DNA Adducts and Carcinogenicity of Nitro-polycyclic Aromatic Hydrocarbons

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We have been interested in the structure-activity relationships of nitro-polycyclic aromatic hydrocarbons (nitro-PAHs), and have focused on the correlation of structural and electronic features with biological activities, including mutagenicity and tumorigenicity. In our studies, we have emphasized 1-, 2-, and 6-nitrobenzo[a]pyrene (nitro-B[a]P) and related compounds, all of which are derived from the potent carcinogen benzo[a]pyrene. While 1-, 2-, and 3-nitro-B[a]P are potent mutagens in Salmonella, 6-nitro-B[a]P is a weak mutagen. In vitro metabolism of 1- and 3-nitro-B[a]P has been found to generate multiple pathways for mutagenic activation. The formation of the corresponding trans-7,8-dihydriodols and 7,8,9,10-tetrahydriodotetros suggests that 1- and 3-nitro-B[a]P trans-7,8-diol-9,10-epoxides are ultimate metabolites of the parent nitro-B[a]Ps. We have isolated a DNA adduct from the reaction between 3-nitro-B[a]P, trans-7,8-diol-9,10-epoxide and calf thymus DNA, and identified it as 10-(deoxyadenosino-N')-7,8,9,10-tetrahydro-7,8,9,10-tetrahydro-3-nitro-B[a]P. The same adduct was identified in vitro metabolism of 1-(deoxyguanosin-N')-7,8,9,10-tetrahydro-3-nitro-B[a]P. The same adduct was obtained from incubating DNA with 3-nitro-B[a]P in the presence of the mammalian nitroreductase, xanthine oxidase, and hypoxanthine. These data indicate that a mammalian nitroreductase can metabolize 3-nitro-B[a]P to an activated derivative that reacts with DNA to give a novel adduct distant from the site of nitrenium ion formation. Similar DNA adducts were obtained from ring-oxidation and nitroreduction of 1-nitro-B[a]P. Metabolism of 6-nitro-B[a]P resulted in a different pattern, and DNA adducts have not been detected from chemical or biologic activation. Based on these results, together with the other findings, we have proposed that orientation of the nitro functional group is a critical structural feature that can affect metabolism, DNA binding, mutagenicity, and tumorigenicity of nitro-PAHs. Additional data from the derivatives of the nitro-B[a]Ps support this hypothesis. — Environ Health Perspect 102(Suppl 6):177-184 (1994)

Key words: nitro-polycyclic aromatic hydrocarbons, structure-activity relationships, nitrobenzo[a]pyrene, DNA adduct, tumorigenicity

Introduction

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are mutagenic and tumorigenic pollutants that are present in a variety of environmental samples and in the food chain (1-10). We have been interested in the structure-activity relationships of nitro-PAHs and have focused on the correlation between the structural and electronic features and the biologic activities, including mutagenicity and tumorigenicity. Currently the nitro-PAHs and alylamines studied by most groups are derived from nontumorigenic or weakly tumorigenic PAHs such as pyrene and fluorene. Our interest has been particularly in the isomeric nitrobenzo[a]-pyrenes (nitro-B[a]Ps) (4,6,17), which are derived from the carcinogenic and mutagenic PAH benz[a]pyrene (B[a]P). B[a]P is the prototype for the study of chemical carcinogenesis of PAHs. Nitro-B[a]Ps can also be considered derivatives of B[a]P. Study of the carcinogenesis of nitro-B[a]Ps and comparison of the results with that of B[a]P will provide useful information on how a nitro group affects the biologic properties of B[a]P. Thus, the study of structure-activity relationships of nitro-B[a]Ps will provide a better understanding not only of the carcinogenicity of nitro-PAHs and alylamines, but of the carcinogenesis of PAHs as well.

Because nitro-PAHs require metabolic activation in order to exert their genotoxic activities (4,6,7), study of structure-activity relationships of nitro-PAHs has to include metabolism, DNA adduct formation, mutagenicity, and tumorigenicity. A number of studies have reported the correlation of structural features and mutagenicity in Salmonella. These include: a) the first half-cell reduction potential (6,12-14), b) the molecular dimensions and degree of aromaticity (4,6,15,16), c) the geometric location and the orientation of the nitro substituent (4,15,17-20), and d) the number of electrons involved in the first step of nitroreduction (21,22). We have studied the metabolism of a series of nitro-PAHs by rat liver microsomes and determined the mutagenicity of the nitro-PAH substrates and their in vitro metabolites in Salmonella. As a consequence, the metabolic activation pathways of these compounds that lead to mutation have been determined, and the structural and electronic features, which can be used to interpret and predict the bacterial mutagenicity of the nitro-PAHs, also have been determined (4,6,18,19,23-26). In this article, we report our studies on the metabolism, mutagenicity, DNA binding, and tumorigenicity of the isomeric nitro-B[a]Ps and related compounds and discuss the structure-activity relationships of nitro-PAHs.

