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An Adenine-DNA Adduct Derived from Nitroreduction of 6-Nitrochrysene is more Resistant to Nucleotide Excision Repair than Guanine-DNA Adducts

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Abstract

Previous studies in rats, mice and *in vitro* systems showed that 6-NC can be metabolically activated by two major pathways: 1) the formation of *N*-hydroxy-6-aminochrysene by nitroreduction to yield three major adducts: *N*-(dG-8-yl)-6-AC, 5-(dG-*N*²-yl)-6-AC and *N*-(dA-8-yl)-6-AC, and 2) the formation of *trans*-1,2-dihydroxy-1,2-dihydro-6-hydroxylaminochrysene (1,2-DHD-6-NHOH-C) by a combination of nitroreduction and ring oxidation pathways to yield: *N*-(dG-8-yl)-1,2-DHD-6-AC, 5-(dG-*N*²-yl)-1,2-DHD-6-AC and *N*-(dA-8-yl)-1,2-DHD-6-AC. These DNA lesions are likely to cause mutations if they are not removed by cellular defense mechanisms before DNA replication occurs. Here we compared for the first time, in HeLa cell extracts *in vitro*, the relative nucleotide excision repair (NER) efficiencies of DNA lesions derived from simple nitroreduction and from a combination of nitroreduction and ring oxidation pathways. We show that the *N*-(dG-8-yl)-1,2-DHD-6-AC adduct is more resistant to NER than the *N*-(dG-8-yl)-6-AC adduct by a factor of ~2. Furthermore, the *N*-(dA-8-yl)-6-AC is much more resistant to repair since its NER efficiency is ~ 8-fold lower than that of the *N*-(dG-8-yl)-6-AC adduct. On the basis of our previous study and the present investigation, lesions derived from 6-NC and benzo[*a*]pyrene can be ranked from the most to the least resistant lesion as follows: *N*-(dA-8-yl)-6-AC > *N*-(dG-8-yl)-1,2-DHD-6-AC > 5-(dG-*N*²-yl)-6-AC ~ *N*-(dG-8-yl)-6-AC ~ (+)-7*R*,8*S*,9*S*,10*S*-benzo[*a*]pyrene diol epoxide-derived *trans-anti*-benzo[*a*]pyrene-*N*²-dG adduct. The slow repair of the various lesions derived from 6-NC and thus their potential persistence in mammalian tissue, could in part account for the powerful carcinogenicity of 6-NC as compared to B[*a*]P in the rat mammary gland.

Introduction

It has been reported that, in addition to genetic disposition, a significant portion of cancer incidence in the USA is related to environmental factors and lifestyle.^{1–6} Chronic exposure to traces of chemical carcinogens in the diet, in polluted air, or in tobacco smoke can be important in the etiology of certain cancers in the presence of host factors that favor the multistep process of carcinogenesis.^{7–9}

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Nitropolycyclic aromatic hydrocarbons (NO₂-PAHs) are ubiquitous environmental contaminants because they are byproducts of combustion of diesel and gasoline fuels, and are therefore present in airborne particulates and in certain foods and beverages.^{10–12} More recently, the International Agency for Research on Cancer (IARC)¹³ assessed the carcinogenicity of some NO₂-PAHs, including 6-nitrochrysene (6-NC) that are well known diesel and gasoline combustion products. Diesel exhaust was classified as “carcinogenic to humans” (Group 1) and gasoline as “possibly carcinogenic to humans” (Group 2B), and 6-NC as “probably carcinogenic to humans” (Group 2A) by the IARC working committee.

6-NC is the most potent carcinogen ever tested in the newborn mouse assay.¹⁴ The carcinogenic potency of 6-NC in the rat mammary gland is not only higher than that of benzo[*a*]pyrene and its bay region diol epoxide, but is also higher than that of the well-known food-derived heterocyclic aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.^{15–18}

