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DNA Radiosensitization: The Search for Repair Refractive Lesions Including Double Strand Breaks and Interstrand Crosslinks

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1. Introduction

More than half of all cancer patients receive radiotherapy (RT) as part of their treatment regimens. The cytotoxicity of ionizing radiation is mainly mediated by the ensuing DNA damage. Double-stranded DNA breaks (DSB) are generally accepted to be the most important lesions for the induction of cell death by ionizing radiation because they are much more difficult to repair than single strand breaks, although their radiation yield is very low, at two orders of magnitude less than that of single strand breaks (SSB) (Hempel & Mildenberger, 1987). The role of other DNA lesions such as base damage and interstrand crosslinks in cell killing has not been yet fully elucidated. Gamma-radiation inflicts DNA damage *via* two separated processes: i) direct interaction with DNA and; ii) indirect damage produced by secondary radicals ($^{\bullet}OH$, $^{\bullet}H$ and e^{-}_{aq}) generated after water radiolysis (Michaels & Hunt, 1978). Among the H_2O derived radicals, hydroxyl radicals ($^{\bullet}OH$) are the species primarily responsible for strand break formation and DNA base damage (> 35%). Hydrated electrons, e^{-}_{aq}, which are generated at a yield comparable to the $^{\bullet}OH$ (G-values of ~ 2.8×10^{-7} mol.J^{-1}), participate in only ~ 8% of the total damage and the exact nature of DNA damage formed *in vivo* by e^{-}_{aq} is obscure (Nabben *et al*., 1982). In vertebrate cells, the majority of RT-induced DSB are repaired by non-homologous end joining (NHEJ) with some contribution from homologous recombination repair (HRR) during the late S and G2 phases of the cell cycle (Jackson, 2002; Takata *et al*., 1998). The repair of other DNA damages, such as interstrand crosslinks (ICL) is more complicated and less understood (Wang, 2007; Moldovan & D'Andrea, 2009). ICLs can be recognized by the nucleotide excision repair (NER) system and it is accepted that ICL repair involves nucleolytic cleavage at or near the site of ICL to produce a suitable substrate that can subsequently be repaired by homologous recombination (HR) (D'Andrea & Grompe, 2003; Moldovan & D'Andrea, 2009a, Liu *et al*., 2010).

An approach to improve the effectiveness of RT is either to enhance the formation of lethal DNA lesions, or to use inhibitors of DNA repair pathways (or both) and thus to render tumor

cells more sensitive to ionizing radiation. A novel strategy to inhibit radiation-induced double strand break repair was recently promoted by using short modified DNA molecules that mimic double strand breaks (Dbaits) and artificially activate the DNA-PK pathway (Quanz *et al.*, 2009). Likewise, using siRNA screening of genes involved in DNA damage repair, Higgins *et al.* (2010) identified POLQ (DNA polymerase θ) as a potential tumor-specific target whose knockdown led to tumor cell–specific radiosensitization. However, with the exception of oxygen-enhancing or mimetic agents and platinum derivatives such as cis-platinum, which amplify DNA damage, the only direct DNA radiosensitizing agents known to date are halogenated uracils, such as 5-bromodeoxyuridine (BrdU). BrdU administration to cultured cells, animals and humans leads to replacement of isosteric thymine by 5-bromouracil during replication and excision repair of DNA. The basic pathway of BrdU radiolysis and DNA strand break formation in solution was described many years ago (Zimbrick *et al.*, 1969a,b). It involves dissociative e^-_{aq} attachment to BrdU, followed by the formation of a 5-uracil-yl σ-radical ($^\bullet$U) and a bromine ion (Br^-, Fig. 1). The reaction is very efficient in air-free solutions, with $k(e^-_{aq} + BrU) = 2.6x10^{10}\ M^{-1}s^{-1}$ and $G(U) = 2.4x10^{-7}$ mol.J^{-1}, but interactions with $^\bullet$OH may intervene and can lead to somewhat different products. BrdU is also an efficient UV-light absorbing phtotosensitizer; the homolytic Br−C bond cleavage, however, results in two

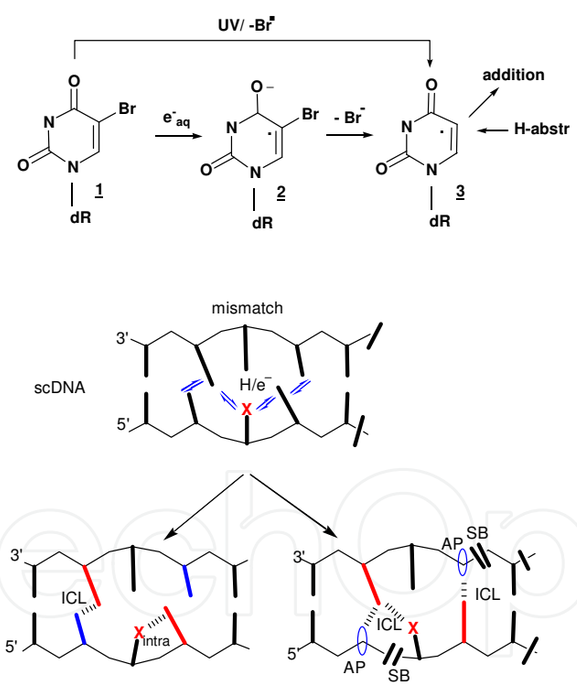

Fig. 1. Primary reaction mechanism of 5-BrdU sensitization and subsequent damages in wobble scDNA. The dynamic structure of scDNA facilitates electron (hydrogen atom) transfer (ET) between the two strands. The ET direction depends on the BrdU local sequence context (e.g. purine *vs.* pyrimidine environment). In the scheme the 5-uracil-yl radical (3) and its products are denoted by X. Modified bases (reduced or oxidized; red and blue), uracil and/or 2'-deoxyribose may further interact between and/or with undamaged bases and oxygen to form various products, including AP-sites, intra- and inter-strand crosslinks (ICL) and strand breaks (SB).

radical species (•U and •Br). It is generally assumed that •U abstracts an H-atom from 2′-deoxyribose to form a strand break or accepts an electron from a neighboring base (inter-base chain e^- transfer (ET), generating an oxidized base), which eventually terminates with the formation of a guanine cation radical, $G^{+\bullet}$ (Nese *et al.*, 1992). •U-radical addition reactions may result in DNA crosslinks (Fig. 1), but this was only recently shown experimentally (Zeng & Wang, 2007). New findings show that electron capture and migration along BrdU-substituted DNA might be contra-thermodynamically selective and, thus far more complicated (Yoshioka *et al.*, 1999; Aflatooni *et al.*, 1998). In cells, radiosensitization by BrdU (typically at ~20-30% thymine replacement) gives about 2-3 fold radiotoxicity enhancement and in most cell types parallels similar increase in the DSB yield, and/or the decreased rate of their repair (Sawada & Okada, 1972). Further characteristics of BrdU-mediated DSB and other DNA damages *in vivo* (*e.g.* DNA crosslinks) are scarcely available.

