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Minireview

Covalent binding of benzo[a]pyrene 7,8-dihydrodiol 9,10-epoxides to DNA: molecular structures, induced mutations and biological consequences

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Abstract

Optical spectroscopic techniques have been used to characterize adducts formed upon reaction of the (+)- and (−)-enantiomers of 7R,8S-dihydroxy 9S,10R-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (*anti*-BPDE) to DNA or synthetic oligonucleotides. The reaction yields preferentially adducts in which the exocyclic aminogroup of deoxyguanosine is bound to the C10 position of the diol epoxide either *cis* ($BPDE_c$-N^2-G adduct) or *trans* ($BPDE_t$-N^2-G adduct) relative to the hydroxyl group at the C9 position. The $BPDE_c$-N^2-G and $BPDE_t$-N^2-G adducts fall into the categories of type I and type II complexes, respectively. Two-dimensional NMR in conjunction with energy minimization computation have provided detailed information on the solution structure of single adducts localized in oligonucleotides. The results demonstrate that the pyrenyl chromophores of both the (+)- and (−)-$BPDE_t$-N^2-G adduct are located in a widened minor groove and directed towards the 5′-end [(+)-$BPDE_t$-N^2-G] or the 3′-end [(−)-$BPDE_t$-N^2-G] of the modified strand. The chromophore of the (+)-$BPDE_c$-N^2-G adduct is quasi-intercalated into the oligonucleotide and associated with a displacement of the deoxyguanosine ring into the minor groove. Replication of racemic or (+)-anti-BPDE modified DNA in mammalian cells leads predominantely to single point mutations of transversion type (GC → TA). The mutagenic specificity however, appears to be determined by the base sequence context and local conformation at the adduct site. Cooperative adduct formation at certain base sequences is suggested by excimer fluorescence, most probably derived from two closely located (+)-$BPDE_t$-N^2-G adducts in adjacent base pairs on opposite DNA-strands.

Key words: Optical spectroscopy; Covalent binding; BPDE; DNA; NMR; Mutations

1. Introduction

An important factor in tumour formation in man is exposure to various physical and chemical agents [1,2]. A prevalent class of potent environmental chemical carcinogens is polycyclic aromatic hydrocarbons (PAHs), which are present in various petroleum and combustion products from heat and power generation and motor vehicle exhausts [3–5]. Since PAHs are generally produced by pyrolysis of organic matters such as in

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tobacco smoking and certain procedures of food preparation, the PAH exposure to humans is extensive [4–6].

Like most chemical carcinogens, PAHs are not active themselves but require metabolic transformation to bay-region diol epoxides (Fig. 1) and subsequent covalent binding of these reactive electrophiles to targets in DNA. Replication and fixation of the resulting damage as a critical mutation(s) is generally believed to initiate the carcinogenic process [7–9].

Bay-region diol epoxides can exist as two diastereomers; the *syn*- or *anti*-form in which the benzylic hydroxyl group and the epoxide oxygen is on the same or different side of the molecule respectively, and each diastereomer as a pair of enantiomers (Fig. 1). Biological studies of all possible isomers from a number of PAH-derived diol epoxides have shown that the *anti*-diastereomers with R,S-diol S,R-epoxide absolute configuration are in most cases the most tumorigenic form [9-11]. Furthermore, the steric conformation of the diol epoxides seems also to be of crucial importance since the most active forms prefer diequatorial orientation of their hydroxyl groups [9–12].

The most thoroughly studied representative of the carcinogenic PAHs is benzo[a]pyrene (BP). Our present knowledge on the molecular mechanisms involved in chemical carcinogenesis, particularly tumour initiation, is largely based on findings with this compound. The purpose of this review is to summarize some of the more recent findings on the molecular and spectroscopic characteristics of BP-diol epoxide (BPDE) DNA adducts. We discuss how the structural features of the adducts may relate to biochemical events such as the formation of mutations. We have particularly tried to extract information on base sequence specific events and structures, mutation mechanisms and protooncogene activation. Other reviews with a strong focus on the molecular properties of BPDE-DNA adducts have been published [13,14].

2. Metabolic activation of benzo[a]pyrene

The enzyme catalyzed formation of bay-region diol epoxides of BP (BPDE) involves epoxidation at the 7,8-position of BP to yield preferentially the BP-7R,8S-epoxide (Fig. 1, step 1). Hydrolysis

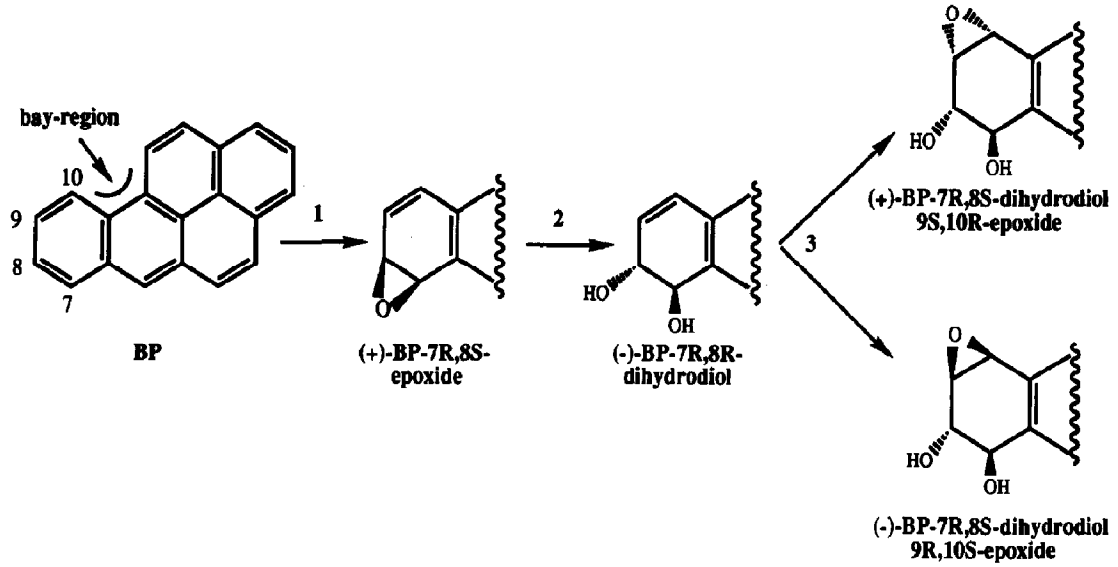

Fig. 1. Metabolic activation of benzo[a]pyrene (BP) to diastereomeric BP-7,8-dihydrodiol 9,10-epoxides via intermediate formation of BP-7,8-oxide and BP-7,8-dihydrodiol.