Several *in vivo* studies in mice and rats, and also *in vitro* assays, have demonstrated that 6-NC can be activated by two major pathways (Figure 1).^{19–21} The first pathway involves simple nitroreduction to form the corresponding 6-hydroxyaminochrysene (N-OH-6-AC); the latter is known to yield three DNA adducts: *N*-(dG-8-yl)-6-AC, 5-(dG-*N*²-yl)-6-AC, and *N*-(dI-8-yl)-6-AC; the latter (inosine) adduct is a product of deamination of the adenine adduct *N*-(dA-8-yl)-6-AC.^{12, 19} The second pathway involves a combination of ring oxidation and nitroreduction, yielding the reactive electrophile, *trans*-1,2-dihydroxy-1,2-dihydro-6-hydroxyaminochrysene (1,2-DHD-6-NHOH-C). We reported previously that incubation of 1,2-DHD-6-NHOH-C with calf thymus DNA resulted in the formation of three adducts.²¹ These adducts were identified by LC/MS combined with ¹HNMR as 5-(dG-*N*²-yl)-1,2-DHD-6-AC, *N*-(dG-8-yl)-1,2-DHD-6-AC and *N*-(dA-8-yl)-1,2-DHD-6-AC (Figure 1). DNA adducts derived via both of these pathways, detected by ³²P-postlabelling analysis, were found in several organs including the mammary gland of rats treated with 6-NC orally²² or by i.p. injection.^{20, 23} In an earlier study, Delclos *et al*²⁴ concluded that the metabolic activation of [³H]6-NC in the neonatal mouse involved a combination of ring oxidation and nitroreduction pathways.

DNA lesions derived from 6-NC are likely to cause mutations if they are not removed by cellular defense mechanisms before DNA replication occurs. We have shown earlier that guanine adducts derived via the simple nitroreduction of 6-NC such as *N*-(dG-8-yl)-6-AC and 5-(dG-*N*²-yl)-6-AC are rather moderate substrates of the human nucleotide excision repair (NER) apparatus in HeLa cell extracts *in vitro*.²⁵ These results suggest that these two DNA adducts may persist in mammalian tissues and thus could contribute to the potent tumorigenic activity of 6-NC. However, nothing is known about the NER activities of the *N*-(dG-8-yl)-1,2-DHD-6-AC derived from the combined ring oxidation and nitroreduction pathways, or the *N*-(dA-8-yl)-6-AC derived via the nitroreduction pathway.

In this work we report that the nitroreduction product of 6-NC, *N*-OH-6-AC, also reacts with adenine either in oligo-2'-deoxyribonucleotides or calf thymus DNA, to yield the adduct *N*-(dA-8-yl)-6-AC (Figure 1). The direct existence of this adduct has not been previously reported, and here we report the synthesis and characterization of a site-specifically modified oligonucleotide containing a single adenine *N*-(dA-8-yl)-6-AC adduct. A further objective of this work is to compare the NER efficiencies of the *N*-(dA-8-yl)-6-AC and *N*-(dG-8-yl)-1,2-DHD-6-AC lesions embedded in identical sequence contexts in cell-free human cell extracts, with the NER efficiency reported previously for the *N*-(dG-8-yl)-6-AC adduct under identical conditions.²⁵ We show here that the NER efficiency associated with the *N*-(dG-8-yl)-1,2-DHD-6-AC is about 50% of the efficiency observed in the case of the previously studied *N*-(dG-8-yl)-6-AC adduct,²⁵ while the efficiency of NER of the *N*-(dA-8-

yl)-6-AC adduct is ~ 8 times lower than *N*-(dG-8-yl)-6-AC, and the levels of dual incision products are close to background.

Materials and Methods

Caution: *6-NC and its known metabolites described here are mutagenic in bacterial and mammalian systems as well as tumorigenic in rodents. Therefore, appropriate safety procedures must be followed when working with these compounds.*

Preparation of *N*-OH-6-AC and 1,2-DHD-6-NHOH-C

These compounds were synthesized as we reported previously.^{21, 26}

Preparation of Modified Oligonucleotides

Briefly, the modified oligonucleotide 5'-CTCTCG*CTTCC (G* = *N*-(dG-8-yl)-1,2-DHD-6-AC) was synthesized by directly reacting 1,2-DHD-6-NHOH-C with the corresponding 11-mer oligonucleotide in aqueous buffer solution as described previously.²¹ The modified oligonucleotide 5'-CTCTCA*CTTCC [A* = *N*-(dA-8-yl)-6-AC] was prepared in a similar manner by reaction of *N*-OH-6-AC with the unmodified oligonucleotide. The purities of the modified 11-mer oligonucleotides containing site-specific *N*-(dG-8-yl)-1,2-DHD-6-AC and *N*-(dA-8-yl)-6-AC were demonstrated using denaturing 15% high resolution gel electrophoresis methods. A small fraction of these modified oligonucleotides were digested to the nucleotide level using a combination of phosphodiesterase I and II exonucleases for use in detailed MS/MS analysis; the structural characterization of the *N*-(dG-8-yl)-1,2-DHD-6-AC is described in detail elsewhere.^{21, 25, 27}

