Université de Sherbrooke

Radiosensibilisation de l'ADN par le 5-bromodéoxyuridine : l'importance de la structure et de la séquence de l'ADN

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Thèse présentée à la Faculté de médecine et des sciences de la santé
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Insanity: Doing the same thing over and over again and expecting different results
- Albert Einstein

The trouble with having an open mind, of course, is that people will insist on coming along and trying to put things in it
- Terry Pratchett

Résumé

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Les dimères interbrins sont des lésions de type complexe, où les deux brins d'ADN sont pontés de façon covalente. Par conséquent, ce type de lésion est très toxique pour la cellule, car il nuit à la séparation des brins d'ADN nécessaire à des processus cruciaux pour la cellule, comme la réplication et la transcription. De plus, des expériences récentes montrent que la réparation des dimères interbrins passe par la formation d'un bris double brin, une autre lésion avec un potentiel toxique élevé. Ce n'est que tout récemment qu'on a montré que la radiation ionisante menait à la formation de dimères interbrins dans l'ADN cellulaire. On en sait donc encore très peu sur les conditions dans lesquelles se produisent les dimères et comment ils sont réparés.

Récemment, à la suite d'une exposition aux radiations ionisantes, notre groupe a mis en évidence la formation de dimères interbrins dans un ADN où une thymidine avait été remplacée par le 5-bromo-2′-désoxyuridine (BrdU). Ces dimères n'étaient formés que lorsque le BrdU se trouvait au centre d'une zone mésappariée. Puisque c'était la première fois que ce type de dommage était observé lors d'une exposition de l'ADN bromé à la radiation ionisante, ma thèse a porté sur l'exploration de la formation du dimère interbrin, particulièrement sur les conditions qui favorisaient sa formation. Les trois articles présentés dans cette thèse montrent que la forme de l'ADN (forme A vs forme B), la séquence, ainsi que le type de radiation employé ont une influence importante sur le type et la fréquence du dommage produit.

Ces résultats montrent qu'on en sait encore très peu sur le mécanisme réel de radiosensibilisation de l'ADN bromé dans les cellules. Cependant, ils mettent aussi en évidence la réactivité distincte des régions mésappariées de l'ADN, ainsi que leur fort potentiel pour la formation de dimères. Or, ces régions mésappariées ne représentent qu'une fraction des structures secondaires et tertiaires de l'ADN présentes dans la cellule.

MOTS-CLÉS : 5-bromodéoxyuridine, dommages à l'ADN, dimères interbrins, structure de l'ADN, radiation ionisante.

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Liste des abréviations

Acide désoxyribonucléique	ADN
Acide éthylène-diamine-tétraacétique	EDTA
Acide ribonucléique	ARN
Adénine	A
Atome d'hydrogène	$H^{\cdot}$
5-bromo-2′-désoxyuridine	BrdU
5-bromouracile	BrU
Chromatographie liquide haute performance	HPLC
Cobalt-60	^{60}Co
Cytosine	C
Électron aqueux	e_{aq}
Électron-volt	eV
Epidermal Growth Factor	EGF
Gray	Gy
Guanine	G
Hydrogène	H_2
Méthyl	$-CH_3$
Oxyde nitreux	N_2O
Paire de bases	pb
Peroxyde d'hydrogène	H_2O_2
Proton	H^+
Radicaux hydroxyles	$^{\bullet}OH$
Réparation par excision des nucléotides	NER
Taux d'hydratation	Γ
Thymine	T
Transfert d'énergie linéaire	TEL
Ultraviolet	UV

Chapitre I – Introduction

I.1 – Structure de l'ADN

"To say that a man is made up of certain chemical elements is a satisfactory description only for those who intend to use him as a fertilizer" – Hermann Joseph Mueller

I.1.1 – Composantes de l'ADN

De la même façon qu'un roman ne se résume pas qu'à un mélange des 26 lettres de l'alphabet, et une symphonie à 8 notes, la vie ne se résume pas à la combinaison aléatoire de A, C, G, et T. Et pourtant... Ce sont des propriétés physiques et chimiques de l'ADN que découlent les caractéristiques qui sont à l'origine de la fonction centrale de cette molécule pour la cellule. La double hélice d'ADN est composée de deux brins antiparallèles, eux-mêmes formés à partir d'une répétition de nucléotides, qui comprend une base, un sucre (désoxyribose) et un groupement phosphate (figure I-1). C'est la succession de désoxyriboses et de groupements phosphates qui forme le squelette de la molécule d'ADN, tandis que les bases correspondent à la partie variable de la molécule, celle où est contenue l'information. Il y a quatre bases dites « natives » dans l'ADN; l'adénine, la guanine, la cytosine et la thymine, symbolisées par les lettres A, G, C et T. Les autres bases, comme la méthylcytosine, correspondent souvent à des modifications épigénétiques ou à des produits de déamination d'une des quatre bases natives.

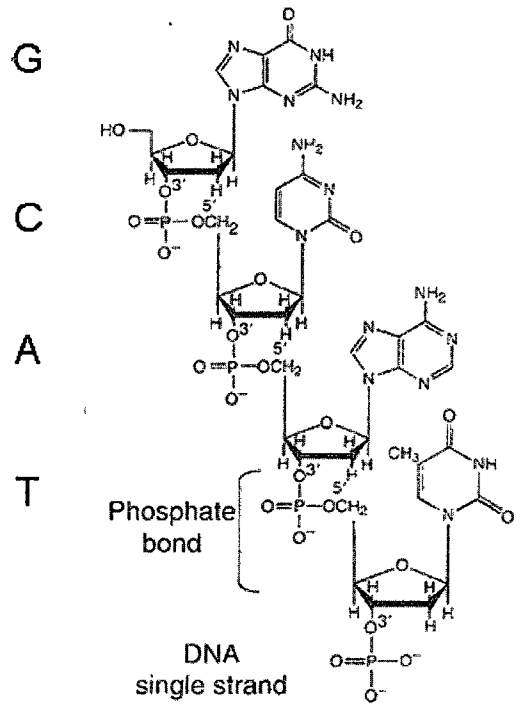

Figure I-1 : Composantes de l'ADN. Modifié à partir de Lehnert, 2007, p 31.

On sépare les quatre bases en deux catégories : les purines (A et G) et les pyrimidines (C et T). L'adénine (A) s'apparie avec la thymine (T), et la guanine (G) avec la cytosine (C). Chaque base d'un brin s'apparie à sa base complémentaire située sur le brin opposé grâce à la formation de ponts hydrogène. Ces ponts hydrogène permettent à l'ADN d'adopter une structure à la fois robuste et flexible, où la proximité des deux brins est maintenue, tout en minimisant l'énergie nécessaire à leur séparation.

I.1.2 – Forme A, B et Z

La double hélice d'ADN est une molécule extrêmement flexible, et peut adopter un nombre impressionnant de configurations en réponse aux pressions de son environnement. La figure I-2 montre trois des formes les mieux connues jusqu'à

présent, soit les formes A, B, et Z. Les caractéristiques de ces formes sont présentées dans le tableau I-1.

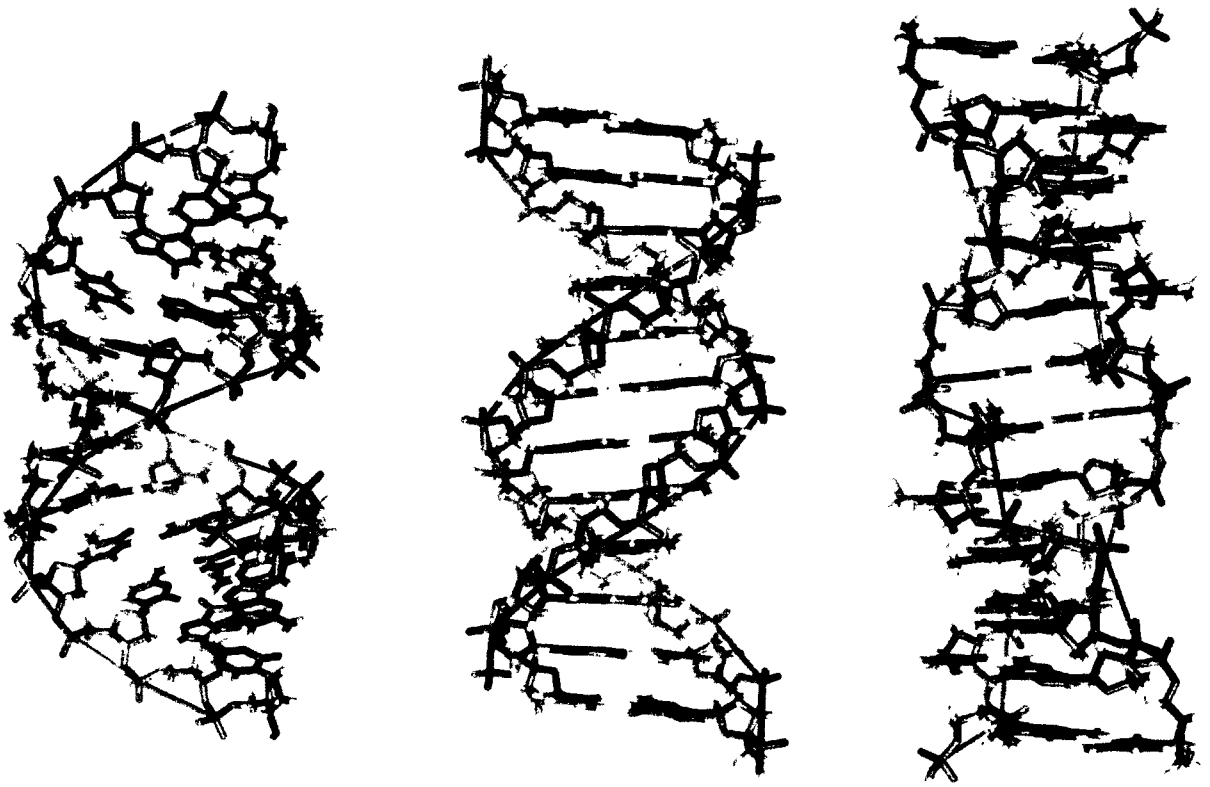

Figure I-2 : Formes A, B et Z de l'ADN (de gauche à droite).