to the corresponding 7R,8S-dihydroxy-7,8-dihydro-BP (Fig. 1, step 2) followed by a second epoxidation at the 9,10-position yield 7R,8S,9S, 10R-BPDE ((+)-*anti*-BPDE) and 7R,8S,9R,10S-BPDE ((−)-*syn*-BPDE) (Fig. 1, step 3). Usually, the *anti*-diasteromer predominates although the actual ratio between *anti*- and *syn*-BPDE may vary depending on the experimental system employed. Similarly, the stereoselective metabolism of BP may also vary so that (−)-*anti*- and (+)-*syn*-BPDE may be formed in certain experimental systems. Extensive studies of *anti*- and *syn*-BPDE and their enantiomers have established that (+)-*anti*-BPDE is by far the most active form in mammalian systems [7,10,11,15] and have led to the suggestion that the high biological potency of this isomer is associated with unique structural properties of the adducts that result from covalent binding of the diol epoxide to DNA [12,13,16].

3. Structures of BPDE-DNA adducts

In analogy with the description of protein structure, DNA as well as DNA-adduct structures can be described at different levels: primary, secondary as well as tertiary structure. For DNA adducts the primary structure may describe the covalent bonding structure, the secondary structure may give a general description of adduct location, such as intercalation or minor groove binding, and the tertiary structure finally should give a three-dimensional description of the atomic coordinates of a DNA-adduct system, solid or in a solvent. For BPDE-DNA adducts, information is now available on all three levels, through various kinds of chemical and biophysical techniques. The heterogeneities observed to varying degrees in all biochemical reactions (*in vitro* or *in vivo*) leading to DNA adduct formation have presented

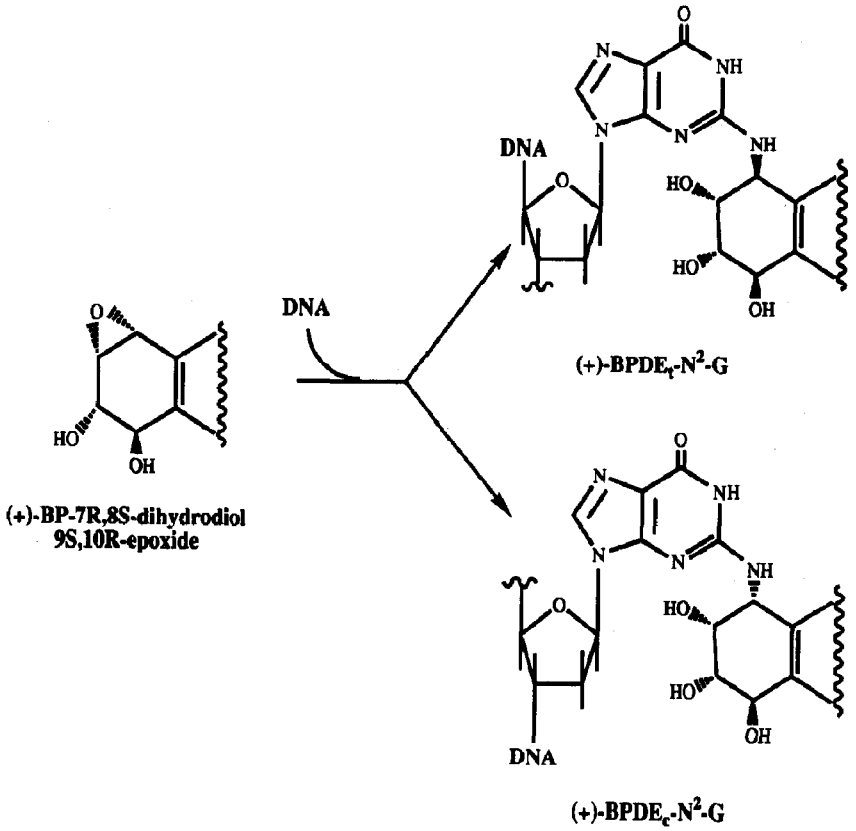

Fig. 2. Reaction of (+)-*anti*-BP-7,8 -dihydrodiol 9,10-epoxide with the exocyclic nitrogen of deooxyguanosine in DNA to yield *trans*- ((+)-BPDE$_t$-N^2-G) and cis- ((+)-BPDE$_c$-N^2-G) adducts.

special problems to define and describe major or average structures, as will be further discussed below.

It was known already from the end of the 1970s that exposure of mammalian cells to BP generally results in covalent binding of preferentially (+)-*anti*-BPDE to the exocyclic nitrogen of deoxyguanosine (G) in DNA (Fig. 2). This reaction seems to be highly selective, although minor binding sites involving mostly deoxyadenosine (A) were also found. The reaction was found to involve predominantly *trans* addition of N^2 of G to the C10 position of the diol epoxide (in the following referred to as (+)-$BPDE_t$-N^2-G adduct) Ref. ([17] and references therein). The stereochemistry of the *trans*- and *cis*-adduct is illustrated in Fig. 2.