Reaction of *N*-OH-6-AC with calf thymus DNA

The basic procedure of El-Bayoumy *et al.*²¹ was followed with some minor modifications. Calf thymus DNA (25 mg) was dissolved in 5 mL of 50 mM sodium phosphate buffer (pH=5.0) and 0.8 ml of a 24 mM solution of *N*-OH-6-AC in dimethyl sulfoxide (DMSO) was added to this solution under nitrogen. After incubation for 30 minutes at 37°C, the mixture was extracted with two volumes of water-saturated ethyl acetate, two volumes of water-saturated *n*-butanol, and finally two volumes of a 24:1 chloroform/isoamyl alcohol solution. Subsequently, NaCl was added to yield a 0.5 M solution, and the DNA was precipitated with 2 volumes of ice-cold ethanol, washed twice with 70% ethyl alcohol/water, then with acetone and finally with ether. Enzymatic digestion of the DNA was achieved by dissolving the DNA in 20 mM sodium succinate, 8 mM CaCl₂ (pH= 6.0), and Micrococcal Nuclease (1500 units) was added to the DNA solution, and the solution was then incubated at 37 °C for 1 h. Subsequently, Nuclease P1 (30 units) was added, and the mixture was incubated at 37 °C for an additional 1.5 h, and then incubated with spleen phosphodiesterase (10 units) and alkaline phosphatase (50 units, 2 units/mg DNA) after adjusting the pH to 9 with 0.1 M Tris (pH 9) buffer. This mixture was incubated at 37 °C for 16–18 h, and then extracted twice with an equal volume of water-saturated *n*-butanol, and was concentrated under reduced pressure. A small amount of DMSO was added to the residue, and the solution was analyzed by HPLC using an Agilent 1200 Series Rapid Resolution LC System equipped with a 250 mm × 4.6 mm C18 column (ACE).

Mass Spectrometry

The modified oligonucleotides were enzymatically digested and the structures of the modified guanine or adenine residues were identified using an Agilent 1100 Series capillary LC/MSD Ion Trap XCT equipped with an electrospray ion source. In the ion trap experiments, 8 µL of the solutions containing the oligonucleotides were injected into a

narrow bore Zorbax SB-C8 column (50 × 1 mm i.d.) and eluted with an isocratic mixture of methanol and water (70:30) with 0.1% formic acid as the mobile phase, at a flow rate of 0.25 mL/min. The mass spectra were recorded in the positive mode. The nebulizer gas pressure was 40 psi, the dry gas flow rate was 8.0 L/min, and the dry temperature was set at 350 °C.

Preparation of oligonucleotide NER substrates

Briefly, the first step was to ensure the purity of the modified 11-mer oligonucleotides by repeated reversed-phase HPLC methods. The purity was further verified using ³²P-endlabelled 11-mer oligonucleotides containing the lesions and denaturing 12% polyacrylamide gels as described.²⁵ The purified 11-mer oligonucleotides 5'-CTCTCG*CTTCC containing the single G* = *N*-(dG-8-yl)-1,2-DHD-6-AC lesion or 5'-CTCTCA*CTTCC containing the single A* = *N*-(dA-8-yl)-6-AC lesion were ³²P-labeled at the 5'-end and incorporated into 135-mer oligonucleotides by standard ligation methods as described elsewhere.^{25, 28} The adducts were situated at the 67th nucleotide position counted from the 5'-end. These internally and radioactively labeled and carcinogen-modified 135-mer sequences were then purified using 12% denaturing polyacrylamide gels and subsequently annealed with their fully complementary 135-mer strands with either C or T opposite the adducts G* or A*, respectively, by heating the solution to ~90°C for 2 min and cooling overnight to 4°C.

Preparation of cell extracts and nucleotide excision repair assays

The HeLa S3 cells (American Type Culture Collection) were grown in culture using standard methods as described.²⁵ The cell extracts were prepared using standard methods as described.²⁹ The 135-mer duplexes (1 nM) containing single *N*-(dG-8-yl)-1,2-DHD-6-AC, *N*-(dA-8-yl)-6-AC, or *N*-(dG-8-yl)-6-AC lesions were then incubated with the cell extracts for specified amounts of time (*cf* Results). After the incubation, the oligonucleotide excision products and intact DNA sequences were desalted by precipitation with 80% (v/v methanol), denatured by treatment with hot formamide (90 °C) for ~ 2 min, and subjected to electrophoresis in denaturing 12% (w/v) polyacrylamide gel. The gels were analyzed by autoradiography using a GE Storm 840 phosphorimager. The NER dual incisions yielded short oligonucleotide fragments 24–32 nt in length containing the ³²P-label and the lesion, that are easily resolved from the 135-mer unreacted oligonucleotides. The yield of dual incision products was determined from densitometry tracings of autoradiographs by summing the total radioactivity in the 24–32 oligonucleotide region and by dividing it by the total radioactivity in the same lane as described in more details elsewhere.^{30–32}