Metabolism, Mutagenicity, and Metabolic Activation Leading to Mutation

Both 1- and 3-nitro-B[a]P have been demonstrated to exhibit potent bacterial and mammalian mutagenicity (8,19,24,25,27-31). The isomeric mixtures also exhibited greater than additive mutational responses in Salmonella typhimurium (32,33). Although 6-nitro-B[a]P is not a direct-acting mutagen in S. typhimurium TA98, it is mutagenic in the presence of S9 activation (8,23,27,28). We have recently determined the mutagenicity of 2-nitro-
B[a]P and found that this isomeric nitro-B[a]P is a potent mutagen in *S. typhimurium* tester strains TA98, TA100, and TA104, both in the presence and in the absence of S9 activation enzymes (16).

Incubation of 3-nitro-B[a]P under aerobic conditions with liver microsomes from untreated rats and from phenobarbital- and 3-methylcholanthrene-treated rats yielded 3-nitro-B[a]P trans-7,8-dihydriodiol and 3-nitro-B[a]P trans-9,10-dihydriodiol as predominant metabolites, and 3-nitro-B[a]P 7,8,9,10-tetrahydriodiol as a minor metabolite (24,26). The identification of 3-nitro-B[a]P 7,8,9,10-tetrahydriodiol as a metabolite suggests that the vicinal bay-region trans-7,8-diol antli-9,10-epoxide was formed during metabolism (24).

Metabolism of 3-nitro-B[a]P under hypoxic conditions yielded 3-amino-B[a]P as a predominant metabolite together with a trace of 3-nitro-B[a]P trans-7,8- and 9,10-dihydriodiol. 3-Nitro-B[a]P and its trans-dihydriodiol metabolites were potent direct-acting mutagens in *S. typhimurium* TA98, and their mutagenicities were greatly reduced in TA98NR and TA98/1,8-DNP₆ (24). Unexpectedly, 3-amino-B[a]P was also a potent direct-acting mutagen. The lower number of revertants produced by 3-nitro-B[a]P and its two trans-dihydriodiol metabolites in TA98NR suggests the involvement of bacterial nitroreduction in both the direct and S9-mediated mutagenicity of these three compounds. The lower mutagenicity in TA98/1,8-DNP₆ of 3-nitro-B[a]P trans-7,8- and 9,10-dihydriodiol in the presence and absence of S9 and of 3-nitro-B[a]P in the presence of S9 suggests that nitroreduction followed by esterification is necessary in order to exert the full mutagenicity of these compounds. However, esterification is not required for the direct-acting mutagenicity of 3-nitro-B[a]P (24). Based on the metabolism and the mutagenicity results of 3-nitro-B[a]P and its metabolites, multiple metabolic activation pathways exist, including nitroreduction, ring-oxidation, and ring-oxidation followed by nitroreduction and esterification. The metabolic profiles, the mutagenicities of the metabolites, and the multiple metabolic activation pathways of 1-nitro-B[a]P are similar to those of 3-nitro-B[a]P (25,34).

In contrast to 1- and 3-nitro-B[a]P, the metabolism of 6-nitro-B[a]P by rat liver microsomes under similar conditions generated 3-hydroxy-6-nitro-B[a]P as the predominant metabolite and the following minor metabolites: 1-hydroxy-6-nitro-B[a]P; 6-nitro-B[a]P 1,9,1-hydroquinone; 6-nitro-B[a]P 3,9-hydroquinone; and B[a]P 3,6-quinone (23). The formation of 1-hydroxy-6-nitro-B[a]P and 6-nitro-B[a]P 1,9-hydroquinone has been shown to be mediated via the 6-nitro-B[a]P 1,2-epoxide intermediate (35). Metabolism of B[a]P under similar conditions produced 3-hydroxy-B[a]P as the predominant metabolite, and both B[a]P trans-4,5- and 7,8-dihydriodiol were also formed in considerable quantity. Comparison of the metabolism patterns between B[a]P and the 1, 3-, and 6-nitro-B[a]P indicates that the nitro group affects the regioselectivity of the cytochrome P450 isozymes (26).