3.1. Optical spectroscopy

A large number of spectroscopic studies have addressed the question of the secondary structures of DNA-BPDE adducts. Adducts arising from reactions of racemic *anti*-BPDE with DNA, or microsome-catalysed reactions of BP with DNA, resulted in heterogeneous adduct formation, although the chemically dominating (+)-$BPDE_t$-N^2-G adduct seemed to be a preferred secondary structure. The nature of this structure was however debated at length, since various kinds of spectroscopic techniques gave apparently conflicting results [13]. The spectroscopic studies have led to the definition of two major types of BPDE adducts, called I and II [14,18]. The preferred secondary structure of (+)-$BPDE_t$-N^2-G adduct corresponds to adduct type II, and seems to be correlated with the strong mutagenic and carcinogenic potency of (+)-*anti*-BPDE. Adducts of type I arise predominantly from less potent BPDE-isomers such as the (−)-*anti*-enantiomer and *syn*-BPDE. In this case the diol epoxides are bound to N^2-G via *cis*-addition of the benzylic C10 position (Fig. 2) [19–22]. Thus, *trans*-adducts are of type II and *cis*-adducts are of type I.

Adducts of type I are characterized by a ≈ 10 nm shift towards longer wavelength of their light absorption (relative to BP-tetraol, the parent chromophore, in water), a negative LD signal and strong fluorescence quenching (about 1% intensity relative to BP-tetraol in water) [14,23,24]. Since a negative LD signal shows that the molecular plane of the chromophore is close to perpendicular to the DNA helix axis, the suggested secondary structure was termed "quasi-intercalative". Also the considerable light absorption wavelength shift after adduct formation is consistent with intercalation-like geometry of the complex [12,13].

Adducts of type II are characterized by ≈ 2 nm shift towards longer wavelength of their light absorption, a positive LD signal and about a factor of 2 less efficient fluorescence quenching, compared to type I adducts (about 2% fluorescence intensity relative to BP-tetraol in water) [14,23,24] The positive LD signal suggested a geometry where the molecular plane of the chromophore makes less than 55° angle with the orientation (DNA helix) axis. This observation could be explained by groove binding, or by local deformation of the DNA helix, so that e.g. an intercalation-like geometry still would allow a small angle between the chromophore plane and the overall orientation axis of the DNA. As will be discussed further below, the preferred secondary structure of this adduct is now firmly established to be minor groove binding (shown by determination of the tertiary structure of a model system), as was originally proposed by Geacintov et al. in 1978 [25]. The adduct site has been described as a dynamically disordered "flexible joint" to account for the decreased stiffness of (+)-*anti*-BPDE modified DNA [26,27].

The structural features of the adducts discussed above have been obtained in systems where pure native or synthetic DNA has been reacted with the diol epoxide thus raising the question about the relevance in more *in vivo* like systems. Very little information is available on this important point. However, it has been shown by Eriksson et al. [28] using LD spectroscopy that (+)-*anti*-BPDE bound to chromatin form adducts consistent with type II complexes.

The highly efficient fluorescence quenching of BPDE in the G-bound adduct form, irrespective of secondary structure of the adduct, has recently been explained in terms of an efficient transient

electron transfer from the guanine base to the BPDE chromophore [29]. Transient light absorption spectra around room temperature after excitation of the BPDE adduct chromophore show characteristic contributions from guanine cation free radicals and pyrene anion free radicals. This mechanism is less efficient at low temperatures (100 K) [30], and also in denatured DNA, where the fluorescence quantum yield is considerably higher than in native DNA [23].

During studies of the fluorescence properties of (+)- and (−)-*anti*-BPDE adducts in poly(dG-dC)·poly(dG-dC), a characteristic long wavelength emission due to BPDE excimers (or possibly exciplexes) was observed (Ref. [31] and unpublished observations). Recent observations using different synthetic polynucleotides as well as DNA as substrate for adduct formation have shown that the excimers are preferentially formed in alternating GC sequences. Model building aided by energy minimization calculations based on the knowledge of the solution structure for a single adduct to be discussed below [32] have shown that the most likely site for excimer fluorescence contains a GC/CG step, where the guanine on either strand has been modified with BPDE [33]. Both BPDEs with partly overlapping pyrenyl moieties already in the ground state are contained within the minor groove, which is widened compared to the standard B form DNA (for adduct geometry, see below). An outline of the proposed conditions for excimer formation is shown in Fig. 3.

The effects of BPDE-adduct formation on polynucleotide conformation transitions has been studied by light absorption, flow LD and CD spectroscopy [27,34–37]. The rate of the NaCl-induced transition from B to Z conformation in BPDE-modified poly(dG-dC)·poly(dG-dC) was shown to be markedly increased by (+)-*anti*-BPDE adducts and to be decreased by (−)-*anti*- and (±)-*syn*-BPDE. The cooperativity of the transition is much less pronounced in the modified polynucleotides compared to the non-modified ones. One suggested conclusion is that the B-like conformation is retained at higher NaCl concentrations in the immediate vicinity of each BPDE adduct [27].

3.2. *NMR spectroscopy*

A tertiary solution structure of a DNA oligonucleotide duplex with a well defined site of (+)-*anti*-BPDE adduct formation has been reported from two-dimensional ^{1}H NMR studies [32]. A single strand of a non-selfcomplementary DNA 11-mer with a centrally placed guanine was reacted with (+)-*anti*-BPDE. The products of the

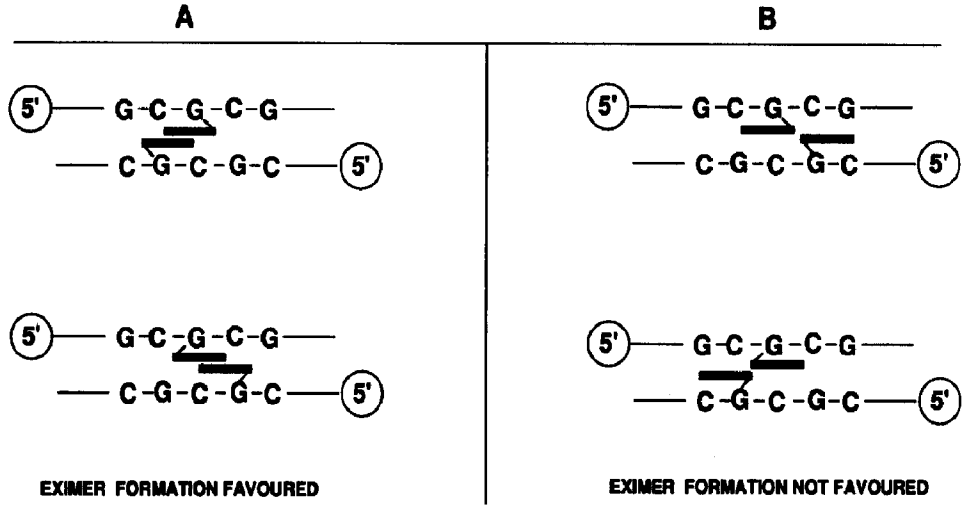

Fig. 3. The proposed requirements for the formation BPDE-excimers. (A) excimer formation of (+)-*anti*-BPDE (top) and (−)-*anti*-BPDE (bottom) favoured. (B) Excimer formation not favoured.