2. DNA-structure/conformation dependent BrdU-sensitized formation of strand breaks

A crucial difference between the radiosensitization of single and double stranded DNA by BrdU was not known until our work with purified *semi*-complementary (mismatched) DNA showing the single stranded specificity of BrdU-induced DNA damage (Cecchini *et al.*, 2004). In parallel experiments, using BrdU-substituted (or not) single-stranded (ssDNA), double-stranded (dsDNA) and mismatched (wobble) semi-complementary (scDNA) DNA we have found that BrdU efficiently sensitizes single stranded BrdU-substituted (brominated) oligonucleotides, but not when these are hybridized to completely complementary oligonucleotides to form normal dsDNA duplexes. We estimate that BrdU radiosensitization efficiency in dsDNA drops up to 20-fold compared to that in ssDNA. Comparative measurements of radiolytic loss of the bromine atom in ssDNA *vs.* dsDNA likewise indicate that this process is greatly suppressed in dsDNA (Cecchini *et al.*, 2004, 2005a). In mismatched, scDNA duplexes, strand brakes are formed in loci encompassing nucleotides surrounding BrdU. However, high efficiency single strand break formation takes place on the brominated strand, or on the opposite, non-brominated strand but have not been detected on both, suggesting that they are mutually exclusive events (Hunting *et al.*, unpublished; Cecchini *et al.*, 2005b). Experiments performed with scDNA bearing variable number of mismatches (from one to five) and containing a single BrdU substitution gave qualitatively similar results. The radiation dose-response for strand break formation was linear for both the brominated and the opposite, non-brominated strand within the single-stranded regions of a standard model scDNA containing a bulge formed by up to five mismatched bases (Dextraze *et al.*, 2007). Interestingly, UVB-irradiation of BrdU-substituted DNA also demonstrated DNA-structure specificity, but in this situation BrdU greatly enhances breakage of only the brominated strand in dsDNA, or either the brominated or the non-brominated strand in the case of scDNA (Cecchini *et al.*, 2005b; Chen *et al.*, 2000). The different effects initiated by radiolysis and photolysis, especially with BrdU-dsDNA, underline the role of DNA structure-conformation properties in solution as a prerequisite for the initial electron-capture by BrdU and/or the propagation of an excess-electron along the polymer after gamma-irradiation.

The importance of DNA structure during sensitization by BrdU is further demonstrated by comparison of the damages induced in A- and B-form DNA. Using brominated 25-mer

oligonucleotides hybridized to complementary or semi-complementary ones with five mismatched bases: AAT(orBU)AA, we have shown that strand breaks are specific for B-DNA, whereas A-DNA only undergoes formation of alkali-labile DNA lesions (Dextraze *et al.*, 2007). Piperidine-sensitive lesions are observed exclusively at the site of BrdU substitution. Generally, the cleavage reaction is a confluence of at least three factors: e^-_{aq} capture, forward electron transfer and charge recombination. The strand break positions migrate along the DNA strand, however, there is a clear preference for the dA 5′-flanking BrdU. Similarly, 5′-dABrdU-sequence preference has been observed in UV-induced BrdU DNA cleavage (Chen *et al.*, 2000). This is to be expected since in B-DNA the 5′ proximal 2′-deoxyribose would be the ultimate H-atom donor to the uracil-5-yl radical, whereas in A-DNA conformational (spatial) restrictions render the same 5′dA 2′-deoxyribose a less-accessible H-donor. Therefore, in A-DNA the uracil-5-yl radical is more likely to abstract H-atoms from other donors (bases), thus oxidizing proximal to the BrdU-site bases which results in alkali-labile site formation.

Sequence-preferential strand break formation was examined in a series of 25-mer scDNA encompassing a central 1- or 5-mer mismatched site with BrdU incorporated (brominated strand) in a purine 5′d(AABrdUAA), 5′d(GGBrdUGG), or a pyrimidine 5′d(ATBrdUTA) environment, and the semi-complimentary (non-brominated) strand contained any of the sequences: 5′d(AATAA), 5′d(CCCCC), 5′d(GGGGG), or 5′d(ATTTA) (twelve permutations in total) (Dextraze *et al.*, 2007; 2009). While there was no significant change between the strand-break yields in ssDNA and typically there were no changes in strand breaks produced on each strand (*i.e.*, brominated *vs*. nonbrominated), two wobble sequence permutations derived from the above pattern: d(GGBrdUGG)^d(GGGGG) and d(ATBrdUTA)^d(ATTTA) produced more breaks on the brominated strand, whereas the generation of breaks was enhanced in the non-brominated strand in the combinations: d(GGBrdUGG)^d(AATAA), d(GGBrdUGG)^d(ATTTA), and d(AABrdUAA)^d(GGGGG). Similarly irradiation of the same scDNA, gave different patterns of interstrand crosslinks (see below).

3. BrdU sensitized formation of interstrand crosslinks (ICL) in mismatched (wobble) DNA duplexes

In addition to increasing strand break generation by ionizing radiation, the presence of bromouracil induces formation of DNA interstrand crosslinks (ICL). This process occurs in single stranded regions within double stranded DNA (*i.e.*, in scDNA) and requires the presence of B-DNA (Dextraze *et al.*, 2007; 2009). Although anticipated, the generation of ICL during radiosensitization with BrdU has not been demonstrated experimentally prior to our work (Cecchini *et al.*, 2005a). Formation of intra-strand crosslinks has been reported in UVB-irradiated BrdU-substituted synthetic DNA (Zeng & Wang, 2006) and in cells (Zeng & Wang, 2007). However, the generation of DNA ICLs by ionizing radiation has been largely ignored in favor to studies on double-strand breaks and their repair. At least part of the problem is technical; it is difficult to detect and quantify ICLs when the same agent forms both strand breaks and ICL, because the analysis of ICL generally involves a denaturing step. Apart from multiple damage events that may cause the disappearance of ICL-products due to DNA backbone cleavage at nucleotides located between the ICL-site and the radiolabel, another factor that obstructs DNA-ICL detection is that ICLs may decompose under prolonged irradiation, especially in the presence of O_2 (Ding & Greenberg, 2007).

Therefore, ICL detection and quantitation implies careful selection of the irradiation conditions. In our experience, the presence of a mild •OH-radical scavenger (20 mM EDTA) sufficiently protects against ICL destruction, and even enhances ICL yields up to 1 kGy irradiation dose. A typical ICL- migration pattern observed in agarose denaturing gel electrophoresis of ^{32}P-labeled scDNA samples containing different mismatched regions and subjected to γ-irradiated (750 Gy) is shown in Fig. 2. The figure exemplifies the fact that, depending on the mismatched-sequence context of the incorporated BrdU, ICL-DNA segments are formed, which differ in their length, structure and yield. Comparative analyses of ICL yields and various electrophoretic-band patterns depending on DNA structure are presented in (Dextraze *et al.*, 2009). Although, no ICL chemical structure identification has been done yet, quantitative data show that d(AABrdUAA)^d(GGGGG) and d(GGBrdUGG)^d(CCCCC) bulge patches are the least prone to crosslink formation in DNA wobbles, while efficient crosslinking takes place in T-enriched bulge structures, *e.g.* d(GGBrdUGG)^d(ATTTA) and d(ATBrdUTA)^d(ATTTA). The calculated total ICL radiation yield (G) in the later sequences (*i.e.* including all ICL bands) is in the range of (1-4) x10^{-8} mol.J^{-1}. Taking into account that G(e^-_{aq}) = 2.75x10^{-7} mol J^{-1} it follows that the formation of interstrand crosslinks in BrdU-scDNA is an event that occurs at least once with every ten solvated electrons produced. Ding and Greenberg (2007) reported γ-radiolysis production of ICL in unsubstituted dsDNA and identified the structure as T[5m-6n]A (Fig. 6B). Under their irradiation conditions the estimated G-value of the single ICL is ~ (3-4) x10^{-4} nmol.J^{-1}. These data underline that BrdU-sensitized ICL formation in mismatched scDNA duplex is much more efficient (> 10^4 - fold) than in normal dsDNA.