La forme B est la mieux connue, car elle est adoptée dans des conditions physiologiques, notamment dans les têtes intactes des spermatozoïdes (Voet et Voet, 1995). La forme A, quant à elle, est retrouvée en présence d'un duplexe ADN/ARN, ou lorsque l'ADN est déshydraté, par exemple en présence de 80 % éthanol. Bien que l'homologie de la forme A dans ces deux conditions ait été remise en question, notamment à cause des différences dans la conformation du sucre (Horton et Finzel, 1996), la forme générale de l'ADN-A demeure la même. La formation d'ADN-Z a surtout été observée au niveau de l'ADN synthétique contenant une grande proportion de guanines en présence de concentrations élevées en sel, ainsi qu'en présence de cytosines méthylées en position C5 (Behe et Felsenfeld, 1981, Thamann *et al.*, 1981).

Tableau I-1 : Caractéristiques des formes de l'ADN.

	A	B	Z
Sens du pas de l'hélice	Droite	Droite	Gauche
Diamètre	~26 Å	~20 Å	~18 Å
Paires de bases/ tour d'hélice	11	10	12
Rotation de l'hélice / paire de bases	33 °	36 °	60 °
Longueur d'hélice / tour	28 Å	34 Å	45 Å
Pliage des sucres	C3'-endo	C2'-endo	C2'-endo (C et T) C3'-endo (A et G)

Dans la cellule, la démonstration de l'existence de l'ADN-Z a été plus ardue. La transition entre la forme B et la forme Z nécessite de l'énergie, qui devient disponible dans certaines conditions dans la cellule, comme le superenroulement négatif de l'ADN observé en aval de l'ARN polymérase durant la transcription (Liu et Wang, 1987). Depuis les premières évidences de l'existence de l'ADN-Z, sa formation lors de la transcription a été corrélée par plusieurs expériences (Herbert et Rich, 1999, Jiang *et al.*, 1991, Lafer *et al.*, 1983). De plus, le groupe de Rich a observé que la protéine ADAR1, une ARN adénosine déaminase, liait spécifiquement la forme Z de l'ADN (Herbert *et al.*, 1995). L'ADN-Z semble impliquée dans plusieurs processus fonctionnels de la cellule, notamment la régulation de l'expression, le positionnement des nucléosomes, le remodelage de la chromatine et la recombinaison (Li *et al.*, 2009).

Ces trois conformations ne sont qu'un exemple des multiples configurations que l'ADN peut adopter en fonction des pressions de son environnement. Plusieurs

protéines présentes dans le noyau de la cellule sont aussi en mesure de modifier la structure de l'ADN, que ce soit pour l'empaqueter (chromatine), ou pour contrôler les différents processus cellulaires qui impliquent l'ADN (transcription, réplication, réparation).

I.1.3 – ADN cellulaire : un environnement dynamique

Lorsqu'on pense à l'ADN, la première image qui nous vient en tête est généralement celle de la double hélice. On oublie souvent que l'ADN est avant tout une structure dynamique, en constant changement. Même la chromatine, l'agencement de protéines et d'ARN qui permet la compression de 4 mètres d'ADN dans un noyau de 5 μm de diamètre, représente un niveau d'organisation supérieur qui affecte autant l'accessibilité de l'ADN aux protéines du noyau que l'organisation générale de l'ADN en régions fonctionnelles.

La figure I-3 montre les niveaux successifs d'organisation de l'ADN cellulaire. La double hélice d'ADN s'enroule autour de protéines appelées histones pour former les nucléosomes. Ces nucléosomes forment à leur tour des solénoïdes de 30 nm de diamètre qui se compactent en filaments. Durant la mitose, ces filaments se condensent encore davantage pour former des chromatides, qui permettent la répartition d'une copie du génome à chaque cellule-fille.

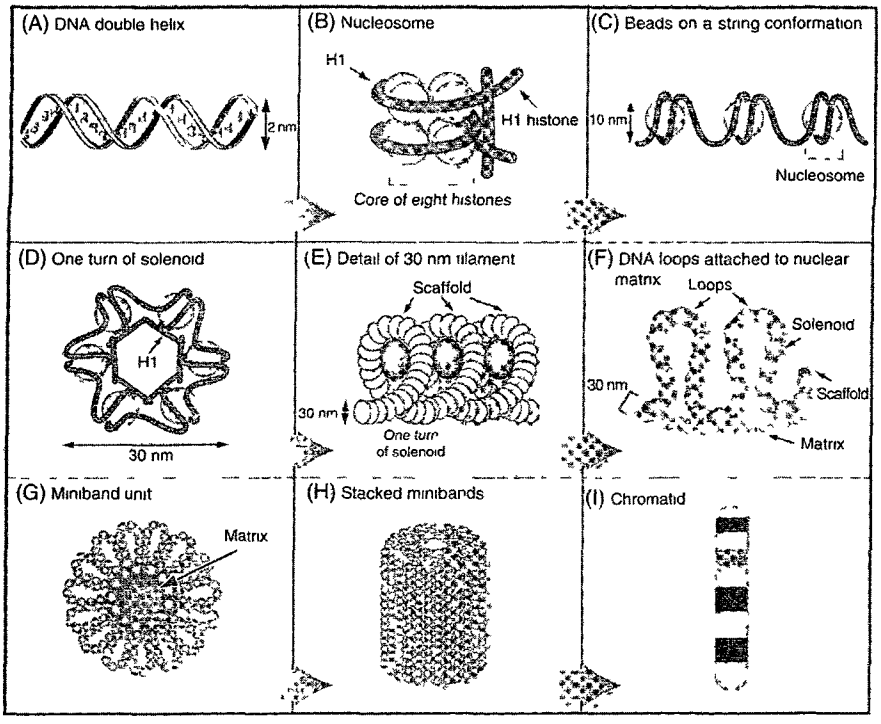

Figure I-3 : Condensation de l'ADN en chromatine. Source : (Lehnert, 2007, p 206)

Cependant, il existe aussi plusieurs processus dynamiques qui impliquent l'ADN dans la cellule. Dans les régions activement transcrites du génome, la structure même de la chromatine est constamment modifiée afin de moduler l'accessibilité de l'ADN aux protéines responsables de la régulation de la transcription. Les modifications post-traductionnelles des histones comptent pour une fraction non négligeable des méthodes utilisées par la cellule pour réguler cette accessibilité, et constituent en quelque sorte un code qui identifie les régions de l'ADN qui doivent être libérées des contraintes stériques de la chromatine pour faciliter la transcription, en fonction des besoins de la cellule. Dans d'autres cas, les histones sont aussi modifiées pour faire entrer en dormance certaines parties du génome (Voir Mellor, 2006, pour une revue de l'impact des modifications nucléosomales sur la transcription).

D'autres phénomènes, qu'ils soient constants (transcription, réparation) ou ponctuels (réplication) font de l'ADN cellulaire une molécule en constants changements.

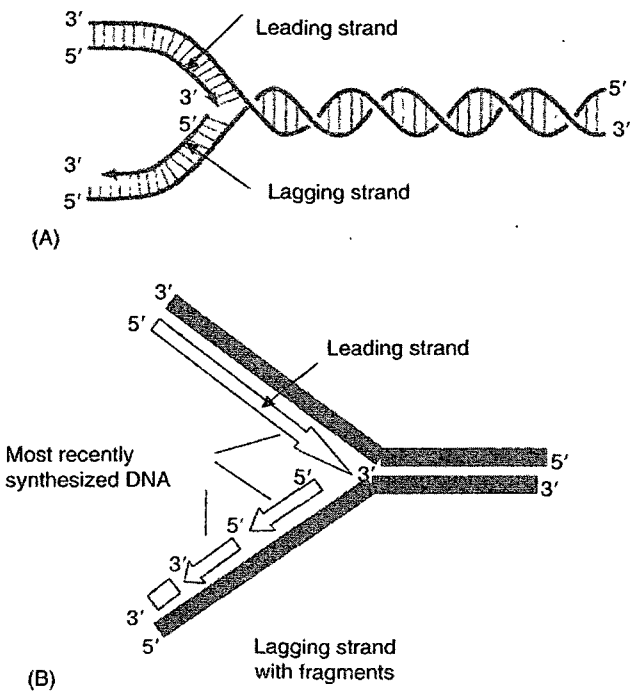

Figure I-4 : Réplication de l'ADN. Source : (Lehnert, 2007, p 35)

Lors de la réplication, les deux brins de la double hélice d'ADN sont séparés afin de permettre la reproduction de l'information contenue dans chaque chromosome. La figure I-4 montre une représentation schématisée de la réplication de l'ADN. Les deux copies ainsi créées permettront la formation de deux cellules-filles avec la même information génétique. Cependant, la figure I-4 ne montre qu'une vision simplifiée de ce qui se produit dans la cellule. En réalité, la réplication est un processus complexe qui nécessite la coordination de dizaines de protéines (Pollard et Earshaw, 2002), même dans les systèmes bactériens, qui sont beaucoup moins complexes. Une hélicase doit séparer les deux brins d'ADN en amont de l'ADN polymérase, l'enzyme responsable de la polymérisation du nouveau brin, et qui comprend elle-même souvent plusieurs sous-unités. D'autres protéines sont responsables de protéger les brins d'ADN des nucléases alors qu'ils sont sous forme simple brin. Finalement, un agencement complexe de protéines est chargé de s'assurer que le brin d'ADN nouvellement synthétisé ne comprend pas d'erreurs, et de réparer celles qui pourraient s'être produites.