reaction were separated by HPLC as previously described [38] and the pure strands with (+)-BPDE$_t$-N^2-G adducts were collected. The modified strand was allowed to form a duplex with its complementary sequence, and two-dimensional nuclear Overhauser effect (NOESY) spectra were recorded. A number of spectral crosspeaks corresponding to intermolecular NOEs were observed and assigned, indicating close spatial proximity (< 5 Å) between certain protons of the 7,8,9-trihydroxy-7,8,9,10-tetrahydropyrene residue and of the DNA.

The results of the NMR study showed that on the average the BPDE chromophore is located in the minor groove of the oligonucleotide, directed towards the 5'-end of the modified strand, and stacking over part of the sugar-phosphate backbone of the unmodified strand. A solution structure was calculated by performing energy minimization computations, while including the NMR derived distance constraints as boundary conditions. At the end of the computations the DNA was found to be retained in a B-like conformation, with all Watson-Crick base pairs still intact. A widening of the minor groove to accomodate the pyrenyl residue of BPDE was observed. The angle between the long axis of the chromophore and the average helix axis was found to be 45°, in reasonable agreement with angles of 26° [26] and 39° [39], evaluated from flow dichroism studies of (+)-*anti*-BPDE adducts in poly(dG-dC)·poly(dG-dC) or DNA.

Using the same oligonucleotide template and NMR methodology, the covalent adducts of the only weakly carcinogenic (−)-enantiomer of anti-BPDE have also been characterized [40]. Recent studies with site- and stereo-specific BPDE-oligonucleotide adduct separation had revealed that the *trans*-adducts of (+)- as well as (−)-*anti*-BPDE preferentially occur in type II binding sites, whereas both *cis*-adducts preferentially occupy type I binding sites [21]. The (−)-BPDE$_t$-G adduct was chosen for further characterization of solution structure. The results again showed minor groove localization of the pyrenyl moiety, but in this case directed towards the 3'-end of the modified strand. The long axis of the chromophore was found to form 40° angle with the average DNA helix axis, in good agreement with the angle observed for the (+)-BPDE$_t$-G adduct as expected for a type II binding site. These observations cannot yet give a conclusive answer to the question of the structural basis for the great difference in tumorigenic activity between

Table 1
Spectroscopic and structural properties of diol epoxide adducts of type I and II in double-stranded DNA and synthetic polynucleotides

	Adduct type I	Adduct type II
light absorbance and fluorescence excitation maxima (cf. BP-tetraol 314, 328, 343 nm) [13,14,23,25]	322, 337, 354 nm	316, 330, 345 nm
excimer fluorescence excitation maxima [31]		320, 335, 351 nm
Linear dichroism signal	negative	positive
fluorescence quantum yield [cf BP-tetraol: 100%] [14,23,24]	1%	2%
binding characteristics	quasi-intercalated [43]	(a) minor groove binding, retained B-form DNA [32,40] (b) dynamically disordered "flexible joint" [26]
examples of adducts in double-stranded DNA	(+)-BPDE$_c$-N^2-G, deoxyguanosine ring displaced into the minor groove [43]	(+)-BPDE$_t$-N^2-G, 5'-direction of chromophore [a] [32]
	N6-A adduct of (+)-*anti*-B[c]PhDE [97]	(−)-BPDE$_t$-N^2-G 3'-direction of chromohore [a] [40]

[a] Pyrenyl residue location relative to the modified strand.

(+)- and (−)-*anti*-BPDE. The different directionality of the adducts could in principle affect in a different way the protein interactions necessary for DNA replication or repair (cf. Refs. [41,42]). Furthermore, differences in total adduct formation, as well as in the *trans / cis* ratios have to be taken into account, as well as the possibility of hitherto undetected minor but biological important other adducts/lesions formed by the reaction of DNA with (+)- or (−)-*anti*-BPDE.

Recent NMR results on the solution structure of type I complexes, resulting from the *cis*-addition of (+)-*anti*-BPDE to N^2-G in a suitable oligonucleotide ((+)-$BPDE_c$-N^2-G adduct) show that the pyrenyl chromophore is quasi-intercalated [43]. This is in agreement with earlier optical studies (21). In this structure the guanine ring of the adduct is displaced into the minor groove of the oligonucleotide.

The well defined solution structures of the (+)-*anti*-BPDE *cis*- and *trans*-adducts have resolved the question of the nature of the type I and II adducts in DNA. The *cis*- and *trans*-adducts (type I and type II, respectively) have different spectroscopic and structural characteristics, which are summarized in Table 1. The distribution of adduct types depends on the experimental conditions. These observations may explain most or all of the heterogenieties observed in a number of spectroscopic studies. Yet other related questions remain to be solved e.g. how the molecular dynamics of the DNA changes at and around the adduct site, the possibility of more global effects on the DNA structure, and possible base sequence influences on structural as well as dynamic issues.

More speculative questions relate to why the (+)-*anti*-BPDE type II adduct structure is correlated with high mutagenic and carcinogenic potency. Is it because the adduct is "hidden" in the minor groove and escapes normal repair systems? Or does this particular geometry disturb a specific function of the DNA in such a way that the end result is the transformation of the cell? Is this kind of adduct particularly efficient in giving rise to specific mutations in protooncogene regions? These questions will be dealt with in sections 5 and 6.