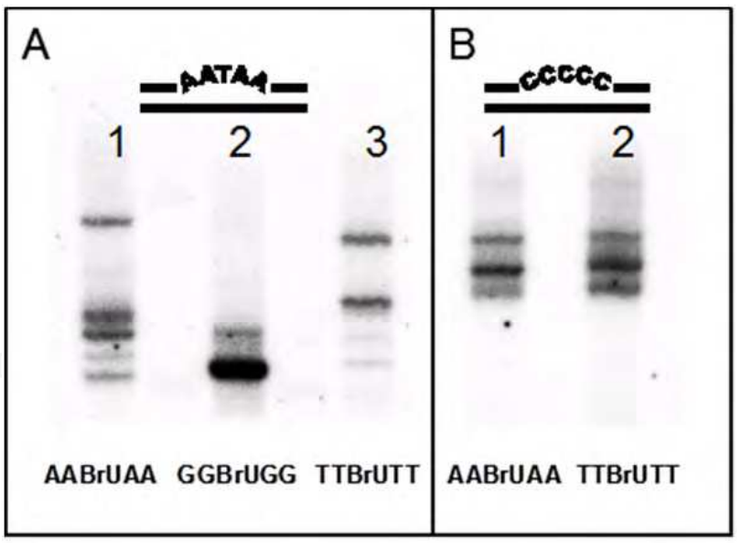

Fig. 2. Sequence-dependent BrdU-radiosensitized formation of ICL in wobble scDNA, as seen by the different electrophoretic mobility of the ICL bands. The central quintet sequence of the brominated strand is shown at the bottom; the opposite strand comprises d(A_2TA_2) or d$(C)_5$ as shown on the top; there is a 5-b.p. central mismatch in all samples, except for lane-3 (panel A) where a single BU^T mismatch is present. The figure exemplifies the role of mismatched sequence (length and composition) on the ICL nature and yields; the individual chemical structures, however have not been yet determined. For a tentative ICL assignment, see Dextraze M.-E., *et al.*, (2009); for recently identified ICL structures, see Fig. 6.

4. Peptide nucleic acids (PNA) as sequence targeted radiosensitizers

In searching for new RT approaches to inflict heavy DNA damage (specific, repair-refractive and lethal) we developed the concept of cell radiosensitization by non-covalently bound DNA radiosensitizers. Our original idea was to use *semi*-complementary BrdU-substituted oligonucleotide vectors which would hybridize to specific genomic sequences and create a mismatch at the site of the bromouracil. In theory, the sequences of the BUdR-loaded oligonucleotide vectors could be designed to efficiently form crosslinks with the target DNA upon radiation, since, as discussed above, the crosslinking efficiency is dependent on the target sequence. However, the use of oligonucleotides as vectors to bring BrUdR close to cellular DNA has many pitfalls (similarly to the antisense RNA applications) and instead we focused on peptide nucleic acids (PNA) as vectors, because PNAs are resistant to degradation and are able to invade DNA duplexes under physiological conditions. To our surprise, PNA were found to efficiently form crosslinks with DNA under ionizing radiation even without bearing halogenated bases.

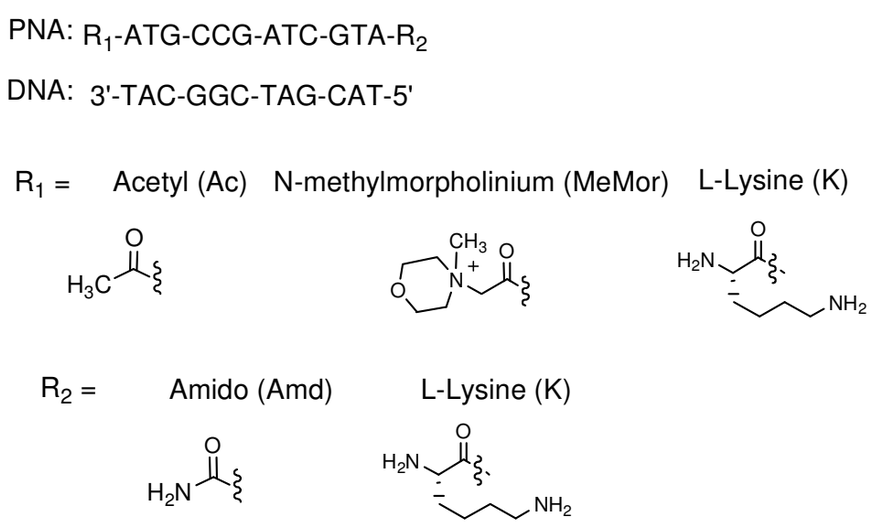

Fig. 3. The 12-mer PNA-DNA heteroduplex sequence (top) used in γ-radiation experiments to induce ICL and the variable N- and C-capping groups (R_1 and R_2) on PNA.

PNAs are nucleic acid analogues with an uncharged peptide-like backbone (Nielsen, 1995; Porcheddu & Giacomelli, 2005; Pellestor *et al*., 2008). PNAs bind strongly to complimentary DNA and RNA sequences. Originally designed as ligands for the recognition of double stranded DNA (Egholm *et al*., 1993; Demidov *et al*., 1995) their unique physicochemical properties allow them to recognize and invade complimentary sequences in specific genes and to interfere with the transcription of that particular gene (antigene strategy) (Nielsen *et al*., 1994; Ray & Norden, 2000; Pooga *et al*., 2001; Cutrona, *et al*., 2000; Doyle *et al*., 2001; Romanelli *et al*., 2001; Kaihatsu *et al*., 2004). The introduction of a bulky charged amino acid (*e.g.* lysine, Fig. 3) improves binding specificity, solubility and cell uptake (Menchise *et al*., 2003). PNAs have several advantages over oligo-deoxyribonucleotides including: greater chemical and biochemical stability (PNAs are not substrates for proteases, peptidases and nucleases), greater affinity towards targets (lack of electrostatic repulsion between hybridizing strands, Fig. 4), and more sequence-specific binding.