Results

Characterization of modified oligonucleotides

After completion of the reactions of 1,2-DHD-6-NHOH-C or *N*-OH-6-AC with the 11-mer oligonucleotides, the reaction mixtures were subjected to further HPLC separation and purification cycles. Following the final purification step and isolation of the modified 11-mer reaction products, the 11-mer sequences were enzymatically digested to the nucleoside dG* level, or to dinucleotides d(A*T)/d(TA*) in the case of the modified adenine residues. The enzymatic digestion products containing the *N*-(dA-8-yl)-6-AC adducts were then characterized by the same mass spectrometric methods³³ that were used to establish the structures of the *N*-(dG-8-yl)-6-AC guanine adducts.²⁵ The structural features of the *N*-(dG-8-yl)-1,2-DHD-6-AC were previously characterized and reported.²¹

A typical elution profile of the modified 11-mer oligonucleotide 5'-CTCTCG*CTTCC with G* = *N*-(dG-8-yl)-1,2-DHD-6-AC is depicted in Figure 2A. The major fraction eluting at

15.1 min exhibited a UV absorption spectrum (shown in the insert) which is consistent with that of the synthetic *N*-(dG-8-yl)-1,2-DHD-6-AC adduct reported earlier.²¹ After the full enzymatic digestion of this oligonucleotide, the nucleoside digestion product dG* was isolated and exhibited the same retention HPLC retention time as the authentic *N*-(dG-8-yl)-1,2-DHD-6-AC product²¹ in HPLC co-elution experiments (data not shown). The LC/MS/MS fragmentation patterns (Figure S1, Supporting Information) were found to be identical to the fragmentation patterns of an authentic *N*-(dG-8-yl)-1,2-DHD-6-AC nucleoside adduct standard described previously.²¹

The reaction of *N*-OH-6-AC with the oligonucleotide 5'-CTCTCACTTCC was carried out under identical conditions used to modify the single guanine residue in 5'-CTCTCGCTTCC to generate the modified oligonucleotide containing a single G* = *N*-(dG-8-yl)-6-AC adduct as described earlier.²⁵ A typical elution profile obtained after a previous HPLC cycle that removed the unmodified oligonucleotide, is shown in Figure 2B. The major fraction eluting at 17.1 min contained the reaction products 5'-CTCTCA*CTTCC with A* = *N*-(dA-8-yl)-6-AC.

MALDI MS/MS fragmentation patterns

N-(dI-8-yl)-6-AC has been previously reported in both target and non-target organs of rodents treated with 6-NC²¹; this adduct is derived from the deamination of *N*-(dA-8-yl)-6-AC. However, the existence of the *N*-(dA-8-yl)-6-AC adduct has not been reported previously and we therefore analyzed the fragmentation patterns of the enzymatically digested oligonucleotide 5'-CTCTCA*CTTCC (Figure 3). Unlike the digestion of oligonucleotides with single *N*-(dG-8-yl)-6-AC guanine adducts, this oligonucleotide containing the *N*-(dA-8-yl)-6-AC adducts proved resistant to digestion to the single nucleoside level. Such phenomena have been observed previously in the case of digestion of oligonucleotides containing single, site-specifically inserted polycyclic aromatic hydrocarbon-deoxyadenosine adducts by 5'- and 3'-exonucleases.³⁴ The digestion of 5'-CTCTCA*CTTCC yielded the end-product 5'-CA* (molar mass 781.3, Figure 3A, B). The major MS/MS fragmentation patterns are depicted in Figures 3C and 3D. Cleavage of the glycosidic bond shown in Figure 3B yields the 6-AC-adenine residue (m/z 376.2, Figure 3A and 3B) that is further fragmented as shown in Figure 3C and 3D. The loss of the C6-NH₂ group from the six-membered ring (b in Figure 3D), assigned to the elimination of (CH₂), yields ions at m/z 348. Further fragmentations in the sequence (c) → (d) → (e) yields fragments of m/z 321, 294 and 267, respectively, due to consecutive HCN (-27 Da) eliminations. Such a fragmentation pattern is typical for adenine,^{35, 36} and is consistent with the structures shown in Figures 3B and 3D. These MS/MS fragmentation patterns are also analogous to those obtained with the authentic *N*-(dG-8-yl)-6-AC standard.²⁵