4. Base-sequence specificity

It is well established that incubating native double-stranded DNA with *anti*-BPDE results in a rapid (ms time scale) formation of non-covalent intercalative complexes followed by a considerably lower rate (s time scale) of covalent binding of the diol epoxide to yield preferentially $BPDE_t$-N^2-G adducts [12,18,44]. It has been suggested that the high preference to form this type of adducts is intimately associated with the formation of physical intercalative complexes which provide the proper reaction geometry between the reactants.

The existence of non-covalent complexes between BPDE and DNA has been clearly demonstrated experimentally [18,45–47] but their relevance for the highly stereoselective covalent binding of *anti*-BPDE to G has not been established. Results obtained from studies with polynucleotides show a non-correlation between non-covalent intercalative complexes and subsequent formation of $BPDE_t$-N^2-G adducts [44]. Poly(dG) · poly(dC) or poly(dG-dC) · poly(dG-dC) in either B- or Z-conformation react with (+)-*anti*-BPDE to yield preferentially $BPDE_t$-N^2-G adducts in high amounts. However, the formation of non-covalent intercalative complexes is comparatively low [44]. In contrast, the formation of non-covalent intercalative complexes is highly promoted in poly(dA-dT) · poly(dA-dT) but with a low yield of adducts [44]. Apparently, quantitative accumulation of non-covalent intercalative complexes is not an absolute requirement for $BPDE_t$-N^2-G adduct formation. However, a role of intercalative non-covalent binding of BPDE in, for instance, prolonging the effective lifetime of the diol epoxide by offering a lipophilic environment and thereby increasing the probability for covalent reaction is certainly possible.

Previous work has demonstrated that the extent of DNA-binding of *anti*-BPDE is directly correlated to the proportion of G's [48]. These results indicated that the binding of the diol epoxide occurred randomly, thus that all guanines were equally reactive. However, recent studies have clearly shown that the correlation between the density of G and adduct-formation is

not complete but depends on factors such as the base-composition adjacent to the target base. For example, Boles and Hogan [49,50] showed that a G within a run of G's is several-fold more reactive towards *anti*-BPDE than a G with non-G neighbors. The method employed is based on the finding that irradiating BPDE-modified DNA with intense laser light at 355 nm cuts DNA at BPDE binding sites. Subsequent fragment analysis by polyacrylamide gel electrophoresis allowed determination of adduct distribution. More recent studies have confirmed these results. Dittrich and Krugh [51] studied the relative reactivity of racemic *anti*-BPDE to restriction fragments of pBR322 plasmid DNA by the same method as Boles and Hogan [49] and observed a 17-fold increased reactivity between *anti*-BPDE and a G in G-rich sequences relative to a single G flanked by pyrimidines. Since the reaction of *anti*-BPDE with DNA mainly results in formation of stable BPDE$_t$-N^2-G adducts it is assumed that the results obtained above reflect the distribution of such adducts.

Rill and Marsch [52] studied the sequence preferences for the formation of alkali-labile DNA-adducts of (+)- or (−)-*anti*-BPDE. The method used is based on treating BPDE-modified plasmid DNA by hot piperidine in order to cause cleavage at adduct sites and subsequent analysis of the fragments by polyacrylamide gels. In agreement with previous results [53] it was concluded that G's on average yielded more alkali-labile adducts than other bases. Since BPDE$_t$-N^2-G adducts are resistent to alkali the method used is selective for adducts formed by reaction of *anti*-BPDE and the N7-position of G (BPDE-N7-G). Furthermore, the reactivities of the G's were nonrandom with respect to base sequence. Both *anti*-BPDE enantiomers showed a strong preference for reaction with the 5'-G in GG doublets but the extent of preference was greatly influenced by the adjacent bases and more extended sequences. It is interesting to note that the results obtained by the different methods are in reasonable agreement. Thus it appears that the sequence preferences for formation of labile BPDE-N7-G and stable BPDE-N^2-G adducts are similar.

Due to the great interest in the mechanism of protooncogene activation, studies have been performed in order to determine whether a preference exists for covalent binding of *anti*-BPDE to the region of the Ha-ras protooncogene comprising codons 12, 13 and 61, codons often altered in the process of tumour formation [54]. Reardon et al. [55], using an approach described by Sagher and Strauss [56] observed an increased formation of *anti*-BPDE adducts with G in codon 12 (GGA) relative to other codons of the rat Ha-ras protooncogene. Furthermore, Dittrich et al. [57] observed in the corresponding human gene that the G's of codons 12 (GGC) and 13 (GGT) were more reactive than the least reactive G in the sequence investigated whereas the G in codon 61 (CAG) was of intermediate reactivity. In contrast to these results, Marien et al. [58] observed only an average reactivity of G in codon 12 for racemic *anti*-BPDE in the human Ha-ras protooncogene. One reason for the obvious discrepancy in results may be related to the methodology used. In the latter study, a sequencing method which relies on the formation of alkali-labile BPDE-N7–G adducts was used.

Geacintov et al. [44] studied the base-sequence dependence of the covalent binding rate constants of racemic *anti*-BPDE to G in various polynucleotides. The results demonstrate that a G flanked by A or C is more reactive than a G flanked by T or G. As demonstrated previously [59] the secondary structure of DNA strongly influences the base specificity for covalent binding of *anti*-BPDE. In a recent study [60], it was shown that the extent of formation of BPDE-N^2-G adducts in a single stranded oligonucleotide was greatly influenced by the bases flanking the G-target. In contrast to what is generally observed in double stranded DNA, pyrimidines rather than purines adjacent to G result in a higher yield of BPDE-N^2-G adducts, in particular *trans*-adducts. It is suggested that the lower preference for G flanked by purines may be due to an unfavorable reaction geometry between *anti*-BPDE and the exocyclic nitrogen of G caused by the purines.

The *trans* / *cis* adduct ratio seems to be strongly affected by the secondary structure and

base sequence. For instance, in native DNA incubated with (+)-*anti*-BPDE the amount of $BPDE_t$-N^2-G adducts equals or exceeds 98% [19,22] whereas in poly(dG-dC) · poly(dG-dC) and poly(dG) · poly(dC) the amount is reduced to about 86% (21). These results indicate the importance of the base sequence in determining whether the covalent reaction will proceed through *trans* or *cis* opening of the epoxide. In a single stranded oligonucleotide with the center sequence 5'-TGCGT it was found that the *trans* / *cis* adduct ratio was significantly decreased relative to double stranded DNA [61].