La réplication n'est qu'un exemple parmi tant d'autres où la structure de l'ADN est modifiée par un phénomène de régulation cellulaire. Pour cette raison, il est crucial de prendre en compte cet aspect du métabolisme de la cellule lorsqu'on étudie les conséquences d'un agent extérieur sur l'ADN, notamment la radiation.

I.2 – Effet des radiations ionisantes sur l'ADN

I.2.1 – Rayonnement ionisant et interaction avec la matière

L'interaction du rayonnement avec la matière a pour conséquence le transfert d'une partie ou de la totalité de l'énergie de la particule initiale le long de sa trajectoire. La notion de TEL (transfert d'énergie linéaire, keV/µm) est utilisée pour caractériser ce dépôt d'énergie en fonction de la distance parcourue dans la matière. Par exemple, le Cobalt-60 (^{60}Co), utilisé en radiothérapie, produit 2 photons avec une énergie moyenne de 1.25 MeV dont le TEL est d'environ 0.2 keV/µm. On distingue ainsi les sources de radiation à haut TEL (particules lourdes, rayons alpha), où la densité des ionisations est élevée le long de la trajectoire, de celles à bas TEL (^{60}Co), où la densité des ionisations est plus faible.

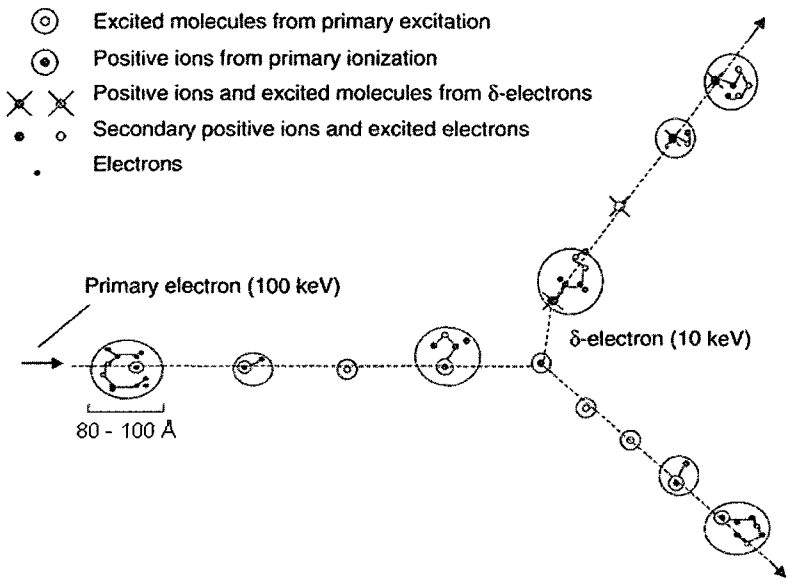

Figure I-5 : Dépôt d'énergie le long de la trajectoire de l'électron primaire. L'électron primaire (ici avec une énergie de 100 keV) dépose son énergie en excitant (points blanc) ou en ionisant (points noirs) la matière qu'il rencontre le long de sa trajectoire. Le dépôt de l'énergie peut ensuite mener à une série d'ionisations et d'excitations secondaires dans un volume de 80 à 100 Å autour du site de l'interaction (appelée grappe). La production d'électrons δ, peu probable pour le rayonnement à faible TEL, peut se produire lorsque les grappes se recoupent avec un rayonnement à haut TEL. Source : (Lehnert, 2007, p 13)

Dans le cas de la radiation électromagnétique (p. ex. les photons), le dépôt de la dose passe par le transfert de l'énergie à des électrons primaires très énergétiques. Il y aura par la suite formation d'espèces réactives le long de la trajectoire de l'électron primaire par des processus d'excitation et d'ionisation (figure I-5). Ces processus créent aussi des électrons de basse énergie ($\leq$20 eV) qui, pour la plupart, ont une énergie qui se trouve sous le seuil d'ionisation. Malgré qu'ils soient produits en très grande quantité, on a longtemps cru qu'ils avaient peu d'importance au niveau de la radiobiologie, puisqu'ils ne possédaient pas suffisamment d'énergie pour ioniser la matière. Cependant, les travaux de Léon Sanche ont montré que les électrons de basse énergie étaient en mesure, par un phénomène d'attachement dissociatif, de causer des dommages dans la matière, notamment l'ADN (Boudaiffa *et al.*, 2000; pour une revue de la littérature, voir Sanche, 2002). Cependant, la chimie des radiations classique

tient surtout compte des produits issus de l'ionisation de la matière le long de la trajectoire incidente.

I.2.2 – Radiolyse de l'eau

Dans les systèmes biologiques, l'eau est une composante majeure; elle représente entre 70 et 80 % du contenu d'une cellule. Ainsi, la probabilité que la radiation interagisse avec l'eau est importante si on la compare aux autres molécules. Pour cette raison, beaucoup d'efforts ont été consacrés à la compréhension des réactions qui se produisent à la suite de cette interaction. Un schéma simplifié de la décomposition de l'eau est présenté à la figure I-6.

Excitation

$H_2O \rightarrow H_2O^*$

$H_2O^* \rightarrow H^{\cdot} + {}^{\cdot}OH$

Ionisation

$H_2O \rightarrow H_2O^{\cdot+} + e^-$

$H_2O^{\cdot+} + H_2O \rightarrow H_3O^+ + {}^{\cdot}OH$

$e^- + nH_2O \rightarrow e^-_{aq}$

Figure I-6 : Radiolyse de l'eau à l'échelle de la picoseconde.

C'est durant l'étape physique ($\sim 10^{-16}$ s) que se produisent les premières étapes de la radiolyse, soit l'excitation et l'ionisation des molécules d'eau le long de la trajectoire incidente. Lors de l'étape physico-chimique ($\leq 10^{-12}$ s), il y a dissociation des molécules d'eau en ${}^{\cdot}OH$, $H^{\cdot}$ et H_3O^+ (voir figure I-6). De plus, les électrons, qui ont été arrachés lors des ionisations initiales, perdent graduellement leur énergie suite à

d'autres ionisations et excitations, et vont ainsi se thermaliser. Il y aura ensuite réorganisation des molécules d'eau autour de l'électron pour former un électron hydraté. À cette étape, la diffusion n'entre pas en jeu dans les différentes réactions. Lors de l'étape subséquente de chimie non homogène qui se déroule au sein des grappes ainsi formées autour des interactions primaires, ce sont ces diverses espèces réactives qui vont diffuser et se combiner pour former les espèces moléculaires des produits de la radiolyse de l'eau telles que H_2 et H_2O_2. À 10^{-6} s, c'est-à-dire à la fin de l'expansion des grappes et au début de l'étape de chimie homogène, les espèces présentes de la radiolyse de l'eau sont, principalement $^{\bullet}OH$, e^-_{aq}, $H^{\bullet}$, H_2O_2, H_2, H_3O^+ et OH^-. Ce sont précisément ces espèces qui réagiront ensuite avec le matériel biologique pour produire l'effet indirect des radiations.

I.2.3 – Effet direct et indirect

Bien qu'on puisse décrire l'effet direct/indirect des radiations sur la matière biologique à différents niveaux (moléculaire, cellulaire, vasculaire), dans le cas de l'ADN on décrit l'effet direct des radiations comme l'interaction directe de la particule incidente avec l'ADN (figure I-7).

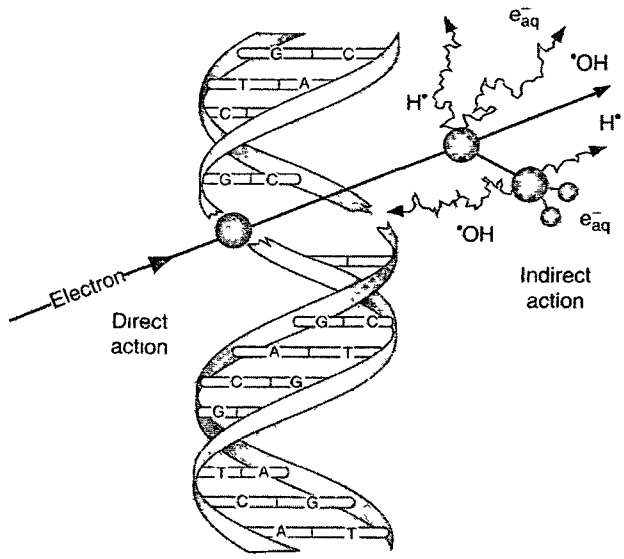

Figure I-7 : Effet direct et indirect des radiations sur l'ADN. Source : (Lehnert, 2007, p 11)

L'effet indirect, lui, implique un dépôt d'énergie aux molécules adjacentes, suivi de la formation d'espèces réactives par excitation ou ionisation. Ce sont les espèces réactives, le plus souvent les produits issus de la radiolyse de l'eau dans le cas du matériel biologique irradié en solution diluée, qui vont ensuite interagir avec l'ADN.