The same approach was used to analyze the products of reaction of *N*-OH-6-AC with calf thymus DNA (Supporting information). Reversed phase HPLC analysis yielded several reaction products that eluted at different time points (Figures S2, S3). The products 5-(dG-*N*²-yl)-6-AC, *N*-(dA-8-yl)-6-AC, and *N*-(dI-8-yl)-6-AC were readily detectable. The *N*-(dI-8-yl)-6-AC adducts arises from the deamination of *N*-(dA-8-yl)-6-AC adduct as shown earlier by El-Bayoumy *et al.*²¹ The enzymatic digestion of adenine adducts in *N*-OH-6-AC - treated calf thymus DNA was incomplete because two fractions with identical masses m/z 796.3 were found that correspond to the mass of the d(TA*)/d(A*T) dinucleotides (Supporting Information, Figures S2, S3, and S4). These results indicate that the phosphodiester bond between the TA* and A*T residues resists cleavage by nucleases. This might explain why the *N*-(dA-8-yl)-6-AC adducts were not observed in *N*-OH-6-AC-treated calf thymus DNA earlier.²¹

In order to further verify these assignments, the oligonucleotide 5'-CCATCGCTACC that contains an AT and a TA sequence, was exposed to *N*-OH-6-AC under conditions similar to those used for the reaction with calf thymus DNA. Similar analysis of the enzymatic digestion of the modified oligonucleotides and LC-MS/MS analysis yielded the same two d(TA*)/d(A*T) fractions with *m/z* 796.3 that were observed in the case of calf thymus DNA treated with *N*-OH-6-AC; the fragmentation patterns were analogous (Figure S5) to those shown in Figure 3 for the digestion products of the oligonucleotide 5'-CTCTCA*CTTCC. Thus, the d(TA*)/d(A*T) enzymatic digestion products of the oligonucleotide 5'-CCATCGCTACC-3' yield the same d(TA*)/d(AT*) masses (Figure S5) and the same fragmentation patterns as those determined after *N*-OH-6-AC-treatment of calf thymus DNA and enzymatic digestion (Figure S4). Taken together, these experiments confirm that *N*-OH-6-AC reacts readily with adenine residues in DNA to form *N*-(dA-8-yl)-6-AC adducts. Therefore, the inosine d(I*T)/d(TI*) deamination products of these adenine adducts were also found in the enzyme-digested calf thymus DNA that had been reacted with *N*-OH-6-AC (Figure S6). Interestingly, adenine appears to be more reactive than guanine in the 5'-CCATCGCTACC sequence context since no *N*-(dG-8-yl)-6-AC adducts were detected when this oligonucleotide was treated with *N*-OH-6-AC.

Nucleotide excision repair assays

The dual excision efficiencies of the *N*-(dG-8-yl)-6-AC and 5-(dG-*N*²-yl)-6-AC adducts in 135-mer duplexes were reported previously.²⁵ The NER efficiencies for these two adducts were found to be approximately the same and about eight times smaller than for a cisplatin-derived G*TG* intrastrand cross-linked lesion. Here we used the *N*-(dG-8-yl)-6-AC adduct as a reference and compared the relative dual incision efficiencies of the *N*-(dG-8-yl)-1,2-DHD-6-AC and *N*-(dA-8-yl)-6-AC adducts relative to this adduct.

After incubating 135-mer duplexes containing either G* = *N*-(dG-8-yl)-1,2-DHD-6-AC or the *N*-(dG-8-yl)-6-AC adducts in HeLa cell extracts, followed by gel electrophoresis, the typical gel phosphorimager patterns shown in Figure 4A were obtained. Prominent bands due to 24–30-mer dual incision oligonucleotide fragments appear and their intensities increase with incubation time. The duplex with the *N*-(dG-8-yl)-1,2-DHD-6-AC lesion is a weaker NER substrate than the one with the reference *N*-(dG-8-yl)-6-AC adduct. A comparison of NER efficiencies of duplexes with the *N*-(dA-8-yl)-6-AC adenine adduct and with the *N*-(dG-8-yl)-6-AC guanine adduct are shown in Figure 4B; the intensity of the latter image was enhanced in order to better evaluate the relative intensities of the weak dual incision bands relative to the background. It is evident that the NER efficiency of the C8-adenine adduct is much weaker than that of the C8-guanine adduct, thus indicating that the *N*-(dA-8-yl)-6-AC adduct is significantly more resistant to NER than the guanine adducts *N*-(dG-8-yl)-6-AC, *N*-(dG-8-yl)-1,2-DHD-6-AC (Figure 4) and 5-(dGN²-yl)-6-AC.²⁵