If and to what extent both *trans* and *cis* BPDE-N7-G adducts are formed has not been studied in detail.

5. Observations on BPDE-induced mutations in mammalian cells

Mutations induced by racemic or enantiomeric pure (+)-*anti*-BPDE have recently been studied in endogenous loci in mammalian cells [62–68]. Independent of system or type of cells employed it can be concluded that the absolute majority of mutations induced by the diol epoxide are base pair substitutions involving GC → TA transversions. Mazur and Glickman [62] examined the distribution of mutations in the *aprt*-locus in chinese hamster ovary (CHO)-cells and concluded that approximately 50% were localized in runs of Gs flanked by As, thus suggesting the existence of mutation "hot-spots". Based on analysis of mutation prone sequences these authors suggested that a substantial proportion (35%) of all base pair substitution mutations could be related to the formation of apurinic sites as a result of labile BPDE-N7-G or BPDE-N3-A adducts. Carothers and Grunberger [63] analyzed the mutations induced by racemic *anti*-BPDE in the endogenous *dhfr*-gene in CHO-cells and also concluded that sequences with Gs flanked by purines are more frequently mutated than sequences with Gs flanked by pyrimidines. These authors suggested that the absolute majority of the BPDE-induced mutations are related to stable BPDE-N^2-G adducts. This view is consistent with the observed DNA-binding specificity of *anti*-BPDE *in vitro*. A purine localized 5' to G promotes formation of stable BPDE-N^2-G adducts whereas an adjacent pyrimidine seems to facilitate the formation of less stable BPDE-N7-G adducts [52,53,69].

The effect of the cell-cycle on the quantitative and qualitative distribution of *anti*-BPDE-induced mutations in the coding region of the *hprt*-gene in diploid human fibroblasts has been studied by Chen et al. [64]. In cells treated in the G1-phase almost 30% were clustered within a unique run of G's whereas in cells exposed to *anti*-BPDE in the S-phase less than 5% were found in that region. Obviously, there is a selective sequence effect on the DNA damage, probably due to a low rate of removal because of inefficient recognition and processing of the damage by the excision repair system. It may be of relevance in this context that homopolymeric stretches of DNA, poly (dG) · poly(dC) may adopt an A-like conformation [70].

The distribution of mutations in the transcribed and non-transcribed strands was found to be dependent on the cell-cycle, suggesting a preferential removal of potentially mutagenic *anti*-BPDE adducts from the transcribed strand. An alternative explanation for the unequal distribution of adducts is that the polymerases δ and α involved in the replication of the leading and lagging strand of DNA, respectively, [71,72] differ in their fidelity and response to BPDE adducts.

Chen et al. [67] studied the mutation pattern in repair-deficient cells and obtained results fully consistent with the view that the strong strand bias of *anti*-BPDE induced mutations in normal cells results from preferential repair of DNA-damage caused by the diol epoxide (see also Ref. [65]).

It should be mentioned that in all studies discussed above racemic *anti*-BPDE has been used. Since both diol epoxide enantiomers are expected to form covalent adducts with DNA, although with a predominance of (+)-anti-BPDE, the contribution of the (−)-enantiomer to the observed mutations is not known.

Andersson et al. [68] analyzed the mutation spectrum in the *hprt*-gene in human lymphocytes following (+)-*anti*-BPDE treatment. In accor-

dance with previous studies [62–67] the great majority of mutations observed were GC → TA transversions. No particular mutation hot-spots were found but a pronounced sequence context specificity was observed. The majority of of mutations in the coding region of the gene were located in AGG triplets, thus confirming the high sensitivity of a G flanked by purines in mutation induction. In agreement with previous results [65,67] a pronounced strand-bias with regard to the mutations involving GC base-pairs was found. All diol epoxide induced mutations were located on the non-transcribed strand. Although the coding region of the *hprt*-gene contains more G's in the non-transcribed strand than in the transcribed strand, this fact is not sufficient to explain the strong mutation bias assuming that the distribution of (+)-*anti*-BPDE-adducts is initially random. Thus, taken together the results indicate a preferential removal of adducts from the transcribed strand.

The qualitative distribution of transversion mutations also depends on the concentration of mutagen used. Wei et al. [66] analyzed mutations in the *hprt*-gene of V-79 cells at a high and low cytotoxic dose and of (+)-*anti*-BPDE. In agreement with the results discussed above, a highly cytotoxic dose of diol epoxide almost exclusively induced mutations at GC base-pairs whereas a low dose revealed a great proportion of mutations involving AT base pairs. In fact, the proportion of mutations involving AT base pairs seems to be inversely correlated to the dose of diol epoxide employed for reasons as yet unknown.

By using a newly developed bacterial plasmid system Mackay et al. [73] and Rodriguez and Loechler [74,75] have shown that the principal BPDE-N^2-G adduct localized 3′ to T exclusively induced GC → TA transversion mutations. Furthermore, the sequence context was shown to have a great influence on the type of single base substitution mutations induced. It was suggested that the sequence context influences the conformation of the BPDE-N^2-G adduct which in turn determines the mutagenic specificity [74,75].

Active *ras*-oncogenes are frequently observed in tumours induced by PAH in experimental animals [54,76]. The activation usually involves codons 12 and 61. With BP it has been shown that the incidence of lung adenomas in mice is significantly increased and that the increase is closely associated with transversion mutations GC → TA involving the first or second G in codon 12 GGT in the Ki-*ras*-protoncogene [77]. Exposure of human fibroblasts in culture to racemic *anti*-BPDE causes activation of the Ha-*ras*-protooncogene by an GC → TA transversion at codon 12 [78]. Modification of the Ha-*ras*-protooncogene *in vitro* by *anti*-BPDE and replication in a mammalian cell system results in a variety of point mutations, predominantly GC → TA and AT → TA transversions in codons 12 and 61 [79]. The majority of mutations were found in codon 61 [80].