Lorsqu'on tient compte des couches d'hydratation de l'ADN en solution, on distingue alors trois types d'effets : l'effet direct, indirect et quasi direct. On qualifie d'effet quasi-direct la production de radicaux dans la couche primaire interne ($\Gamma \leq 9$ H_2O/nucléotide) car ils sont immédiatement transférés à l'ADN. La couche primaire d'hydratation se distingue de la couche secondaire par la faible mobilité des molécules d'eau (temps de résidence supérieur à la picoseconde) et la densité plus importante de ces molécules. La portion interne de la couche primaire comprend les molécules d'eau qui sont étroitement liés à la molécule d'ADN et qui demeurent associées à l'ADN même à 0 % d'humidité (Feig et Pettitt, 1998). Ce n'est donc pas surprenant que la production de radicaux hydroxyles ne soit observée que dans la couche primaire externe à partir de $\Gamma \approx 9$ H_2O/nucléotide (Debije *et al.*, 2000, La Vere *et al.*, 1996). La couche secondaire d'hydratation commence à $\Gamma \approx 20$

H_2O/nucléotide, et il est impossible de la distinguer du reste des molécules d'eau en solution par la mobilité ou la densité des molécules d'eau (Mroczka et Bernhard, 1993, Saenger, 1984, Wang *et al.*, 1993).

I.2.4 – Interaction des radicaux avec l'ADN

Suite à l'interaction de la radiation avec la matière biologique et la génération de radicaux qui attaquent l'ADN, plusieurs types de dommages sont produits. Suite à des expériences menées avec des capteurs de radicaux, on estime que 60-70% des dommages subis par la cellule proviennent de l'interaction des radicaux $^{\bullet}OH$ avec l'ADN (Chapman *et al.*, 1973, Roots et Okada, 1972), alors que les électrons solvatés, qui créent surtout des lésions aux bases, ont peu ou pas d'effet sur la mortalité cellulaire. Les radicaux $^{\bullet}OH$ interagissent avec les doubles liaisons des bases et sont d'excellents abstracteurs d'atomes d'hydrogène sur le désoxyribose ($k_{OH} = 2.3 \times 10^9$ L mol^{-1} s^{-1}, Motohashi et Saito, 1993), ce qui mène à la formation de bases modifiées et à la formation de cassures.

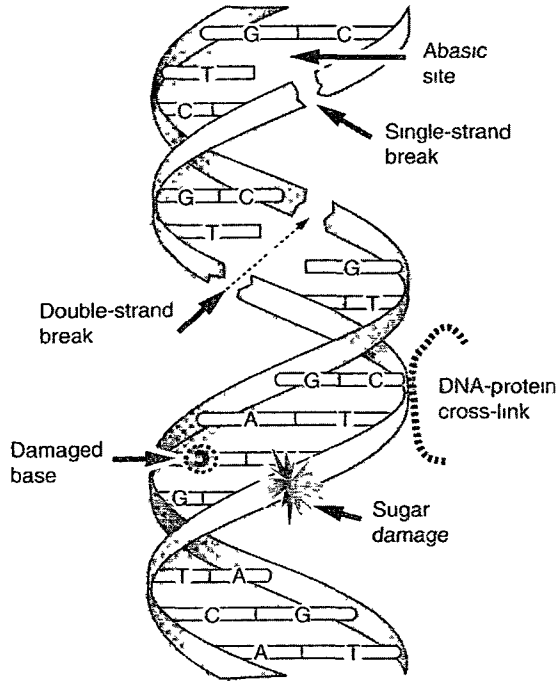

Figure I-8 : Types de dommages formés par l'exposition de l'ADN aux radiations ionisantes. Source : (Lehnert, 2007, p 124)

Parmi les dommages subis par l'ADN cellulaire à la suite d'une exposition aux radiations ionisantes, on compte deux grandes catégories, qui sont schématisées à la figure I-8 :

- les dommages simples :
    - site abasique
    - base modifiée
    - dommage au sucre
    - bris simple brin
- les dommages complexes
    - lésions multiples (incluant les bris double brin)
    - pontage ADN-protéine
    - pontage ADN-ADN

La figure I-9 montre la fréquence de ces différents dommages subis par la cellule lors d'une exposition à 1 Gy d'une source de radiation à faible TEL (Lehnert, 2007). Bien

que les lésions simples soient les plus nombreuses, ce sont les lésions complexes, incluant les bris double brin, qui sont principalement responsables de la mort cellulaire.

TABLE 6.1

Estimation of the Number of Early Physical and Biochemical Changes That Occur When Mammalian Cells Are Irradiated with 1 Gy of Low LET Radiation

Initial Physical Damage	
Ionization in the cell nucleus	~1000,000
Ionization directly in DNA	~2000
Excitation directly in DNA	~2000
Selected Biochemical Damage	
Damaged bases	1000–2000
Damaged sugars	1200
DNA single-strand breaks (SSBs)	1000
Alkali-labile sites	250
Double-strand breaks (DSBs)	40
DNA-protein cross-links (DPC)	150
Selected Cellular Effects	
Lethal events	~0.2–0.8
Chromosome aberrations	~1
Hypoxanthine phosphoribosyltransferase (Hprt) aberrations	10^{-5}

Figure I-9: Fréquence des dommages subis par l'ADN cellulaire. Source : (Lehnert, 2007, p 125)

Les lésions multiples comprennent les bris double brin, et sont majoritairement formés par un mécanisme bi-radicalaire (Milligan *et al.*, 1995), soit deux radicaux qui réagissent à proximité l'un de l'autre pour former deux lésions. Ces lésions sont particulièrement toxiques pour la cellule, car elles compliquent le processus de réparation.

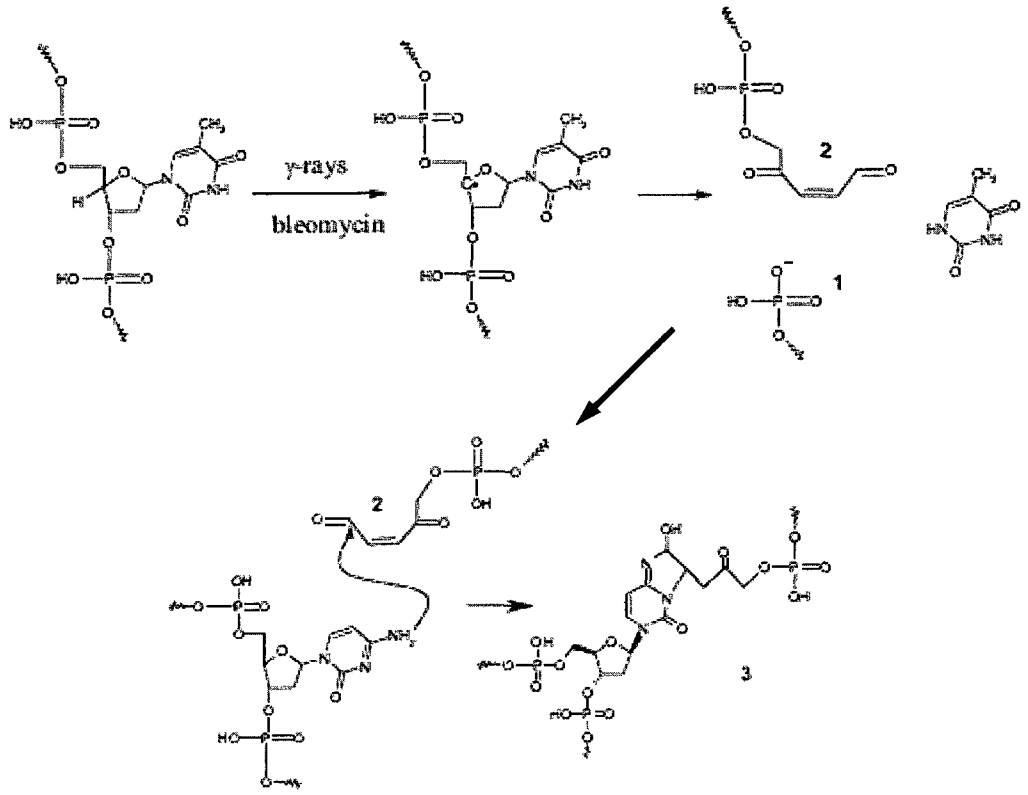

Figure I-10 : Mécanisme de formation d'un dimère interbrin par les radiations ionisantes. Modifié à partir de Regulus *et al.*, 2007. Suite à l'exposition de l'ADN aux radiations ionisantes, les radicaux hydroxyles issus de la radiolyse de l'eau font l'abstraction d'atomes d'hydrogène, ce qui entraîne la formation d'un radical sur le désoxyribose en position C4'. Ce radical mène ensuite à la formation du produit 2 par un processus de β-élimination, en plus de la libération de la base et de l'extrémité 3', ce qui produit une cassure franche. Une cytosine située sur le brin en face peut alors attaquer 2 pour former le dimère interbrin.

