidium bromide-
stained gels, and the lower panel are autoradiographs of the same gels. In (D) the relative
repair capacity was calculated from (B) and (C) [9].
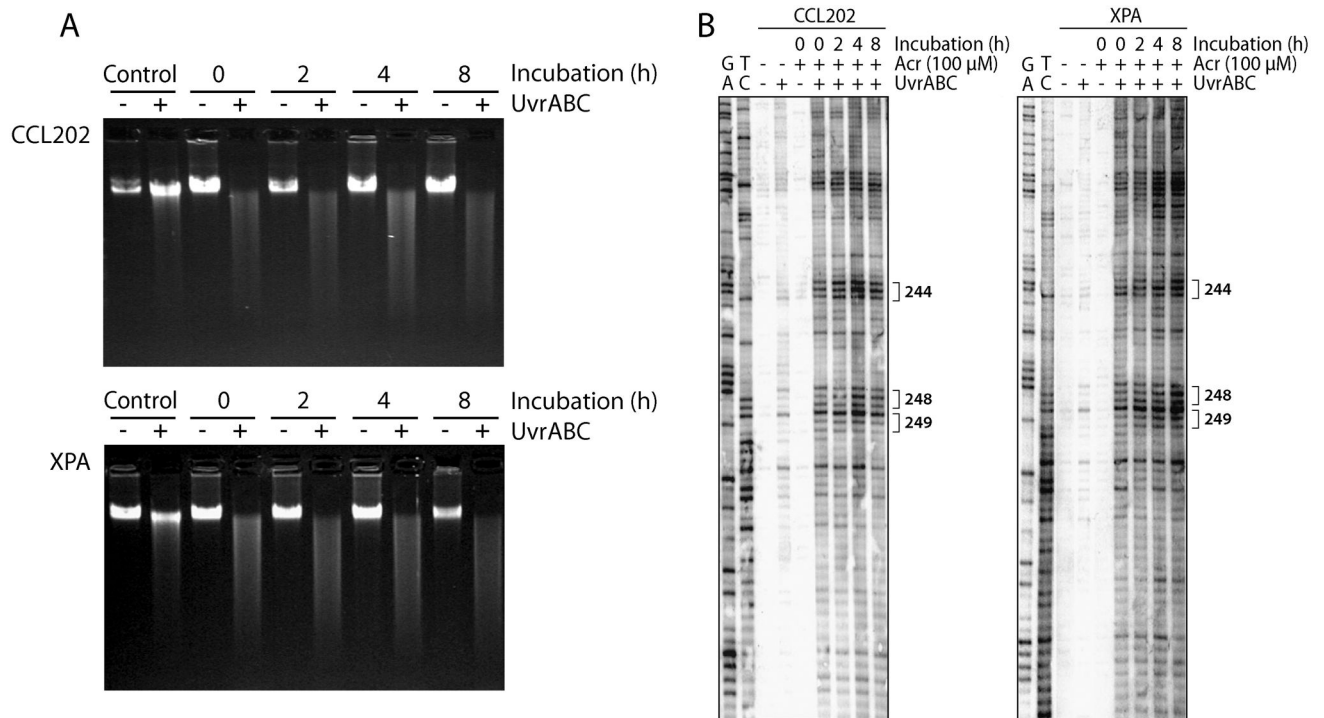
Figure 8.
Lack of repair of Acr-induced DNA damage in the p53 gene (A) as well as in genome overall (B) in normal human NER proficient NHLF (CCL-202) and NER deficient XPA cells. Exponentially growing cells at 70% confluency were treated with Acr (100 μM) for 6 h, then incubated in fresh medium without Acr for 0, 2, 4, and 8 h. The genomic DNAs were isolated and reacted with UvrABC which can cut Acr-dG adducts [9, 11]. Acr-DNA adducts formed in exon 7 of the p53 gene were mapped at the nucleotide level by the UvrABC-LMPCR method as previously described [9]. Note: Acr-DNA adducts were not repaired at the genome level and at the codons 244, 248 and 249 of the p53 gene.

Table 1

Mutations in *supF* gene induced by Acr-modifications under pH 8 and 9.8 conditions manifested in normal human bronchial epithelial cells (NHBE) and normal human lung fibroblasts (CCL202) [*].

Cell lines	pH	8	8	8	9.8	9.8
	Acr (mM)	0	0.5	2.5	2.5	5
NHBE	No. mutants/Total colonies	3/25120	128/9940	31/1483	2/15624	23/56448
	Mutation frequency ($\times 10^4$)	1.2	128.8	209.0	1.3	4.1
CCL202	No. mutants/Total colonies	11/101440	78/51216	53/9920	45/74400	114/75360
	Mutation frequency ($\times 10^4$)	1.1	15.2	53.4	6.0	15.1

[*] Acr-DNA modifications and *supF* mutation detection are the same as previously described (9,11)