BP is a weak tumour initiator in comparison to, for instance 7,12-dimethylbenz[a]anthracene (DMBA). It has been suggested that the difference in potency is due to the lower binding of (+)-*anti*-BPDE to A relative to the binding to this base of the corresponding diol epoxide of DMBA [81,82]. In fact, the incidence of skin tumours in mice following topical application of BP seems to be correlated with the extent of DNA binding of *anti*-BPDE to A rather than to binding to G [83]. In this context it is interesting to note that *in vitro* replication of an oligonucleotide previously modified with 7-bromobenz[a] anthracene at A and G results in incorporation of A opposite both type of adducts [84]. In contrast, Hruszkewycs et al. [42] found that an oligonucleotide carrying G-adducts of (+)- or (−)-*anti*-BPDE resulted in very low, if any, incorporation of A opposite the adducts. However, in a similar study Shibutani et al. [85] observed a higher miscoding potential (preferential incorporation of A) of the *trans*- and *cis*-adducts of (−)-*anti*-BPDE than the corresponding adducts derived from (+)-*anti*-BPDE. These variation in experimental observations may derive from variations in the *in vitro* replication system used.

In addition to protooncogene activation chemical carcinogenesis seems to require inactivation of so called tumour suppressor genes; genes essential for adequate and proper control of cell growth and differentiation. The p53 suppressor gene has recieved great interest recently since a

number of different tumours in humans suspected to be caused by exposure to chemical carcinogens have been shown to carry mutated p53 genes [86]. Interestingly, the type of mutation observed (transition or transversion) seems to reflect the suspected carcinogenic agent (Ref. [87] and references therein). With regard to PAH, tumours in organs in which PAH most likely plays a role, such as cigarette smoke induced lung cancer, are associated with GC → TA transversion mutations in the p53 gene [88].

Previous studies on the mutagenicity of all possible BPDE isomers in mammalian cells have clearly demonstrated that the enantiomers of *syn*-BPDE and (−)-*anti*-BPDE are inferior as mutagens relative to (+)-*anti*-BPDE [9]. However, no systematic study similar to the ones described above on type and location of mutations induced by these isomers of BPDE have been performed.

6. Possible mechanisms for (+)-*anti*-BPDE induced mutations. Comparison with other carcinogens

In addition to (+)-*anti*-BPDE other bulky carcinogens such as the diol epoxide derivative of benzo[c]phenanthrene ((−)-*anti*-B[c]PhDE), aflatoxin B_1-*exo*-8,9-epoxide (AFB_1-8,9-epoxide), the N-hydroxy derivatives of aromatic amines or amides like aminofluorene (AF) and N-acetylaminofluorene (AAF) or nitro-PAH (e.g. 1-nitro- and 1,6-dinitropyrene) predominately induce transversion mutations (GC → TA or AT → TA) in spite of the difference in binding specificity [89–92]. As discussed above, (+)-*anti*-BPDE forms mainly *trans*-adducts with the exocyclic nitrogen of G whereas (−)-*anti*-B[c]PhDE forms adducts with the exocyclic nitrogens of both G and A [93]. AFB_1-8,9-epoxide seems to react exclusively with the N7 in G [94], whereas the N-hydroxy derivatives of AF and AAF or the nitropyrenes demonstrate a preference for the C8 position in G [95,96].

As discussed, the pyrenyl residue of the (+)-*anti*-$BPDE_I$-N^2-G adduct is localized in the minor groove with known geometry [32], thus demonstrating no rotation of the modified G from normal *anti*- to *syn*-conformation. Regarding the adducts of *anti*-B[c]PhDE, less structural information is available. Because of stereochemical similarities, *trans*-addition (−)-*anti*-B[c]PhDE to N^2-G can be expected to result in type II adducts with similar characteristics as adducts of (+)-*anti*-BPDE. On the other hand a recent NMR study shows that (+)-*anti*-B[c]PhDE bound to N^6-A results in a complex in which the phenanthryl residue is intercalated [97]. If, and to what extent (−)-*anti*-B[c]PhDE bound to N^6-A also results in intercalative complexes is not known. At this stage complexes of type II cannot be excluded. However, if type II adducts to the exocyclic aminogroup of A are formed the chromophores are expected be localized in the major groove.

The features of the N7-G complex of AFB_1-8,9-epoxide (AFB_1-N7-G adduct) and the C8-G complexes of the N-hydroxy-derivatives of AF and AAF (AF-C8-G and AAF-C8-G adduct, respectively) have been studied by two-dimensional NMR [96,98,99]. The results for the AFB1-N7-G adduct demonstrate a high degree of binding homogeneity and show that the AFB_1-residue is intercalated above the 5′-side of the modified G [96]. The situation with the AF-C8-G adduct is more complex. The results on a fully complementary duplex (C opposite the AF-C8-G adduct) demonstrate a high degree of conformational heterogeneity of the adducts and no single adduct could be resolved. However, by replacing C with A opposite the AF-C8-G adduct a considerably increased homogeneity was observed [98]. Recent results on the solution structure of an AAF-C8-G adduct opposite C demonstrate that the duplex adduct is in a quasi-intercalated state and associated with base displacement [99].

It can be concluded that different bulky carcinogens exhibit a high preference for binding to G and, dependent on the compound, quite selected positions. Certain compounds, notable some members of the PAH-diol epoxides, bind to a large extent to A in addition to G (see above). The resulting adducts greatly differ in their structural features. However, independent of this fact all the adducts induce single point mutations,

particularly transversions. The mechanism(s) underlying the fixation of a potentially mutagenic adduct/lesion into a mutation is far from understood. Several alternatives can, however, be proposed. Before discussing these alternatives in greater details, the properties of the adducts/lesions with respect to replication fall into several categories, (A): *the adduct is miscoding*, (B): *the adduct is noncoding*, and (C): *the adduct is an intermediate in the formation of the ultimate premutagenic lesion.*

A miscoding adduct (alternative A) implies that the DNA-replication machinery attempts to read the modified base but does so inaccurately. A noncoding adduct (alternative B) implies that the DNA-replication machinery is unable to read the modified base and bypasses the lesion by incorporating a base which can be complementary or noncomplementary. Abasic sites (alternative C) may result from decomposition of labile adducts (e.g. BPDE-N7-G and AFB_1 -N7-G adducts). Replication of DNA containing apurinic sites leads to preferential insertion of A opposite the lesion [100]. An outline of alternative pathways for the formation of GC → TA transversion mutations is shown in Fig. 4.