Examples of densitometry tracings of the lanes of the gels in Figures 4A and 4B at the 45 min incubation time points are compared for each of the three adducts in Figure 5. In Figure 5A, the intensities of the bands due to the NER excision fragments are significantly greater than the bands in the background due to unspecified nuclease activity observed in these cell extracts. Analogous phosphorimager patterns obtained in the case of 135-mer duplexes with either the *N*-(dA-8-yl)-6-AC adducts or the reference adduct *N*-(dG-8-yl)-6-AC are shown in Figure 5B. In the case of the adenine adduct, the vertical scale was magnified by a factor of 3 for a better comparison of the intensities of the NER bands relative to the background (Figure 5B).

Cell extracts prepared on different days are characterized by different NER activities. To take this variability into account, the NER efficiency of the 135-mer duplex with the *N*-(dG-8-yl)-6-AC adduct, measured at the 45 minute time point, was used as a reference in

each experiment. An arbitrary value of 100 was assigned to this efficiency in each set of experiments conducted with the same cell extract (the absolute NER values in different cell extracts varied from ~ 2 – 4%). The efficiencies at other time points and all other adducts were then calculated relative to this value of 100.³² Such independent experiments with different cell extracts were conducted four times. These averages and their standard deviations obtained at each incubation time point were used to construct the plots of the NER efficiencies for the three DNA adducts as a function of incubation time (Figure 6). This quantitative analysis shows that the NER efficiencies depend linearly on the incubation time at least up to 45 min as reported previously in the case of benzo[*a*]pyrene diol epoxide-derived guanine adduct, *N*-(dG-8-yl)-6-AC and 5-(dG-*N*²-yl)-6-AC adducts in similar in vitro NER experiments with 135-mer duplexes.³² The relative rates of NER dual incision product formation, assuming a linear time dependence and regression analysis of the data points in Figure 6, are summarized in Table 1. The results indicate that the *N*-(dG-8-yl)-6-AC guanine adduct derived from nitroreduction of 6-NC is the best NER substrate in this series. The relative rate of excision is about two-fold smaller in the case of the *N*-(dA-8-yl)-1,2-DHD-6-AC adduct, and 8 ± 1 times smaller in the case of the adenine adduct *N*-(dG-8-yl)-6-AC derived from the simple nitroreduction of 6-NC.

Finally, we note that the extensive degradation at the site of the lesion at nucleotide position 67 revealed by the intense bands at that position in the gels (Figure 4) does not result from cleavage during the cell extract experiments, but as a result of cleavage induced by the hot formamide treatment of the samples used to denature the DNA samples after incubation in cell extracts and prior to the implementation of the gel electrophoresis experiments, as discussed earlier.²⁵

Discussion

In previous studies the existence of *N*-(dG-8-yl)-6-AC, 5-(dG-*N*²-yl)-6-AC, and *N*-(dG-8-yl)-1,2-DHD-6-AC derived from the nitroreduction and/or combination of nitroreduction and ring oxidation pathways of 6-NC, was reported.^{10–12, 24, 25} In the present work, we demonstrate that activation of 6-NC by the nitroreduction pathway readily gives rise to the adenine adducts *N*-(dA-8-yl)-6-AC in calf thymus DNA and in oligonucleotides containing adenine residues.

Reaction of DNA with *N*-hydroxyamines derived from the reduction of several NO₂-PAH or from the *N*-oxidation of aromatic amines yields C8-guanine, *N*²-deoxyguanosine and C8-deoxyadenosine adducts.³⁷ In general, the type and levels of the lesion depend on the structure of the parent compound.³⁸ For example, it has been reported that activation of 2-nitropyrene (2-NP) results in the formation of C8-guanine and C8-adenine lesions in DNA³⁹ while its isomer, 1-nitropyrene (1-NP), yields only C8-guanine adducts. These results³⁹ suggest that the adenine adduct may contribute to the powerful mutagenicity of 2-NP as compared to 1-NP.³⁸

The relative contributions of C8-guanine and C8-adenine residues derived from the metabolic activation of 6-NC to the mutagenic burden of cells have not yet been evaluated. However, the mutagenic activities of DNA adducts can manifest themselves only if they are not removed by DNA repair mechanisms before cellular DNA replication occurs. It is therefore of interest to evaluate and compare the relative NER efficiencies of the different 6-NC-derived guanine and adenine adducts.