The major structural difference in these nitro-B[a]P isomers is that while 1- and 3-nitro-B[a]P have their nitro group preferentially adopting a parallel (co-planar) orientation, 6-nitro-B[a]P has its nitro group in a perpendicular orientation (14,17). Nitro orientation may be the critical factor that affects the regioselectivity of the cytochrome P450 isozymes.

**Nitro Orientation and Mutagenicity in Salmonella**

More than 40 structurally related nitro-PAHs were used to determine the relationships among direct-acting mutagenicity, orientation of the nitro group, and reduction potential of the nitro group (13,14,17). The compounds consisted of isomeric mono nitro and dinitro B[a]P, benzo[a]pyrenes, their derivatives, and other nitro-PAHs containing from two to five aromatic rings. A general finding is that nitro-PAHs with their nitro substituent oriented perpendicular to the aromatic system exhibit either very weak or no direct-acting mutagenicity in *S. typhimurium* strains TA98 and TA100. However, if a nitro-PAH of this type has a relatively low first half-wave reduction potential, it may be direct-acting (14).

Furthermore, a positive correlation between the first half-wave reduction potential and direct-acting mutagenicity is found only when the compounds are structurally similar (14). Nitro-PAHs with a perpendicular nitro orientation always have a higher (absolute value) first half-wave reduction potential than the isomer(s) with a parallel orientation.

**Nitro Orientation and Tumorigenicity**

Comparison of the tumorigenicity of a series of nitro-PAHs and their parent PAHs in the newborn mouse assay indicates that addition of a nitro group to a PAH can drastically alter its tumorigenicity. Based on the newborn mouse assay studied so far, we propose that when a nitro group is introduced to a carcinogenic bay-region containing PAH and the nitro group of the resulting nitro-PAH preferentially adopts a perpendicular or nearly perpendicular orientation, the tumorigenicity of this nitro-PAH is weaker than the parent PAH. Examples include conversion of benzo[a]anthracene to 7-nitrobenzo[a]anthracene, benzo[a]pyrene to 6-nitrobenzo[a]pyrene, and dibenzo[a,h]anthracene to 7-nitro dibenzo[a,h]anthracene. As previously reported by Wislocki et al. (36), the percentages of animals bearing liver tumors induced by 7-nitrobenzo[a]anthracene, benzo[a]anthracene, 6-nitrobenzo[a]pyrene, and benzo[a]pyrene are 28, 79, 28, and 49%, respectively (36). Similarly, the percentages of animals bearing liver nodules induced by 7-nitro dibenzo[a,h]anthracene and dibenzo[a,h]anthracene are 42 and 100%, respectively (unpublished data). Because attempts to prepare DNA adducts by chemical reduction of these compounds followed by reaction with calf thymus DNA failed, the nitro functional group is unlikely to be activated by reductive metabolism (unpublished data). Study of the oxidative metabolism of these compounds indicates that the formation of the bay-region diolepoxide may not always be diminished. For example, while metabolism of benzo[a]anthracene by mouse liver microsomes generated benzo[a]anthracene trans-3,4-dihydriodiol in a yield of less than 4% of the total metabolites, metabolism of 7-nitrobenzo[a]anthracene under similar conditions resulted in 7-nitrobenzo[a]-anthracene trans-3,4-dihydriodiol in a much higher yield (37). These data suggest that formation of the 7-nitrobenzo[a]-anthracene bay-region diolepoxide should be no less than that of the benzo[a]anthracene bay-region diolepoxide.

**DNA Adduct Formation**

Covalent binding of the activated metastabes of chemical carcinogens to cellular DNA is considered the critical step in the multistage process leading to tumor formation. We have studied the DNA adduct formation of a number of nitro-PAHs (38-41). The studies on 1- and 3-nitro-B[a]P include (a) preparation of DNA adducts by chemical reduction of nitro-B[a]Ps to form the N-hydroxyaminobenzo[a]P (n) in situ followed by reaction with calf thymus DNA; (b) use of the resulting adducts as standards to identify the DNA adducts formed from anaerobic incubation of nitro-B[a]Ps *in vitro*; (c) preparation of DNA adducts from...
Figure 1. Formation of 6-(deoxyguanosin-N²-yl)-3-aminobenzo[a]pyrene from nitroreduction of 3-nitro-B[a]P.

Figure 2. The proposed SN1 mechanism for the 6-(deoxyguanosin-N²-yl)-3-aminobenzo[a]pyrene formation.

Figure 3. Dewar reactivity numbers for substitution of polycyclic aromatic hydrocarbons.