Dans le cas des pontages ADN-ADN, ce n'est que récemment qu'on a établi que ces dommages étaient produits par les radiations ionisantes, qui jusque-là n'avaient été observés qu'à la suite d'une exposition aux rayons UVA ou à des agents thérapeutiques comme la mitomycin C. En effet, les groupes de Cadet et Greenberg ont établi en 2007 qu'il y avait formation d'un dimère interbrin par les radicaux OH dans l'ADN synthétique et dans les cellules (Regulus *et al.*, 2007, Sczepanski *et al.*, 2009a). Ce dimère interbrin est d'autant plus intéressant qu'il inclut aussi une cassure simple brin, et s'inscrit donc dans la catégorie des lésions multiples, très

dommageables pour la cellule (figure I-10). De plus, le groupe de Greenberg a récemment montré que la réparation de ce dimère transitait par la formation d'une cassure double brin par le complexe UvrABC dans un système bactérien (Sczepanski *et al.*, 2009b).

Cependant, de façon générale, on en sait encore très peu sur la formation et la réparation des dimères interbrins causés par les radiations ionisantes, entre autres parce que leur identification dans la cellule est très récente. La majeure partie de l'information connue à ce jour a été recueillie à la suite d'une exposition à des agents thérapeutiques (souvent des agents alkylants bifonctionnels) ou aux rayons ultraviolets (UVA). Néanmoins, ces travaux ont déjà montré que la réparation des dimères interbrins nécessitait la coordination de plusieurs systèmes de réparation (Noll *et al.*, 2006) comme la recombinaison homologue et la réparation par excision des nucléotides (NER). De plus, l'analyse de la réparation des dimères interbrins est compliquée par le fait qu'ils n'altèrent pas la structure de l'ADN de façon identique; certains, comme la mitomycin C, ont peu ou pas d'effet sur la structure de la double hélice (Rajski et Williams, 1998), tandis que les dimères produits par le cis-dismminedichloroplatine créèent un renflement important dans l'ADN (désenroulement de 79°, fléchissement de 45°, Malinge *et al.*, 1994), ce qui rend le dimère plus susceptible d'être reconnu par la NER (Noll *et al.*, 2006). Ainsi, il reste encore beaucoup à faire afin de mieux comprendre comment les dimères interbrins sont formés par la radiation ionisante, comment ils sont réparés, et quelle est leur importance dans l'impact global de l'exposition de la cellule aux radiations.

I.3 – Le 5-Bromodéoxyuridine

I.3.1 – Structures et propriétés

Le 5-bromo-2'-désoxyuridine (BrdU) est un analogue halogéné de la thymidine qui peut s'incorporer dans l'ADN lors de la réplication et ainsi se substituer aux thymidines (figure I-11). Bien qu'il soit aujourd'hui surtout utilisé pour la détection de la réplication des cellules (Eisch et Mandyam, 2007, Leif *et al.*, 2004, Quinones-Hinojosa *et al.*, 2005), le BrdU a d'abord été développé en tant que photo- et radiosensibilisateur (Dewey et Humphrey, 1965, Sano *et al.*, 1968). L'intérêt pour le BrdU en tant que radiosensibilisateur découle de trois principales caractéristiques.

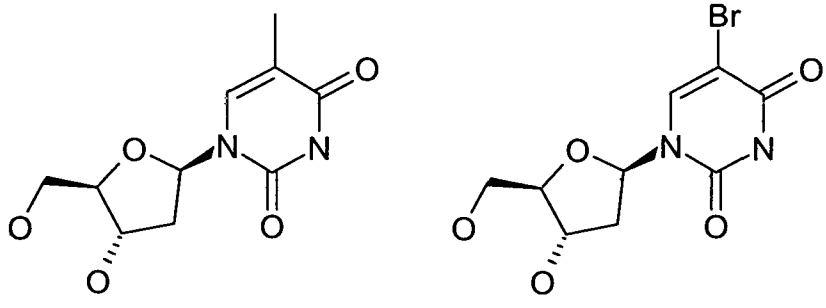

Figure I-11 : Structure de la désoxythymidine et du BrdU.

La première est que la taille du brome est semblable à celle du groupement $-CH_3$ de la thymidine (r = 0.195 et 0.20 nm, respectivement), ce qui lui permet d'être incorporé dans l'ADN cellulaire durant la réplication. La seconde est que le BrdU, une fois qu'il est incorporé dans l'ADN cellulaire, ne présente qu'un faible effet toxique; même lorsque 50% des thymidines sont remplacées par le BrdU, on ne constate qu'une faible diminution de la survie cellulaire (Iliakis *et al.*, 1989, Szybalski, 1974). Enfin, la troisième caractéristique du BrdU est que l'électronégativité du brome en fait un bon capteur d'électrons (k_{eaq} = 1.6 x 10^{10} dm^3 mol^{-1} s^{-1} à pH 7.0, Patterson et Bansal, 1972) et un bon groupement partant, ce qui permet la création d'un radical uridin-5-yl très réactif, notamment pour ce qui est de l'abstraction d'atomes d'hydrogène ($k_{2\text{-PrOH}}$ = 4.1 x 10^7 dm^3 mol^{-1} s^{-1}, Mertens et Sonntag, 1994). La figure I-12 décrit le mécanisme de formation d'une cassure à l'ADN causée par l'exposition aux rayons UV d'un ADN bromé. Le mécanisme est généralement supposé être semblable pour une irradiation aux rayons ionisants, notamment en ce qui a trait à l'abstraction d'un atome d'hydrogène sur le sucre du nucléotide en 5'.

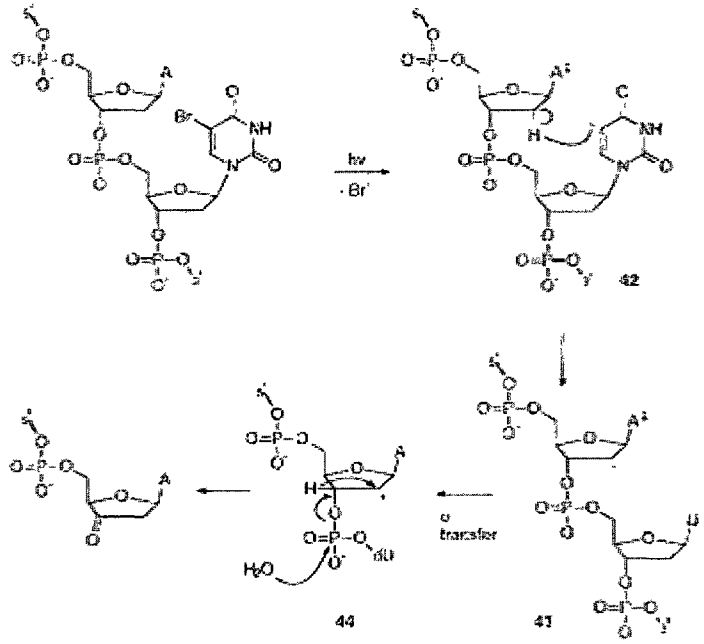

Figure I-12 : Mécanisme de formation des cassures dans l'ADN bromé exposé aux rayons UV. À la suite de l'exposition aux rayons UV (313 nm), un électron est transféré de la base en 5′ du BrdU. Il y a ensuite départ de l'anion brome et formation d'un radical uridine-5-yl. Ce radical fait l'abstraction d'un atome d'hydrogène sur la position C1′ du sucre en 5′ (C2′ dans le cas de la radiation ionisante). Il y a ensuite transfert d'un hydrogène et formation d'une cassure par la libération de l'extrémité 3′ du désoxyribose.

D'autres analogues halogénés de la thymine sont aussi de bons capteurs d'électrons, dans l'ordre suivant : fluorouracile < chlorouracile < bromouracile. Cependant, le 5-fluorouracile n'est pas incorporé dans l'ADN, et le chlorouracile forme moins de radical uracil-yl que le bromouracile (Bansal *et al.*, 1972, Patterson et Bansal, 1972). Pour cette raison, c'est surtout le BrdU qui a fait l'objet d'études de radiosensibilisation.

I.3.2 – Radiosensibilisateur : essais précliniques

Il y a déjà plus de 40 ans que le BrdU a été identifié en tant que radiosensibilisateur (Dewey et Humphrey, 1965). En effet, l'ADN cellulaire où une partie des thymidines a été remplacée par le BrdU subit une augmentation des cassures simples et doubles (Limoli et Ward, 1993, Saffhill et Ockey, 1985) et des aberrations chromosomiques (Dewey *et al.*, 1966) après une exposition aux radiations ionisantes. Le mécanisme de radiosensibilisation de l'ADN bromé passe principalement par l'attachement dissociatif d'un électron sur le BrdU, puis par le départ d'un anion brome et la formation du radical uridin-5-yl (Wang *et al.*, 2006). Comme il a été mentionné plus haut, ce radical est très réactif, et va donc faire l'abstraction d'un atome d'hydrogène sur le C1′ ou le C2′ du déoxyribose situé en 5′ du BrdU pour donner l'uridine (Schyman *et al.*, 2007). Le ribose radicalaire ainsi créé mène ensuite à la formation de cassures. À l'origine, on croyait que seuls les électrons aqueux issus de la radiolyse de l'eau étaient responsables de la formation de cassures, mais des données récentes du groupe de Léon Sanche ont montré qu'il était possible que les électrons de basse énergie interviennent aussi dans le mécanisme de radiosensibilisation (Abdoul-Carime *et al.*, 2000b, Dugal *et al.*, 2000, Li *et al.*, 2003). Toutefois, il est important de noter que la contribution des électrons aqueux à la radiosensibilisation de l'ADN bromé dans les cellules est probablement la plus importante, puisqu'on assiste à la disparition de la majorité du nombre de cassures double en présence d'acétone, un capteur d'électrons aqueux (Webb *et al.*, 1993).