Based on the results of the present study and those reported previously, the *N*-(dG-8-yl)-6-AC and 5-(dG-*N*²-yl)-6-AC adducts are approximately as resistant to human NER and as poorly repaired in human cell extracts as the highly genotoxic 10*S* (+)-*trans*-B[*a*]P-*N*²-dG

adduct derived from the biologically relevant (+)-7R,8S,9S,10R-benzo[*a*]pyrene diol epoxide enantiomer.²⁵ However, the *N*-(dA-8-yl)-6-AC adduct is ~ 8 times more resistant to NER than the *N*-(dG-8-yl)-6-AC lesion. The *N*-(dG-8-yl)-6-AC lesion is more resistant to NER than the *cis*-Pt adduct by a factor of about 15,²⁵ and is a better substrate than the *N*-(dG-8-yl)-1,2-DHD-6-AC lesion by a factor of ~ 2. Taken together, lesions derived from 6-NC and B[*a*]P can be ranked from the most to the least resistant lesion as follows: *N*-(dA-8-yl)-6-AC > *N*-(dG-8-yl)-1,2-DHD-6-AC > *N*-(dA-8-yl)-6-AC ~ 5-(dG-*N*²-yl)-6-AC ~ (+)-*trans*-B[*a*]P-*N*²-dG. The results reported here demonstrate that the slow repair – though to a varied extent – of the various lesions derived from 6-NC and thus their potential persistence in mammalian tissue, could in part account for the powerful carcinogenic activity of 6-NC as compared to B[*a*]P in the rat mammary gland.^{17, 18}

In a recent study, we have shown that adducts derived from the binding of dibenzo[*a,l*]pyrene diol epoxide to *N*⁶-adenine are strongly resistant to NER in human HeLa cell extracts, while the guanine adducts are moderate to strong substrates of NER in the same assays.⁴⁰ Furthermore, there are several established examples of DNA lesions that are resistant to NER; these include adenine adducts such as 7-(deoxyadenosin-*N*⁶-yl) aristolactam adducts derived from aristolochic acid,^{41, 42} 3-(deoxyguanosin-*N*²-yl)-2-acetylaminofluorene (dG(*N*²)-AAF),⁴³ and other fjord PAH-*N*⁶-adenine adducts,⁴⁴ and DNA adducts derived from 3-nitrobenzanthrone,⁴⁵ and 2-nitrofluorene.⁴⁶ Thus, the 6-NC-derived adenine adduct constitutes yet another example of resistance of bulky DNA adducts to the human nucleotide excision repair system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NO₂-PAH	nitropolynuclear aromatic hydrocarbons
6-NC	6-nitrochrysene
<i>N</i>-OH-6-AC	6-hydroxylaminochrysene
1,2-DHD-6-NC	<i>trans</i> -1,2-dihydroxy-1,2-dihydro-6-nitrochrysene
1,2-DHD-6-NHOH-C	<i>trans</i> -1,2-dihydroxy-1,2-dihydro-6-hydroxylaminochrysene
<i>N</i>-(dG-8-yl)-6-AC	<i>N</i> -(deoxyguanosin-8-yl)-6-aminochrysene
5-(dG-<i>N</i>²-yl)-6-AC	5-(deoxyguanosin- <i>N</i> ² -yl)-6-aminochrysene
<i>N</i>-(dA-8-yl)-6-AC	<i>N</i> -(deoxyadenosine-8-yl)-6-aminochrysene
<i>N</i>-(dA-8-yl)-1,2-DHD-6-AC	<i>N</i> -(deoxyadenosin-8-yl)-1,2-dihydroxy-1,2-dihydro-6-aminochrysene
5-(dG-<i>N</i>²-yl)-1,2-DHD-6-AC	5-(deoxyguanosin- <i>N</i> ² -yl)-1,2-dihydroxy-1,2-dihydro-6-aminochrysene

N*-(dI-8-yl)-1,2-DHD-6-AC**N*-(deoxyinosin-8-yl)-1,2-dihydroxy-1,2-dihydro-6-aminochrysene**

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Figure 1.

Metabolic activation of 6-nitrochrysene (6-NC) *in vivo* (rats and mice) and *in vitro* systems is depicted in this figure. Literature data demonstrated that 6-NC can be activated by two major pathways. The first pathway involves simple nitroreduction to form the corresponding N-OH-6-AC; the latter is known to yield three DNA adducts (see the right side of the figure). The second pathway involves a combination of ring oxidation and nitroreduction yielding 1,2-DHD-6-NHOH-C; the latter is known to yield three DNA adducts (see the left side of the figure).

Figure 2.