The chemical reaction of nitro-B[a]P trans-7,8-diol-anti-9,10-epoxides with calf thymus DNA; and d) identification of the DNA adducts derived from nitro-B[a]P diol-epoxides in vitro. Attempts to prepare DNA adducts of 6-nitro-B[a]P under similar conditions failed (e.g., to prepare the N-hydroxy-6-amino-B[a]P in situ followed by reaction with calf thymus DNA).

DNA Adducts from Nitroreduction of 3-Nitrobenzo[a]pyrene

N-Hydroxy-3-amino-B[a]P, prepared in situ from reduction of 3-nitro-B[a]P, was reacted with calf thymus DNA. After enzymatic digestion of the DNA, the resulting modified nucleosides were analyzed by thermal HPLC-MS and high resolution proton NMR spectroscopy, and the adduct was identified as 6-(deoxyguanosin-N²-yl)-3-aminobenzo[a]P (42) (Figure 1). To avoid steric hindrance, this adduct has its deoxyguanosinyl moiety perpendicular or nearly perpendicular to the 3-aminobenzo[a]pyrenyl aromatic moiety. The same adduct was also formed from in vitro incubations of [3H]3-nitro-B[a]P with xanthine oxidase and rat liver microsomes in the presence of calf thymus DNA under hypoxic conditions (42). These results suggest that nitroreduction of 3-nitro-B[a]P to N-hydroxy-3-amino-B[a]P may be a metabolic activation pathway in mammalian systems. Incubation of S. typhimurium TA98 with [3H]3-nitro-B[a]P was performed, and the same adduct was detected as the predominant product (unpublished data). Thus, because 3-nitro-B[a]P is a potent direct-acting mutagen (24,28), these results suggested that 3-nitro-B[a]P is metabolized by bacterial nitroreductase(s) to N-hydroxy-3-amino-B[a]P, and the identified DNA adduct, 6-(deoxyguanosin-N²-yl)-3-aminobenzo[a]P, should be the species responsible for the mutation induction by 3-nitro-B[a]P. However, contrary to the potent mutagenicity, the preliminary results of the newborn mouse tumorigenicity assay indicates that 3-nitro-B[a]P is weakly tumorigenic; only 5 out of 24 mice developed hepatic nodules (43). These results suggest that either this adduct is not formed, or if it is formed, due to its bulky structure, it is rapidly repaired by the DNA repair enzymes.

Due to the long-range migration involved in the formation of 6-(deoxyguanosin-N²-yl)-3-aminobenzo[a]P, its formation likely involves an SN1 mechanism that includes a) formation of the nitrenium ion by removal of the hydroxyl anion from N-hydroxy-3-amino-B[a]P, b)
isomerization of this nitrenium ion to the energetically more stable carboxylation with the positive charge localized at the C6 position, c) attack by this carboxylation on calf thymus DNA, and d) isomerization and hydride migration to form the adduct (Figure 2). If the nitrenium ion [NH+] can be considered as a substituent to the B[a]P molecule, this long distance migration may be explicable by perturbation molecular orbital theory. Based on the simplified molecular orbital methods, Dewar reactivity numbers are very useful for prediction of substitution of PAHs (44). As shown in Figure 3, C6 of B[a]P has the lowest Dewar reactivity number (1.15) not only in B[a]P, but among the other PAHs shown in Figure 3 as well. The smaller the Dewar activity number, the more preferably the carbocation is formed (localized at that position), and thus it is more favorable for substitution or migration of the positive charge to occur at that position. This may account for the occurrence of this novel migration. Herreno-Saenz et al. reported that reaction of N-hydroxy-3-aminochrysene, formed in situ, with calf thymus DNA generated the N-(deoxyguanosin-C8-yl)-3-aminochrysene as the predominant adduct (45). Binding of calf thymus DNA to N-hydroxy-1-aminoarene formed from nitroreduction of 1-nitropyrene gave N-(deoxyguanosin-C8-yl)-1-aminoarene as the predominant adduct, and no other adducts were formed. The lower Dewar activity numbers for chrysene and pyrene, both at the C6 position, are 1.67 and 1.51, respectively, which are much higher than the 1.15 at the C6 position of B[a]P (Figure 3). Thus, the results seem to be consistent with the prediction by the simplified molecular orbital theory. Determining whether this electronic feature is the critical factor in directing the type of DNA adduct formed warrants further investigation.

**Figure 4.** Formation of the DNA adduct 10-(deoxyguanosin-N2-yl)-7,8-trihydroxy-7,8,9,10-tetrahydro-3-nitrobenz[a]pyrene (dG-3-nitro-B[a]P-DE) from 3-nitro-B[a]P trans-7,8-diol-anti-9,10-epoxide.