D'autres facteurs affectent la radiosensibilisation des cellules par le BrdU, notamment le pourcentage de remplacement de la thymidine (Ling et Ward, 1990), la structure de la chromatine (Lawrence *et al.*, 1995), le temps de doublement de la lignée cellulaire, ainsi que la phase de croissance (exponentielle vs plateau) où se produit l'irradiation (Iliakis *et al.*, 1989, Wang *et al.*, 1994). La présence ou l'absence d'oxygène influence aussi grandement la production de dommages, d'abord parce que l'oxygène agit à titre de capteur d'électrons (k_{eaq} = 1.9 x 10^{10} dm^3 mol^{-1} s^{-1}, Buxton *et al.*, 1988), mais aussi parce que l'oxygène a démontré une influence importante sur le type de dommage produit, notamment en fixant les dommages causés à l'ADN par l'addition d'un radical superoxide. Tous ces facteurs et bien d'autres ont joué un rôle important dans

l'établissement des protocoles cliniques utilisant le BrdU en tant que radiosensibilisateur (Coleman et Mitchell, 1999, McGinn et Kinsella, 1992).

I.3.3 – Radiosensibilisateur : essais cliniques

Le but premier de la radiothérapie est de cibler spécifiquement les cellules cancéreuses, tout en épargnant les cellules saines environnantes. Puisque la cible principale des radiations est l'ADN, beaucoup de radiosensibilisateurs ont été développés au cours des dernières années qui ciblent principalement l'ADN des cellules cancéreuses. À l'origine, l'utilisation du BrdU en tant que radiosensibilisateur utilisé dans le traitement du cancer a soulevé beaucoup d'intérêt, car c'est l'un des rares à s'incorporer dans l'ADN cellulaire. Puisque le BrdU est incorporé durant la réplication, la théorie voulait que les cellules tumorales, qui se divisent plus rapidement, incorporent plus de BrdU dans leur ADN et soient donc plus sensibilisées que les cellules saines environnantes. Bien que les essais cliniques aient montré des résultats encourageants quant à l'efficacité des pyrimidines halogénées en tant que radiosensibilisateur (Kinsella *et al.*, 1984, Levin *et al.*, 1995, Phillips *et al.*, 1991, Urtasun *et al.*, 1996), certains problèmes ont été révélés. Entres autres, la photosensibilité accrue des patients a été traitée en minimisant l'exposition au soleil et en prescrivant une crème protectrice topique. La déhalogénation du BrdU par le foie a été minimisée en injectant directement le BrdU dans la région de la tumeur. Cependant, deux facteurs sont demeurés problématiques au cours des différents essais cliniques : le peu de connaissances concernant la vitesse de réplication des cellules saines environnantes, ainsi que l'incapacité de mesurer facilement l'incorporation réelle du BrdU dans les cellules. Ce premier facteur pourrait expliquer pourquoi l'un des premiers essais cliniques a montré un effet positif pour la combinaison du BrdU et de la radiation (Sano *et al.*, 1965), alors qu'un autre n'a montré aucun effet (Bagshaw *et al.*, 1967), en plus de noter une augmentation du dommage aux tissus normaux. Dans ce dernier cas, le champ d'irradiation couvrait aussi la muqueuse de la cavité

buccale et du pharynx, qui se divise plus rapidement que les cellules tumorales. Ainsi, les cellules saines ont incorporé plus de BrdU que les cellules tumorales. Une autre étude de phase I a utilisé une approche inusitée pour une tumeur entourée de cellules saines qui se divisaient plus rapidement (Eisbruch *et al.*, 1999). En effet, ces cellules incorporent plus de BrdU et seraient donc normalement plus sensibilisées aux radiations que les cellules tumorales. Cependant, pour la même raison, le BrdU est dilué plus rapidement lorsque l'infusion est stoppée, tandis que les cellules tumorales maintiennent encore un taux de remplacement élevé plusieurs jours après l'arrêt, au moment où est prévue l'irradiation.

Plusieurs études ont montré un effet positif de l'utilisation du BrdU en tant que radiosensibilisateur sur la survie des patients atteints de différents types de cancer. Cependant, une étude de phase III parue en 2004, où le BrdU était utilisé en combinaison avec un agent chimiothérapeutique, n'a pas réussi à montrer une amélioration de la survie des patients (Prados *et al.*, 2004). Après la parution de cette étude, les travaux sur l'utilisation du BrdU en clinique ont à toute fin cessés.

I.3.4 – Réévaluation du mécanisme de radiosensibilisation du BrdU

Malgré l'arrêt des travaux cliniques sur le BrdU, l'intérêt pour le radiosensibilisateur est demeuré présent, entres autres parce que c'est l'un des rares agents à sensibiliser directement l'ADN, et aussi à cause de son faible niveau de toxicité. C'est pour cette raison que notre groupe a entrepris de réévaluer le mécanisme moléculaire de radiosensibilisation de l'ADN par le BrdU, en utilisant un modèle simple constitué de courts oligonucléotides synthétiques. C'est ainsi qu'en 2004, notre groupe a montré que l'effet radiosensibilisateur du BrdU dépendait de l'état d'hybridation de l'ADN. En effet, après irradiation dans des conditions hypoxiques, nous avons observé un plus grand rendement au niveau de la formation de cassures dans un ADN simple brin où une thymidine avait été substitué par un BrdU que dans un ADN double brin

(Cecchini *et al.*, 2004). Plus étonnant encore, la présence d'un mésappariement de 5 bases autour du site de modification favorisait la production de cassures par rapport à un ADN double brin, et entraînait la formation d'un nouveau type de dommage, qui n'avait jamais été observé jusque là pour un ADN bromé : le dimère interbrin (Cecchini *et al.*, 2005).

Ainsi, mon projet avait pour but d'approfondir nos connaissances sur la formation du dimère interbrin dans l'ADN bromé soumis aux radiations ionisantes, avec une attention particulière sur l'importance de la structure et de la séquence de l'ADN au site de la substitution. Cette information permettrait non seulement de mieux comprendre le mécanisme de radiosensibilisation du BrdU pour un éventuel recommencement des essais cliniques, mais fournirait aussi de l'information sur la réactivité des régions mésappariées de l'ADN. Les résultats obtenus sont présentés dans les sections suivantes.

Chapitre II – 1er article

5-Bromodeoxyuridine Radiosensitization: Conformation-dependent DNA damage.

Auteurs de l'article : Marie-Eve Dextraze, J. Richard Wagner and Darel J. Hunting

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Avant-propos :

Contribution à la rédaction et à l'expérimentation :

J'ai effectué la totalité des expériences présentées dans cet article. J'ai aussi rédigé cet article, sous la supervision de mes co-directeurs, Richard Wagner et Darel Hunting.

Résumé :

Dans cet article, nous présentons l'effet de la conformation de l'ADN sur la production de dommage à l'ADN bromé. À l'aide d'un modèle expérimental où l'ADN est déshydraté puis graduellement réhydraté, nous montrons que la forme B de l'ADN est nécessaire pour la production de bris et de cassures, la forme A ne produisant que des lésions qui peuvent être révélées par un traitement alcalin. Cet article vient appuyer notre hypothèse, qui soutient que l'environnement où le radical initial est créé – et plus particulièrement la structure de l'ADN dans cet article – lors

de l'irradiation de l'ADN a un effet important sur la létalité du dommage qui est produit.

5-Bromodeoxyuridine Radiosensitization: Conformation-Dependent DNA Damage

† *This work was supported by the National Cancer Institute of Canada.*

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ABBREVIATIONS A, Adenine; AB, bromodeoxyuridine-substituted oligonucleotide; AT, unsubstituted oligonucleotide; BrdU, 5-bromodeoxyuridine; CldC, 5-chlorodeoxycytidine; CldU, 5-chlorodeoxyuridine; dCMP, deoxycytidine monophosphate; DSBs, double strand breaks; DSc, complementary double stranded; DSsc, semi-complementary double stranded; $dU^{\cdot}$, Deoxyuridinyl radical; e^{-}, electron; e_{aq}, solvated electron; e_{kin}, kinetic electron; EDTA, ethylenediaminetetraacetic acid; Γ, Gamma (H_2O/nucleotide); ICL, Interstrand cross-link; als, alkali-labile DNA lesion; sb, strand break; SS, single stranded; T, thymine; TA, complementary oligonucleotide; UHV, ultrahigh vacuum.

ABSTRACT DNA structure has recently emerged as one of the key factors governing the ability of 5-bromodeoxyuridine (BrdU) to radiosensitize DNA. Here, we report the dependence of the specific damage induced by BrdU for different DNA conformations. Strand breaks are specific for B-form DNA, whereas A-DNA only undergoes formation of piperidine-sensitive DNA lesions. Interstrand cross-links are only found in semi-complementary B-DNA. DNA conformation was altered by gradually rehydrating lyophilized DNA samples, which induces an A- to B-form transition. These results were also validated by irradiating DNA in solution, in the presence or absence of 80% ethanol to induce an A- or B-form, respectively. Alkali-labile DNA lesions were revealed using hot piperidine to transform both base and sugar lesions into strand breaks. We also analyzed the location of damage as a function of DNA structure: piperidine-sensitive lesions were observed exclusively at the site of BrdU substitution, whereas strand breaks were able to migrate along the DNA strand, with a clear preference for the adenine 5′ of the BrdU. Thus, not only the hybridization state but also the DNA conformation affect the degree of sensitization by BrdU by influencing the amount and type of damage produced. Although clinical trials using BrdU as a radiosensitizer have been disappointing up to this point, these new findings point to several key features of BrdU radiosensitization that may alter future radiotherapeutic studies.