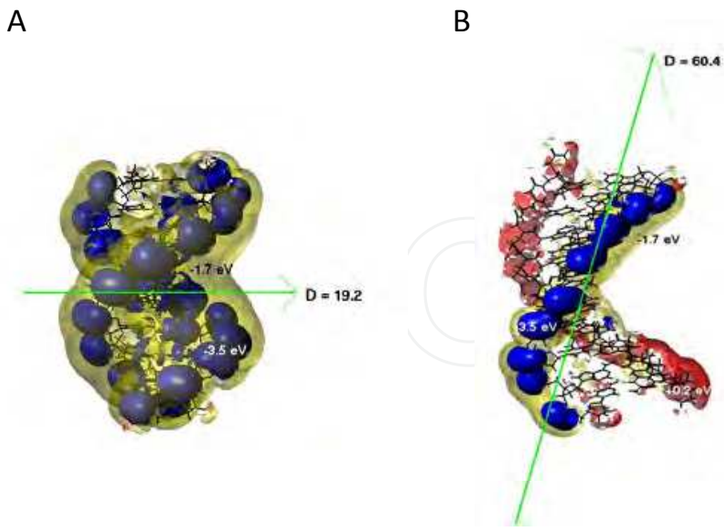

Fig. 4. Electrostatic potentials surrounding 10-mer 3D-models of $(DNA)_2$ and DNA-PNA duplexes, calculated using Sybyl modeling interface and dielectric constant, D = 4. Color-codes as indicated: electronegative potentials -3.5 eV, -1.7 eV (dark blue and yellow) and electropositive +0.2 eV (red). (A) Symmetric electronegative potential surfaces along the two strands of the DNA duplex expand over all backbone atoms (blue) and the two grooves (yellow). (B) Asymmetric isopotential surfaces around PNA-DNA duplex. The electropositive/neutral PNA backbone region (red) extends over the minor and major groove atoms (0 - 0.2 eV at distances ~ 0.1 Å), but the DNA backbone atoms remain enveloped by a negative surface (-3.5 eV). The resultant dipole momenta (green vectors) are 19.2 D and 60.4 D for the DNA-DNA and PNA-DNA duplexes, respectively. In the latter case it is oriented diagonally from PNA to DNA strand and can be a driving force for e^-_{aq} interaction with accessible PNA backbone and groove atoms.

We have studied hybridization of DNA oligonucleotides with PNA, where PNA bear (or not) N- or C-terminal amino groups (-NH_2, lysine, or methylmorpholinium) (Fig. 3, Gantchev *et al.*, 2009). After γ-irradiation (typically 750 Gy) of PNA-DNA heteroduplexes, those with PNA containing free amino group ends formed ICLs (Fig. 5). The multiple bands in each lane represent different crosslinked products and match the number of available amino groups in each heteroduplex. The ICL-formation efficiency is high, G = (5-8) $x10^{-8}$ $mol.J^{-1}$. This G-value even exceeds the ICL yields observed after irradiation BrdU-substituted wobble DNA under identical conditions. Using selective scavengers it was shown that ICL formation in PNA-DNA heteroduplexes strongly depends on the availability of solvated electrons (e^-_{aq}), but proceeds only with a concomitant presence of •OH radicals (Gantchev *et al.*, 2009). Thus, it appears that PNA-DNA ICLs arise in a concerted free radical mechanisms resembling those involved in DNA multiply damaged sites (MDS). By hybridizing 12-mer PNAs with shorter (11-mer), or longer (up to 16-mer) complementary oligo-deoxyribonucleotides thus creating unpaired (single-stranded) regions (deletions and overhangs) at one, or both duplex ends we compared sequence effects on the cross-linking reaction (*e.g.* dT vs. dA termini), the susceptibility of duplex ends to radiation damage, *etc*. The 3′- and 5′- DNA terminal dT nucleotides proved to be of most importance for the efficient ICL formation. Since hydrolysis of N-glycosidic bonds in γ-

damaged nucleotides and/or direct 2′-deoxiribose oxidation yields AP (apurinic/apyrimidinic) abasic sites as a common DNA lesion; we also assessed the role of AP-sites in the PNA-DNA ICL formation using synthetic AP-containing oligo-deoxyribonucletides. We found that presence of AP-sites at different positions of the DNA strand (3′- or 5′-end, and/or penultimate to the ends) results in ICL formation without radiation, but instead required addition of a strong reductant, *e.g.* $NaCNBH_3$. The electrophoretic gel-mobility of thus formed ICL bands resembled that of γ-radiation generated ones. Therefore, we concluded that AP-sites on the DNA strand are the likely partners of the free NH_2, or α- and ε-amino groups of Lys at the PNA ends in the formation of DNA-PNA crosslinks *via* a Schiff-base reaction, followed by imine reduction.

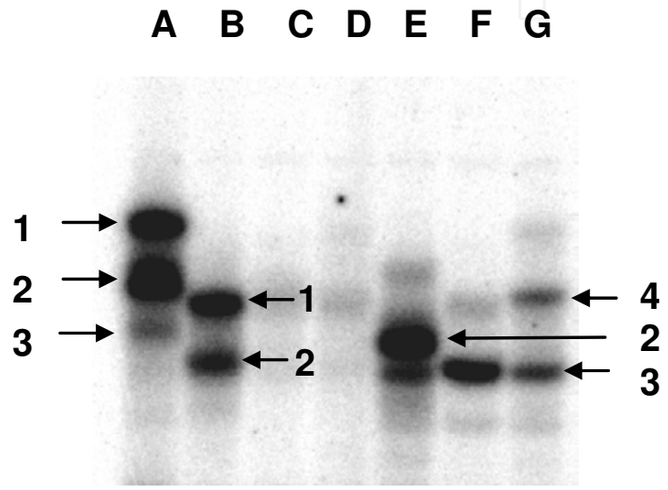

Fig. 5. Electrophoretic migration of bands representing the covalent PNA-DNA dimers (ICL). Aqueous solutions of ^{32}P-DNA hybridized with various PNA were γ-irradiated (750 Gy) under N_2 – atmosphere and in the presence of 25 mM EDTA. Samples differ only by the type of capping groups on PNA ends (R_1 and R_2, Fig. 3). Lanes from left to right: (A) NH_2-K-PNA; (B) NH_2-PNA; (C) Ac-PNA; (D) MeMor-K-PNA; (E) NH_2-PNA-K; (F) Ac-PNA-K; and (G) MeMor-PNA-K; (1- 4): different crosslinked products involving the different amino groups. No ICL are formed with Ac-PNA (C) and MeMor-K-PNA (D). Adapted from Gantchev *et al.*, (2009).