**Table 1.** Comparison of the stability and chemical activities between B[a]P trans-7,8-diol-anti-9,10-epoxide and 3-nitro-B[a]P trans-7,8-diol-anti-9,10-epoxide.

| Stability | Low | High |
|-----------|-----|------|
| Hydrolysis | THF/H2O | Fast | Low |
| Buffer   | Fast | Slow |
| Buffer/DNA | Fast | Slow |
| Toxicity in vivo | High | Low |
| DNA binding | Low | High |

**Figure 5.** Structures of 9-nitroanthracene 1,2-epoxide and 9-nitroanthracene 3,4-epoxide.
Figure 6. Proposed possible metabolic activation of 1-nitrobenzo[a]pyrene leading to tumor initiation.

The covalent binding of a nitro group to the B[a]P molecule may alter the molecule and its presence to a PAH molecule increases the molecular size. Consequently, addition of a nitro group to the B[a]P trans-7,8-diol-anti-9,10-epoxide molecule may alter the intercalation efficiency to the double helical DNA. Thus, it is important to determine, prior to covalent binding, whether intercalation of 3-nitro-B[a]P trans-7,8-diol-anti-9,10-epoxide with the double helical DNA is critical in covalent binding. It is also known that DNA catalyzes the hydrolysis of B[a]P trans-7,8-diol-anti-9,10-epoxide to the corresponding tetrahydrodiol and thus decreases the binding efficiency of B[a]P trans-7,8-diol-anti-9,10-epoxide to DNA. Therefore, it is also important to determine if DNA also catalyzes the hydrolysis of 3-nitro-B[a]P trans-7,8-diol-anti-9,10-epoxide to tetrahydrodiol. All of these areas are now under investigation.

Metabolic Activation of Nitro-B[a]P Leading to Tumor Induction

It is important to determine whether or not tumorigenicity also correlates with bacterial mutagenicity. Both 1- and 3-nitro-B[a]P are weak tumorigens when tested in the newborn mouse bioassay but are potent mutagens in the Salmonella mutagenicity assay. Thus, their tumorigenicity does not correlate with mutagenicity. The proposed mechanism of bioactivation of 1-nitro-B[a]P leading to tumor initiation is shown in Figure 6. The DNA adducts derived from nitroreduction and from the diol-epoxide have been prepared. The next step is to determine whether they are formed in vivo, and, if they are formed, to determine the quantity and the rate of repair.

Hydrolysis of B[a]P trans-7,8-diol-anti-9,10-epoxide to the tetrahydrodiol is a detoxification pathway. However, this may not be true for 1-nitro-B[a]P because 1-nitro-B[a]P tetrahydrodiol may be activated by nitroreduction to form the corresponding N-hydroxyamino species, which may covalently bind to DNA, and lead to tumor initiation. Similarly, 1-nitro-B[a]P trans-7,8-dihydridiol can also be activated via nitroreduction. Because 3-nitro-B[a]P trans-9,10-dihydridiol is also a major metabolite, nitroreduction and ring-oxidation followed by nitroreduction may be activation pathways for tumor initiation. Similar activation pathways for 3-nitro-B[a]P are proposed.

Conclusions

Nitro orientation has been demonstrated to be an important structural feature in determining the metabolism pattern, bacterial mutagenicity, DNA binding, and tumorigenicity of nitro-PAHs, which exhibit mutagenicity drastically different from their parent PAHs. Nitro-PAHs also exhibit markedly different mutagenicity among the geometric isomers. In general, a nitro-PAH with its nitro substituent adopting a perpendicular orientation exhibits either very weak or no direct-acting mutagenicity in S. typhimurium TA98 and TA100. However, if a nitro-PAH of this type has a relatively low first half-wave reduction potential, it may be direct-acting. The correlation described above is valid only when the compounds are structurally similar.

Covalent binding of DNA to the N-hydroxy-3-amino-B[a]P formed in situ results in 6-(deoxyguanosin-N2-yl)-3-amino-B[a]P which is formed via long-range migration. To direct the formation of this adduct, it is important to determine the mechanism of its formation as well as the determining factor(s) such as electronic and structural features of the molecule and the active site of the double helical DNA.

Nitro-PAHs exhibit tumorigenicity drastically different from their parent PAHs. Nitro-PAHs also exhibit markedly different tumorigenicity among the geometric isomers. When a nitro group is introduced to a carcinogenic bay-region containing PAH and the nitro group of the resulting nitro-PAH preferentially adopts a perpendicular or nearly perpendicular orientation, the tumorigenicity of this nitro-PAH is weaker than the parent PAH.

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