5-bromodeoxyuridine (BrdU), an analogue of thymidine, radiosensitizes cells (1-2) leading to single- and double strand breaks (3-4), chromosomal aberrations (5) and cell death. The mechanism for single strand break formation involves electron attachment to BrdU, followed by the departure of a bromide anion and generation of a uracil-5-yl radical that further reacts to create strand breaks. Although the radiosensitizing activity of BrdU was discovered more than forty years ago, clinical studies have given disappointing results (6-10), failing to show a survival advantage for patients with a range of tumor types. Nevertheless, the relatively low toxicity of BrdU as well as its rare ability to directly radiosensitize DNA suggest that further studies are warranted. We therefore decided to re-explore the molecular basis of BrdU radiosensitization in order to reach a better understanding of the conditions that favor BrdU-related damage, and especially the structural requirements to maximize DNA sensitization by BrdU.

Changes in DNA structure with increasing levels of hydration have been thoroughly documented. Variations in structure (11-13), compaction, (14-15) and reactivity to ionizing radiation (16-21) as a function of hydration have been extensively studied in the last decades. At low levels of hydration ($0 < \Gamma < 6$, where Γ represents the number of water molecules per nucleotide), Na-DNA adopts a B-like conformation, which changes to an A-form at higher levels ($6 < \Gamma < 20$). At these lower levels of hydration, radiation can ionize the water of hydration, but the initial ionization holes appear to be transferred to DNA in what is described as the quasi-direct effect. Thus, hydroxyl radicals are not observed below $\Gamma \approx 9$ (22-23), in what corresponds to the inner primary hydration shell. Hydroxyl radical formation has been identified in the outer primary hydration shell, which contains an additional 11–12 moles of water per mole of nucleotide. The secondary hydration shell is formed at higher levels of hydration ($\Gamma > 20$), and is indistinguishable from bulk water (12, 17-18, 22).

The importance of DNA structure for sensitization by BrdU has already been demonstrated by our group (24-26). It was shown that hybridization decreased

sensitization by up to 20-fold compared to that of single stranded DNA. Moreover, a hybridized BrdU-substituted oligonucleotide with a 5-base mismatch produced an interstrand cross-link (ICL) that was dependent on the presence of a mismatch. Here, we examine other aspects of DNA conformation that affect BrdU sensitization. First, we increased the hydration level, thereby changing DNA conformation, and found that strand breaks for BrdU-substituted DNA are specific for B-form DNA, and are not found in A-form DNA. We also investigated the effect of DNA conformation in solution by using ethanol to dehydrate DNA and produce an A-conformation. For both experimental approaches, irradiation of A-DNA produced BrdU-specific alkali-labile lesions, as revealed by treatment with hot piperidine, whereas BrdU-specific strand breaks were only found in B-DNA. ICL production also displayed the same specificity for the B-conformation.

Description	Sequence	Abbreviation
Non brominated single stranded oligonucleotide	5' C-G-A-G-T-A-C-T-G-C-A-A-T-A-A-C-G-T-G-T-A-C-A-G-C 3'	SS AT*
Non brominated complementary oligonucleotide	5' C-G-A-G-T-A-C-T-G-C-A-A-T-A-A-C-G-T-G-T-A-C-A-G-C 3' 3' G-C-T-C-A-T-G-A-C-G-T-T-A-T-T-G-C-A-C-A-T-G-T-C-G 5'	DSc AT*//TA
Non brominated semi-complementary oligonucleotide	5' C-G-A-G-T-A-C-T-G-C-A-A-T-A-A-C-G-T-G-T-A-C-A-G-C 3' 3' G-C-T-C-A-T-G-A-C-G-\A-A-T-A-A/G-C-A-C-A-T-G-T-C-G 5'	DSsc AT*//AT
Brominated single stranded oligonucleotide	5' C-G-A-G-T-A-C-T-G-C-A-A-B-A-A-C-G-T-G-T-A-C-A-G-C 3'	SS AB*
Brominated complementary oligonucleotide	5' C-G-A-G-T-A-C-T-G-C-A-A-B-A-A-C-G-T-G-T-A-C-A-G-C 3' 3' G-C-T-C-A-T-G-A-C-G-T-T-A-T-T-G-C-A-C-A-T-G-T-C-G 5'	DSc AB*//TA
Brominated semi-complementary oligonucleotide	5' C-G-A-G-T-A-C-T-G-C-A-A-B-A-A-C-G-T-G-T-A-C-A-G-C 3' 3' G-C-T-C-A-T-G-A-C-G-\A-A-T-A-A/G-C-A-C-A-T-G-T-C-G 5'	DSsc AB*//AT

Figure II-1 : Sequences of the brominated (B) and non-brominated (T) oligonucleotides. The asterisk (*) indicates the labeled strand. The hybridization state is indicated as follows: single-stranded (SS), double stranded, complementary (DSc), and semi-complementary (DSsc).

MATERIALS AND METHOD

Oligonucleotides

5-bromodeoxyuridine modified and nonmodified oligonucleotides were purchased from the University Core DNA Services (University of Calgary, AB, Canada). Sequences are shown in Figure II-1. Oligonucleotides were end-labeled with ^{32}P [γ-ATP] using T4 polynucleotide kinase (Amersham Pharmacia biotech). Labeling was carried at an initial oligonucleotide concentration of 1 μM for 45 min at 37 °C with 10 U of kinase. The enzyme was inactivated by heating for 10 min at 75 °C. Oligonucleotides were diluted to 400 nM and purified on a G50 Sephadex microcolumn. Hybridization was carried out at a final concentration of 100 nM of the labeled strand and a 2-fold excess of the unlabeled strand. Samples were heated to 82 °C for 5 min then cooled slowly for 3 h. Hybridization controls were carried out as described in Cecchini et al. (25). Deionized, sterile water was used in all experimental protocols.

Hydration of DNA samples

Aliquots of the initial labeling reaction were diluted to a final concentration of 20 nM in phosphate buffer (10 mM, pH 7.5). DNA samples were pipetted into 0.5 mL Eppendorf tubes and dried using a Speedvad evaporator for 45 min, and then hydrated by placing the tubes in scintillation vials containing various saturated solutions, in order to control the relative humidity. Scintillation vials contained roughly 0.5 g of crystals and 4 mL of the corresponding saturated solution. Samples were hydrated for 24 h at 4 °C before irradiation. Saturated solutions yielded levels of relative humidity similar to those reported by Stokes (27) and were measured using a VWR

Hygrometer: K_2CO_3 (≈ 45%), NaCl (≈ 76%), KCl (≈ 84%), H_2O (≈ 99%). The relationship between relative humidity and Γ, as determined by Huttermann (14, 16), was used in the present study. In addition, this relationship between steady-state hydration levels and relative humidity was confirmed by our group using plasmid DNA (28). Under these conditions, control samples of dried, unirradiated samples showed less than 1% breaks. Control samples of deoxygenated DNA solutions were irradiated in the presence of 50 mM EDTA (pH 8.0) in order to prevent hydroxyl radical degradation, leaving only BrdU-specific strand breaks, as described in Cecchini et al. (25).

Ethanol experiments

Single stranded (SS AB* and SS AT*), double stranded (DSc AB*//TA and DSc AT*//TA), and hybridized semi-complementary (DSsc AB*//AT and DSsc AT*//AT) oligonucleotides (20 nM final concentration, in 10 mM phosphate buffer at pH 7.5) were deoxygenated by bubbling for 1 min with N_2 and irradiated in water or 80% ethanol. Certain experiments were conducted in the presence of O_2 or N_2O to scavenge solvated electrons. All samples contained EDTA (25 mM, pH 8.0) to scavenge hydroxyl radicals, whether ethanol was present or absent.

Irradiation and treatment of DNA samples

DNA was irradiated in a Gammacell 220 (^{60}Co) with either 300 or 2400 Gy at a dose/rate of 3.06 Gy/min. After irradiation, hydrated samples were redissolved at their original concentration by adding 20 μL of water and gently pipetting the solution to resuspend the DNA. Samples from ethanol experiments were dried with a Speedvac and resuspended in 20 μL of water. For both experiments (hydration and ethanol), 10 μl was taken from each sample and treated with 10% hot piperidine (30 min at 90 °C) to reveal alkali-labile DNA lesions.

Gel electrophoresis and analysis

Samples were loaded on a 20% denaturing (7 M urea) polyacrylamide gel (35 × 43 cm). A molecular weight ladder was generated by a G+A Maxam & Gilbert sequencing treatment (29). Electrophoresis was carried out at 40 W for 2 h 45, with a 30 min pre-run at 45 W. The gel was exposed overnight in a Phosphor Screen cassette (Molecular Dynamics, Inc.), and scanned with a fluorescence scanner (Storm, Molecular Dynamics Inc.). The bands were quantified using ImageQuant software (Molecular Dynamics) as described in Cecchini et al. (24).