5. Mechanistic and structural aspects of the ICL formation in DNA duplexes and DNA-PNA heteroduplexes

In comparison to inter-strand crosslinks (ICLs), the intra-strand DNA crosslinks have been better studied, mostly as part of locally multiply damage sites (MDS, clustered/tandem DNA lesions). Recently, Wang and co-workers (Lin *et al.*, 2010) found that the exposure of BrdU-substituted telomer G-quadruplex DNA to UVA light results in the formation of G[8-5]U intra-strand crosslink (Fig. 6A). This finding presents evidence that free radical reactions, involving 5-uracil-yl-radical (•U) can also be a source of ICL, albeit not in completely dsDNA duplexes. Thus, Ding & Greenberg (2010) reported radiolytic formation of ICL in anaerobic solutions by BrdU-mimetic iodo-aryl nucleotides incorporated into synthetic dsDNA duplex. Similarly to BrdU-dsDNA irradiation, in these model systems

stand breaks and alkali-labile lesions were also induced at the halogenated site and at the flanking nucleotides. The conclusions from this study highlight the importance of the local DNA structure (wobble *vs.* normal duplex) in terms of Watson-Crick pairing restrictions that likely prevent the 5-uracil-yl σ-radical (in contrast to the aryl radical) interaction with the opposite strand bases to produce ICL. The chemical structures of several DNA inter-strand crosslinks have been reported only recently. Several groups have focused on the identification of ICL structures synthesized in model systems using light- and/or oxidation-sensitive precursors (Hong *et al.*, 2006; Weng *et al.*, 2007; Peng *et al.*, 2008; Kim & Hong, 2008; Op de Beeck & Madder, 2011). A few structures have been definitively identified in natural conditions and, to our knowledge, only one in cellular DNA (Regulus *et al.*, 2007) (Fig. 6C). However, the authors in Regulus *et al.*, (2007) failed to present evidence that this crosslink is exclusively an inter-strand crosslink in cells. Mechanistically, the sole ICL-structure that is exclusively generated with the participation of a primary radiation-induced 5-(2′-deoxyuridinyl) methyl free radical, a product of •OH-induced hydrogen-atom abstraction from thymine, is the T[5m-6n]A crosslink (Ding & Greenberg, 2007; Ding *et al.*, 2008) (Fig. 6B).

A common pathway for ICL formation in dsDNA is the condensation reaction between aldehydes (*e.g.* in abasic DNA sites) and exocyclic amines of opposite bases. Under γ-radiation abasic (AP-apurinic/apyrimidinic) sites are generated either *via* direct H-atom abstraction by •OH radicals from 2′-deoxyribose or after oxidative base damage followed by N-glycosidic bond-cleavage. Oxidation of each of the five positions in 2′-deoxyribose in DNA is possible, but under γ-radiation the best known reactions involve H-atom abstraction at C1′-, C2′- and C4′-positions. The 4′-keto abasic site formed after C4′-oxidation (C4′-AP) is generally known as the "native" abasic site (Chen & Stubbe 2004). Subsequent interactions of sugar radicals with oxygen and/or elimination reactions give a variety of closed-cycle/open-chain aldehydic products, accompanied (or not) by DNA strand-cleavage (Dedon, 2008). One of the first reported, and structurally identified DNA-ICL generated by γ-radiation and/or selective 4′-position 2′-deoxyribose oxidation by bleomycin in model systems and in cells (Fig. 6C; Regulus *et al.*, 2007; Cadet *et al.*, 2010) is produced *via* electrophilic interaction between 2′-deoxypentos-4-ulose abasic site (opened C4′-AP) and N4-dC. Formation of this ICL is accompanied by a DNA strand break. In a series of works, Greenberg and collaborators studied the ICL formation with participation of oxidized native C4′-AP (Sczepanski *et al.*, 2008, 2009a) and 5′-(2-phosphoryl-1,4-dioxobutane, DOB) (Guan & Greenberg, 2009) sites. DOB is produced concomitantly with a single-strand break byDNA-damaging agents capable of abstracting an H-atom from the C5′-position. When oxidized C4′-AP was opposed by dA, a single crosslink formation occurred exclusively with an adjacent dA on the 5′-side. The crosslink formation was attributed to condensation of C4′-AP with the N6-amino group of dA and less favorably with N4-amino group of dC, but not with dG or dT. Interestingly, C4′-AP produced ICLs in which both strands are either intact or ICLs, where the C4-AP containing strand was cleaved (3′ to the AP-site), while DOB-ICLs were always accompanied by an adjacent to the AP-site single-strand break, and thus constituting a clustered type (MDS) lesion.

In contrast, following γ-radiation of BrdU-substituted wobble-DNA (scDNA) duplexes multiple crosslinked products were generated which impedes their chemical identification (Cecchini et al., 2005a; Dextraze et al., 2009). However, in DNA-PNA heteroduplexes, because the amino-groups attached to PNA are exogenous and could be omitted/varied,