Alternative mechanisms can be proposed to explain the preference for induction of GC → TA transversion mutations by bulky carcinogens. In the case the adduct is miscoding, rotation of the adducted base from *anti* to *syn* conformation allows, for instance, that the the imino form of A can be incorporated opposite G [101]. 2D NMR studies combined with energy minimization calculation on an oligonucleotide containing a single AF-C8-G adduct demonstrated that the adduct forms a stable base pair with A provided that the adducted base adopts the *syn*-conformation [98]. This may indicate that the base pair composed of A opposite AF-C8-G is energetically more favorable than the C opposite AF-C8-G [98,102]

A change from *anti* to *syn* conformation of the adducted base and subsequent base pairing with A may be a mechanism by which (+)-*anti*-BPDE causes GC → TA transversion mutations.

We recently found [103] that gradual addition of the full complementary strand to an oligonucleotide containing one $BPDE_t$-N^2-G adduct steadily decreased the fluorescence intensity until it became constant at a stoichiometric ratio of 1:1 modified/unmodified oligonucleotides (cf. also Ref. [21]). This is consistent with the localization of the pyrenyl chromophore in the minor groove and, as a consequence, more efficient interactions with double-stranded complementary DNA leading to fluorescence quenching. In contrast, replacing the complementary strand with a strand containing A opposite the $BPDE_t$-N^2-G adduct resulted in the opposite effect, i.e. the fluorescence intensity increased several-fold until it became constant at oligonucleotide ratios equal to or exceeding 1:1. It has been suggested [104] that base-pairing between A and G requires the imino tautomer of A and the *syn*-conformation of G. The *anti*- to *syn*-transition of G may be facilitated by adduct formation and would result in

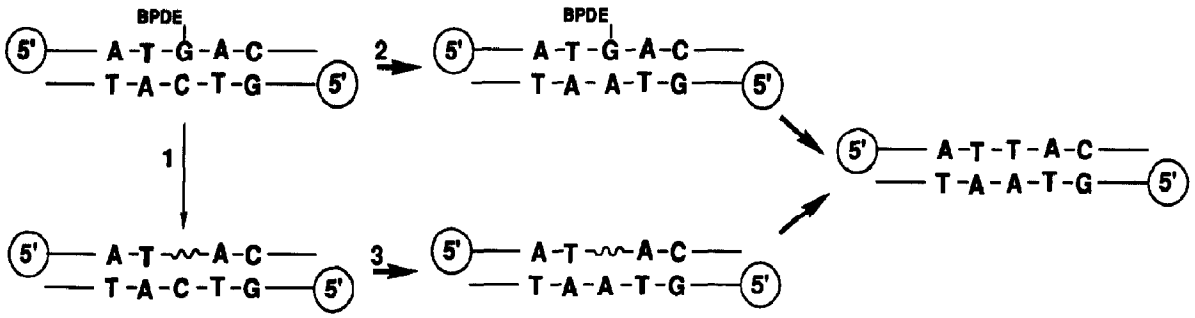

Fig. 4. Alternative mechanisms by which BPDE covalently bound to deoxyguanosine in DNA causes a GC → TA transversion mutation. The adduct gives rise to an apurinic site (1) prior to replication and A is preferentially inserted opposite the lesion. The adduct is intact during replication (2) and A is preferentially inserted opposite the miscoding or noncoding adduct.

major groove localization of the BPDE$_t$-N^2-G adduct opposite A. One would expect that major groove localization of the chromophore would lead to to less efficient DNA interactions relative to minor groove binding, hence explaining the increased fluorescence intensity.

7. Conclusions

The studies on *anti*-BPDE induced mutations in endogenous mammalian genes clearly show that the dominating event is transversion mutations involving GC base pairs flanked by purines and, dependent on dose of diol epoxide used, AT base pairs. The preference for the pur-G-pur sequence context strongly favour stable BPDE$_t$-N^2-G adducts as the premutagenic lesion rather than cis-adducts or labile BPDE-N-7-G adducts. In addition to monomeric adducts, the existence of clustered adducts in alternating GC-sequences is suggested by the observation of excimer fluorescence.

The three-dimensional structure of the BPDE$_t$-N^2-G has been elucidated and shows the adduct residing in a widened minor groove of an otherwise retained B-like DNA conformation. The marked bias with regard to mutations in the transcribed and non-transcribed strand of DNA strongly implies preferential removal of premutagenic BPDE adducts from the transcribed strand prior to DNA replication. The remaining adducts seem to play a crucial role in protooncogene activation and tumour suppressor gene inactivation by inducing transversion mutations involving both G and A.

Thus, BPDE-induced carcinogenesis is a prime exampel of a situation where the biological consequences of carcinogen exposure can be understood in considerable detail in terms of chemical and biomolecular events. Although not directly presenting a way to cure the disease the understanding of the molecular biology behind the transformation of the cell this knowledge may contribute to our understanding of how to avoid it.

8. Perspectives

Over the last years remarkable progress has been made in the characterization of structural properties of various covalent DNA adducts of potent carcinogens. As the numbers of reported structures of this kind continues to increase there seem to be certain characteristic features becoming apparent: minor groove binding or quasi-intercalation. Future work may bring better clarification of the common denominators characterizing the disturbance of normal DNA structure and dynamics by potent carcinogens. From the biological side an important body of knowledge is appearing on induction of various critical point mutations caused by these adducts and how these relate to protooncogene activation and suppressor gene inactivation. In the variable chain of events involved in chemical carcinogenesis there are certainly still substantial gaps of our knowledge, for instance the role of the DNA-repair systems, the disturbances to the cell replication system, the fixation of DNA-lesions into mutations, etc. However, the studies of the BP-induced chain of events is a good illustration of how these processes may be dissected and elucidated in some molecular detail.

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