(A) Product of reaction of 1,2-DHD-6-NHOH-C with the oligonucleotide 5'-CTCTCGCTTCC. The modified oligonucleotides were first separated from the unmodified oligonucleotides in a first HPLC purification step as described, and then subjected to a second purification step as shown in this panel. Elution conditions: 12% – 24% acetonitrile-triethylamine acetate solution, 1 ml/min, ACE C18 model PFP column. The fraction eluting at 15.1 min contains the modified oligonucleotide 5'-CTCTCG*CTTCC with G* = N-(dG-8-yl)-1,2-DHD-6-AC. The UV spectrum is shown in the inset.

(B) Product of reaction of *N*-OH-6-AC with the oligonucleotide 5'-CTCTCACTTCC. The modified oligonucleotides were first separated from the unmodified oligonucleotides in a first HPLC purification step as described, and then subjected to a second purification step as shown in this panel. The elution conditions were the same as in (A). The fraction eluting at 17.1 [min contains the modified oligonucleotide 5'-CTCTCA*CTTCC with A* = *N*-(dA-8-yl)-6-AC. The UV spectrum is shown in the inset.

Figure 3.

MALDI MS/MS fragmentation patterns of a CA*/A*C sample derived from the enzymatic digestion of the 5'-CTCTCA*CTTCC sample (Fig. 2B). (A) Molar mass of the CA*/A*C fragment. (B) Chemical structure of the CA* fragment. (C) MS/MS fragmentation pattern of the m/z 376 Da fragment that results from the cleavage of the glycosidic bond of the modified adenine residue (see the text for details). (D) Chemical structure of *N*-(dA-8-yl)-6-AC.

Figure 4.

(A) Example of a comparison of nucleotide excision repair of 135-mer duplexes as a function of incubation time in HeLa cell extracts. (A) Comparison of NER activities of duplexes with $X^* = G^* = N\text{-(dG-8-yl)-6-AC}$ and $G^* = N\text{-(dG-8-yl)-1,2-DHD-6-AC}$ embedded at the 67th position counted from the 5'-ends of 135-mer duplexes in the sequence context 5'-.....CTCTCX*CTTCC.....Lane M: Oligonucleotide size markers; the lengths, and number of nucleotides are indicated next to lane M.

(B) Comparison of NER activities of duplexes with $X^* = G^* = N\text{-(dG-8-yl)-6-AC}$ and $X^* = A^* = N\text{-(dA-8-yl)-6-AC}$ adducts embedded in otherwise identical 135-mer duplexes. The

autoradiograph was intensified in order to view the weak-intensity bands in the case of the *N*-(dA-8-yl)-6-AC sample.

Figure 5.

Densitometry tracings of the 45-min time points of the gels shown in Figure 4. (A) The $G^* = N\text{-(dG-8-yl)-6-AC}$ and $G^* = N\text{-(dG-8-yl)-1,2-DHD-6-AC}$ samples. (B) The $G^* = N\text{-(dG-8-yl)-6-AC}$ and $A^* = N\text{-(dA-8-yl)-6-AC}$ samples (the vertical scale has been increased by a factor of three). The vertical scales have been normalized to one another to compensate for small loading differences in the individual lanes.

Figure 6.

Time dependence of relative NER efficiencies as a function of incubation time of 135-mer duplexes containing single lesions in HeLa cell extracts. The averages and standard deviations were obtained from four-five independent experiments with different HeLa cell extracts. In each individual experiment, the NER efficiency observed in the case of the *N*-(dG-8-yl)-6-AC adduct at the 45 min incubation time point was used as the reference value against which all other data points were normalized, obtained in the same experiment using the same cell extract. In this manner, the effects of variable NER activities of different cell extracts were eliminated. In different extracts, the fractions of duplexes that were doubly incised varied from 4.5 to 8.2 % at the *N*-(dG-8-yl)-6-AC adduct 45 min time point. In the example shown, the maximum fraction of duplexes incised was 4.5%. Squares, *N*-(dG-8-yl)-6-AC; triangles, *N*-(dG-8-yl)-1,2-DHD-6-AC; circles, *N*-(dA-8-yl)-6-AC.

Table 1

Initial rates of dual incisions of different adducts embedded in 135-mer duplexes in HeLa cell extracts (from Figure 6).

Adduct	% Duplexes incised min ⁻¹
<i>N</i> -(dG-8-yl)-6-AC	0.10 ± 0.01
<i>N</i> -(dG-8-yl)-1,2-DHD-6-AC	0.051 ± 0.005
<i>N</i> -(dA-8-yl)-6-AC	0.014 ± 0.002