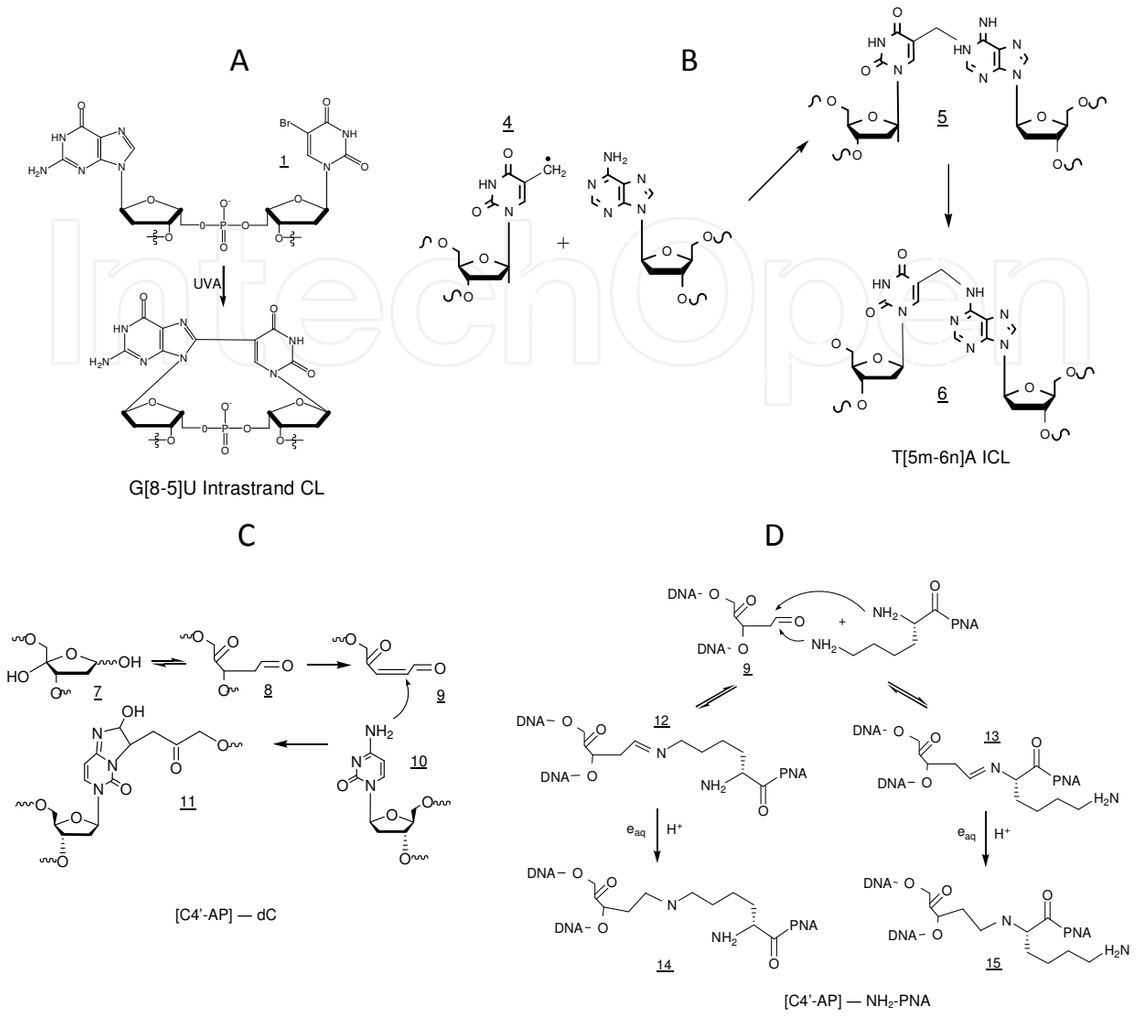

Fig. 6. Chemical structures of known intra- and interstrand crosslinks as generated in model DNA systems, UV and ionizing radiation: (A) The structure of G[8-5]U intrastrand crosslink; the only known crosslink formed *via* direct addition to •U-radical (3). This pathway was not found for ICL formation in normal dsDNA, probably due to steric restrictions, however interstrand crosslinks generated with the participation of 5-uracil-yl radical are possible in wobble scDNA (Fig. 1); (B) The formation of T[5m-6n]A ICL initiated by •OH radical H-abstraction from 5-CH_3dT (4); the intermediate product is addition to N1 (5), which is further rearranged to the final product (6), (Ding *et al.*, 2008); (C) The ICL (11) formed *via* condensation reaction of exocyclic NH_2-group of dC with β-elimination product (9) of an oxidized C4'-AP abasic site (7 and 8). This ICL is associated with a SSB formation, (Regulus *et al.*, 2007) and; (D) Reaction of C4'-AP native abasic site (9) with L-Lys-capped PNA resulting in the formation of PNA-DNA interstrand crosslinks *via* Schiff base (12 and 13). Two free NH_2-groups are equally reactive which can produce two ICLs of different structure (14 and 15). The abasic sites are formed by γ-radiation oxidation of DNA (*e.g.* by •OH radicals). The concomitant solvated electrons, e_{aq}^- are the essential reducing species required to convert Schiff bases in irreversible ICLs, (Gantchev *et al.*, 2009).

together with the synthetically positioned AP-sites on DNA the participation of these two entities in the ICL formation was positively identified (Gantchev *et al.*, 2009). Our data are consistent with a mechanism of ICL formation that involves formation of Schiff bases between PNA-amino functional groups and radiation-damage induced AP-sites on DNA. This type of covalent bonding is widely accepted to take place in the formation of covalent links between NH_2-peptide (protein) groups and damaged (aldehydic) DNA sites, albeit in the presence of an exogenous reducing agent (Mazumder *et al.*, 1996). The new finding is that apart from the prerequisite •OH-mediated, or direct γ-damage of DNA (formation of AP-sites), γ-radiation also provides reducing equivalents to transform the initially formed Schiff base linkage into a more stable reduced bond (amine), *i.e.* to produce irreversible ICL (Fig. 6D). This presents a typical case of radiation-induced MDS, where the synergism of the interactions of •OH, e^-_{aq}, and possibly even •H radicals on PNA-DNA results in ICL. The 3D-modeling (Gantchev *et al.*, 2009) confirms experimental data that open-chain C4'-AP at several DNA-strand end, or penultimate positions are structurally allowed to form covalent bonds with the ε- and α-amino groups of opposite Lys residues, or PNA NH_2-terminal groups and in all cases although, intra-helical ICLs are solvent accessible (*e.g.* the transient Schiff bases are available for interaction with e^-_{aq}). Importantly, if dsDNA duplexes are compared, the e^-_{aq} and solvent accessibility holds for the open structures in BrdU-DNA bubbles in scDNA, only (see below).

Solvated electrons (e^-_{aq}) are indispensible species for the formation of both strand breaks and interstrand crosslinks sensitized by BrdU in scDNA and crosslinks in DNA-PNA heteroduplexes. The e^-_{aq} interaction rate with oxygen, $k(e^-_{aq} + O_2) = 2x10^{10}$ $M^{-1}s^{-1}$ is high, therefore hypoxic experimental conditions are important. The radiosensitization properties of BrdU are based on its ability to undergo dissociative electron transfer (ET) which is initiated by electron capture either from solution, or following excess ET from surrounding DNA bases (Fig. 1). The classical reducibility (electron affinity, EA) trend of nucleobases is BrU>U~T>C>A>G (Aflatooni *et al.*, 1998; Richardson *et al.*, 2004), with BrU being only ~ 40 mV easier to reduce than thymidine (Gaballah *et al.*, 2005). Using the approach described by Michaels & Hunt (1978) and quantitation of BrdU-mediated damage in mismatched duplexes, we calculated a value for $k(e^-_{aq}$ + BrdU-scDNA) of ~$2x10^{10}$ $M^{-1}s^{-1}$. This value is particularly interesting in that the rate constant for BrdU interaction with e^-_{aq} in mismatched, scDNA (single-stranded regions of the duplex) is practically the same as for the free base BrU in solution (Zimbrick *et al.*, 1969a) (*i.e.* essentially diffusion controlled) and, about two orders of magnitude higher than in normal dsDNA. Based on our results from the irradiation of solutions containing PNA-DNA hybrids, we calculated a rate constant for the formation of PNA-DNA crosslinks, assuming only interactions with hydrated electrons, equal to: $k(e^-_{aq}$ + PNA) ~ $5x10^9$ $M^{-1}s^{-1}$, which is also high. While high rate interaction of e^-_{aq} with PNA-DNA hetereoduplexes can be attributed to the lack of electrostatic repulsion (see Fig. 4 and legend), the increased rate of interaction with wobble scDNA is less obvious. We hypothesized that e^-_{aq} may have a restricted access to BrdU when incorporated in a normal DNA duplex, although the Br-atom is partially solvent-exposed in the major groove. To address this issue we applied molecular modeling and nanosecond scale molecular dynamics (MD) simulations, where the excess electron in solution was modeled as a localized $e^-(H_2O)_6$ anionic cluster (Gantchev & Hunting, 2008, 2009). We compared the dynamics and interactions of e^-_{aq} with dsDNA containing a normal BrdU•dA pair in the center of the duplex (dsDNA) with that of a wobble DNA containing a single mismatched

BrdU^dT pair (scDNA), *i.e.* replacing dA with dT. Rather unexpectedly we found that the occupancy of the close-to-DNA space for scDNA and dsDNA at cut-off distance <5 Å was 0.7% vs. 1.6%, respectively (from a total of 4,000 MD configurations). However, the electron interacted with a larger number of individual bases in scDNA. For instance, in dsDNA, the electron moved closely toward only four nucleobases, all from the non-brominated DNA strand, while in scDNA eleven nucleobases from both strands were found to come within reach of e_{aq}^-. The different clustering of the electron (occupation of close to DNA sites) in both duplexes is presented graphically in Fig. 7 (see legend for details). Notably, BrU incorporated in the central (sixth) position of both DNA duplexes, was approached by e_{aq}^- several times in scDNA only. Likewise only in scDNA, the e_{aq}^- preferentially occupied close sites and formed contacts with the two most perturbed thymidines (dT5 and dT7) flanking BrdU. At present, there is no explanation for the disparity of e_{aq}^- interactions with dsDNA *vs*. scDNA, other than the different dynamic structure of the isosteric DNA sequences under study, including the dynamics of structured water and Debay-Hückel layers (Gantchev & Hunting, 2008, 2009). The exposure of wobble-pair pyrimidine carbonyl groups into the DNA grooves results in excess solvation of the mismatched pairs (Sherer & Cramer, 2004).

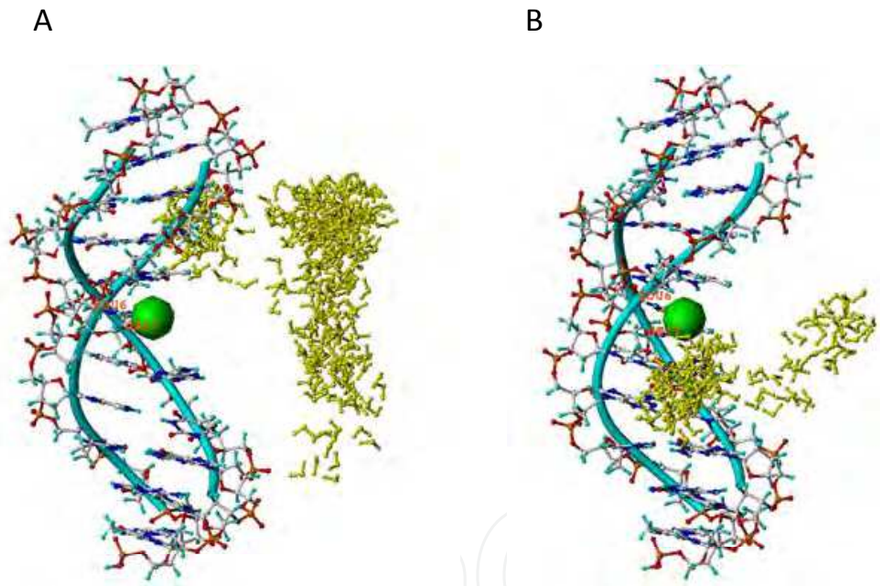

Fig. 7. Superimposed snapshots from ns MD of regular (left) and mismatched (right) 11-mer DNA containing a Watson-Crick BrU6•A17 normal pair, or BrU^T17 wobble pair, respectively. The e_{aq}^- is represented by a $e^-[H_2O]_6$ anionic cluster. The shown dynamic states are selected by the rule, distance $|e_{aq}^- - \text{nucleobase}| < 5$ Å. From the total of 59 states for normal DNA, there are no configurations where e_{aq}^- is close to the central BrU6•A17 base pair. In contrast, in the wobble scDNA from the all 22 states obeying the same selection rule, ~ 65% show close approach to the wobble BrU6^T17 pair. The electron resides most often in the vicinity of the flanking T7 base and less frequently approaches BrU6 and T17. Hydration water and counterions are not shown; BrU•A and BrU^T are in the middle and labeled. Color code: Br-vdW sphere (green); DNA backbone (cyan); nucleotide atoms (standard color); e_{aq}^- (yellow). For details see: Gantchev & Hunting, (2008, 2009).

In addition, the incorporated mismatched pairs (T^T or BU^T) alter the dynamics of the neighboring bases due to incomplete 5′-stacking. Together with the narrowing of the minor groove these phenomena bring the two strands closer which creates conditions for cross-strand (cs) stacking and single and multiple cross-strand (cs) H-bonding not only within the mismatched regions, but also encompassing penultimate nucleotides to create extended "zipper-like" motives (Špačková *et al*., 2000). The properties of single-mismatch scDNA duplexes, including the effect of the nearest sequence context (*e.g.* presence of T-tract DNA) have been discussed elsewhere (Gantchev *et al*., 2005). A schematic presentation of the most often formed cross-strand inter-base contacts is given in Fig. 8. The close presence of e_{aq}^-, although causes dynamic instability and fluctuations around the mismatched BrdU^dT pair does not abolish, but in contrast, provokes additional frequent cs-H-bonding interactions (Gantchev & Hunting, 2009). All these findings are important in terms of facilitated charge-transfer along UV-, or γ-activated BrdU-scDNA. The intrahelical electron or hole transfer to BrdU and/or •U-yl radical are the next important factor that is largely expected to control the efficiency (and location) of the ensuing DNA damage; the formation of DSB and ICL. Indeed, recently a more effective electron transfer has been reported for mismatched duplexes than for fully complementary DNA (Ito *et al*., 2009). Using a two electron acceptor DNA model system with incorporated BrdA, BrdG, BrdU and TT-dimer Fazio *et al.* (Fazio *et al*., 2011) were able to estimate the absolute electron-hoping rates in DNA and have shown that the electron transfer is more efficient in 5′ → 3′ direction. As mentioned, in unsubstituted DNA pyrimidine rather than purine bases have been considered as trapping sites for excess electrons. This is illustrated by resonant free electron attachment experiments (Stokes *et al*., 2007) which show that both thymine and cytosine form stable valence anions for low energy electrons, *i.e.* both thymine and cytosine possess positive adiabatic electron affinities. However, recently a stabile anionic state of adenine (A^-) has been detected (Haranczyk *et al*., 2007). Subsequently, this finding has been shown to have a pronounced effect in the ultrafast ET in DNA and on dissociative bond cleavage (Wang *et al*., 2009), including ET to BrdU from A^- acting as primary trap of radiolysis-generated pre-hydrated electrons (Wang *et al*.., 2010). These new developments in the field add to the existing puzzles of the precise determination of successive chain events leading to multiple BrdU-sensitized damages (DSB and ICL) in wobble scDNA.

Repair of interstrand crosslinks (ICLs) requires multiple strand incisions to separate the two covalently linked DNA strands. It is unclear how these incisions are generated. DNA double-strand breaks (DSBs) have been identified as intermediates in ICL repair, but eukaryotic enzymes responsible for producing these intermediates are not well known (Wang, 2007; Moldovan & D'Andrea, 2009a,b; D'Andrea & Grompe, 2003; Liu *et al*., 2010; Hanada *et al*., 2006). Ongoing research shows that in cell free model systems ICLs of different chemical structure exert different effects during repair and some may be difficult to repair. The repair refractive character of a particular ICL resulting from the C4′-AP abasic site and identified to occur as a clustered ICL-SSB lesion (Sczepanski *et al*., 2008, 2009a) was recently demonstrated to give rise to even more toxic DSBs when subjected to NER (Sczepanski *et al*.., 2009b). Likewise, during UvrABC nucleotide excision repair of the well-defined T[5m-6n]A single-lesion crosslink imbedded in dsDNA (Fig. 6B, Ding *et al*., 2008), DSB were produced in almost 30% of the excision events (Peng *et al*., 2010).

DNA packing into chromatin adds to the complexity of DNA damage recognition and removal, because the highly condensed chromatin is, in general, refractory to DNA repair (Hara *et al*., 2000; Thoma, 2005). In order to grant access to DNA repair machinery, the

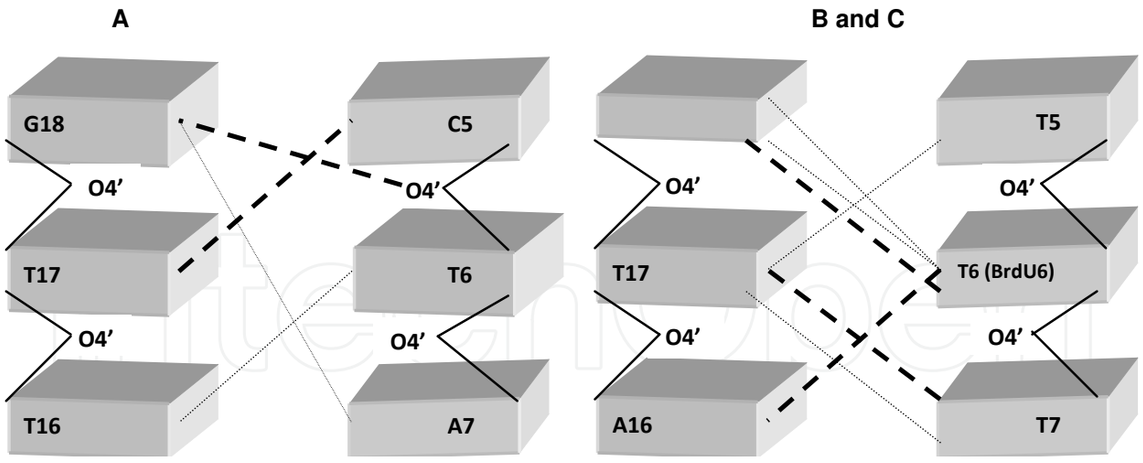

Fig. 8. Nearest-neighbour sequence effects in wobble semi-complementary DNA (scDNA) as observed by MD (adapted from Gantchev *et al*. 2005). Schematic presentation of the frequent cross-strand (cs) inter-base contacts formed in the studied 11-mer DNA duplexes containing a single mismatch: T^T or T^BrU, incorporated in the central triplets: d(CTA)•(TTG) (A), d(TTT)•(ATA) (B) and d(TBrdUT)•(ATA) (C): bold dashed lines (most frequently observed cs H-bons), dotted lines (less frequent cs H-bonds). Note that cs contacts in (C) coincide with those observed also in the presence of e^-_{aq} (Gantchev & Hunting, 2009). These data underline the importance of the wobble DNA dynamic structure for both, interstrand ET and high-frequency opposite-strand atom encounter for the generation of (asymmetric) ICL (Fig. 1).

chromatin response to DNA damage involves activation of ATP-dependent chromatin-remodeling complexes and histone post-translational modification pathways (Peterson & Côte, 2004; Nag & Smerdon, 2009; Méndez-Acuña *et al*., 2010). Again, DSBs recognition and repair in the context of chromatin rearrangement is better studied and understood at the expense of other DNA damages, such as ICLs. One crucial chromatin modification, the phosphorylation of the histone variant H2AX (γH2AX) is perhaps the best example of a histone modification in response to DSB induction in DNA (Van Attikum & Gasser, 2005). Despite the progress achieved in understanding of the repair of certain UV-induced DNA damages (intra-strand crosslinks), *e.g.* cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine photoadducts (6-4 PP), or the acetylaminofluorene-guanine (AAF-G) covalent adduct, little is known about the effects of other bulky DNA lesions (*e.g.* ICLs) on the nucleosome structural dynamics and its interplay with the versatile NER pathway (Smerdon & Lieberman, 1978; Pehrson, 1995; Gaillard et al., 2003; Gospodinov & Herceg, 2011). There is a consensus that NER functionality depends primarily on the damage recognition step, which in turn depends on the degree of DNA helix distortion induced by a particular lesion (Cai *et al*., 2007). It has been hypothesized that structurally different interstrand crosslinks would affect chromatin remodeling and damage recognition in different ways, and some ICLs might retain their refractive character to recognition/repair, or at least will exert an altered repair efficacy. Thus, a recent *in vivo* study (Hlavin *et al*., 2010) confirmed that the structure of synthetic interstrand crosslinks between mismatched bases affects the repair rate (in this case, transcription coupled NER). It can be further hypothesized that PNA-patches hybridized to DNA (*e.g.*, > 8-10 b.p.; PNA- invaded DNA strands, PNA-DNA triple helices, and/or DNA-PNA covalent adducts) would be

difficult to repair without a loss of DNA information, and/or would require major chromatin remodeling. Indeed, Faruqi *et al*. (1998) designed PNAs that bind to the *sup*FG1 mutation reporter gene and found that 8- or 10- b.p. PNA bound to this site induces mutations at frequencies in the range of 0.1%, well above the *in vivo* background. Later, the same group (Kim *et al*., 2006) demonstrated that a psoralen-*bis*-PNA conjugate directs the formation of a photoadduct at the 5'-TpA step of the PNA binding site to the same *sup*FG1 gene. In mammalian cells, the UV-generated PNA-targeted psoralen phtoadducts induced mutations as high as 0.46%, *i.e.* 6.5-fold above the background.

6. Conclusion

In conclusion, there is a need to better understand the parameters which control the formation and repair of complex DNA lesions, such as interstrand crosslinks. Such complex, repair refractive lesions may offer a means to selectively kill tumor cells by taking advantage of either enhanced formation or reduced repair within the tumor environment.

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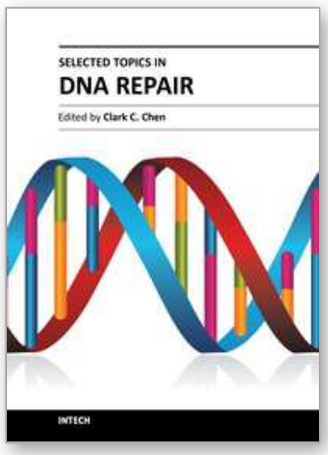

Selected Topics in DNA Repair

Edited by Prof. Clark Chen

ISBN 978-953-307-606-5
Hard cover, 572 pages
Publisher InTech
Published online 26, October, 2011
Published in print edition October, 2011

This book is intended for students and scientists working in the field of DNA repair, focusing on a number of topics ranging from DNA damaging agents and mechanistic insights to methods in DNA repair and insights into therapeutic strategies. These topics demonstrate how scientific ideas are developed, tested, dialogued, and matured as it is meant to discuss key concepts in DNA repair. The book should serve as a supplementary text in courses and seminars as well as a general reference for biologists with an interest in DNA repair.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Tsvetan G. Gantchev, Marie-Eve Dextraze and Darel J. Hunting (2011). DNA Radiosensitization: The Search for Repair Refractive Lesions Including Double Strand Breaks and Interstrand Crosslinks, Selected Topics in DNA Repair, Prof. Clark Chen (Ed.), ISBN: 978-953-307-606-5, InTech, Available from: http://www.intechopen.com/books/selected-topics-in-dna-repair/dna-radiosensitization-the-search-for-repair-refractive-lesions-including-double-strand-breaks-and-i

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