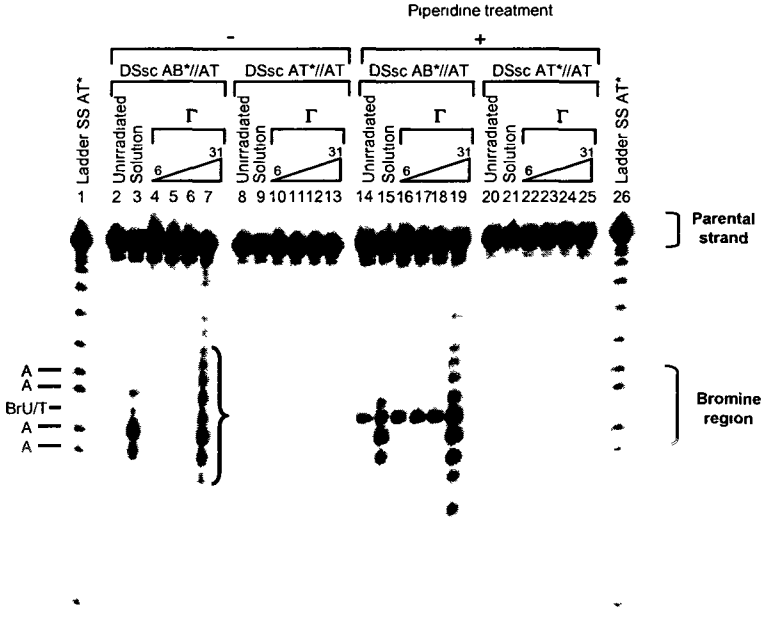

Figure II-2 : Strand breaks and base lesions as a function of hydration. Hybridized, semi-complementary DNA, unsubstituted (DSsc AT*//AT) or substituted (DSsc AB*//AT) with BrdU was irradiated with increasing levels of hydration (from $\Gamma \approx 6$ to $\Gamma \approx 31$). Lanes 2, 8, 14 and 20 are unirradiated controls, whereas lanes 3, 9, 15, and 21

are positive controls, irradiated in solution under a nitrogen atmosphere with EDTA in phosphate buffer. Lanes 14 to 25 were treated with hot piperidine to reveal alkali-labile DNA lesions. (Note: the portion of the gel between the wells and the parental oligonucleotide is not shown)

RESULTS

Hydration and BrdU sensitization

We first examined the role of DNA structure in BrdU sensitization by gradually increasing the DNA hydration level from $\Gamma \approx 6$ to $\Gamma \approx 31$. Although BrdU is a well-known radiosensitizer in solution (30-32) and in cells (33-35), we found no BrdU-specific strand breaks when a hybridized, semi-complementary oligonucleotide (DSsc AB*//AT, Figure II-1) was hydrated between $\Gamma \approx 6$ and $\Gamma \approx 21$ (Figure II-2, lanes 4–6 and 10–12 and Figure II-3). Specific BrdU sensitization (strand breaks and ICLs) was only found when the hydration level reached $\Gamma \approx 31$ (Figure II-2, lane 7), where there was a 5-fold increase in damage in DNA substituted with BrdU, compared to that in unsubstituted DNA (Figure II-3). BrdU-specific ICLs were only found at $\Gamma \approx 31$ (not shown). Because it has been reported that dehalogenation (36-37), uracil-5-yl radical production (38-41), and base fragmentation (42-43) occur in dehydrated BrdU-substituted DNA, we decided to test whether other types of BrdU specific damage occurred at lower hydration levels. Thus, we treated irradiated DNA with hot piperidine to reveal alkali-labile DNA lesions.

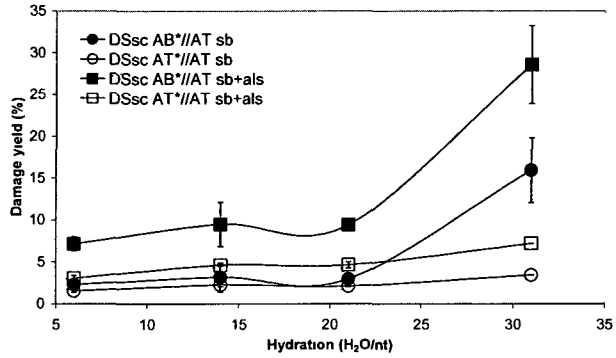

Figure II-3 : Damage yield as a function of DNA hydration. Strand breaks (sb; circles: ○, ●) and strand breaks + alkali-labile DNA lesions (sb + als); squares: □, ■) were measured for unsubstituted DNA (DSsc AT*//AT, open symbols: ○, □) and BrdU-substituted DNA (DSsc AB*//AT, filled symbols: ●, ■) in phosphate buffer.

Treatment with hot piperidine revealed DNA lesions that were created between $\Gamma \approx 6$ and $\Gamma \approx 31$ only in substituted DNA (lanes 14-19 of Figure II-2 and Figure II-3). Although treatment with piperidine revealed low levels of DNA lesions present in unirradiated substituted oligonucleotides (Figure II-2, lane 14), irradiation substantially increased the amount of DNA damage. Both strand breaks and alkali-labile lesions are quite specific for BrdU because irradiation of unsubstituted oligonucleotides induced strand breaks in fewer than 4% of the molecules under these conditions, compared to 16% with substituted DNA (Figure II-3). The only notable damage not specific to BrdU was the creation of alkali-labile DNA lesions at the central thymidine between $\Gamma \approx 14$ and $\Gamma \approx 31$ (Figure II-2, lanes 23-25). Although BrdU-specific degradation at $\Gamma \approx 31$ is concentrated in the mismatch region (Figure II-2, lane 7), leaving the double stranded portion of the oligonucleotide relatively unharmed, it extends farther on either side of the mismatch than in oligonucleotides irradiated in solution (lane 3). To further investigate this, we examined damage localization as a function of DNA sequence. Panel A of Figure II-4 shows a clear bias for strand scission on the adenine 5′ of the BrdU, both in solution and in hydrated DNA, in accordance with previous studies (24). The second adenine 5′ of the BrdU (i.e., AABrU) is also affected, albeit to a lesser extent. Damage migration 3′ of the

BrdU was also observed, but occurred predominantly in hydrated DNA (Figure II-4, panel A). Treatment with hot piperidine revealed that alkali-labile DNA lesions arise virtually exclusively at the BrdU site (Figure II-4, panel B).

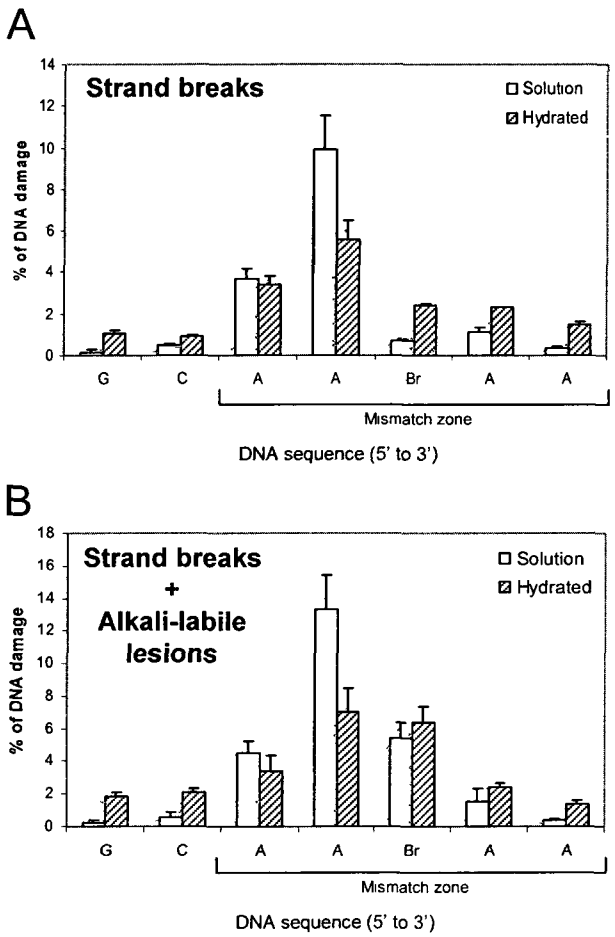

Figure II-4 : Damage as a function of DNA sequence. The relative yield of damage was measured for each base of the mismatch (AABrAA, see Figure II-1 for the complete sequence) and for two bases 5′ of the mismatched, substituted oligonucleotide (DSsc AB*//AT). Strand breaks (panel A) and strand breaks + alkali-labile DNA lesions (panel B) were measured for both solution and hydrated DNA ($\Gamma \approx 31$, in phosphate buffer). Base lesions and sugar damage were revealed using hot piperidine. The background signal was removed by subtracting damage at each nucleotide of the unsubstituted oligonucleotide (DSsc AT*//AT).

The role of DNA structure in BrdU sensitization in solution

Single stranded (SS AB* and SS AT*, Figure II-1), double stranded (DSc AB*//TA and DSc AT*//TA, Figure II-1), and semi-complementary (DSsc AB*//AT and DSsc AT*//AT, Figure II-1) oligonucleotides were irradiated in the presence or absence of 80% ethanol to induce an A- or B-form DNA, respectively. Single stranded oligonucleotides substituted with BrdU showed no evidence of conformation dependent strand break formation (Figure II-5, panel A). However, for hybridized, semi-complementary DNA, an 8-fold increase in strand breaks in the BrdU region was observed when BrdU-DNA was in a B-form rather than in A-form (Figure II-5, panel C). Similar results were obtained for double stranded DNA (not shown). Treatment with hot piperidine exposed a similar tendency for alkali-labile DNA lesions. ICLs were also specific to B-form DNA, with a 4-fold increase (Figure II-6) compared to A-DNA. Substitution with BrdU always produced substantially more damage than in unsubstituted oligonucleotides, under all conditions (Figure II-5, panels B and D). Irradiation in the presence of an electron scavenger (N_2O ; $k_e = 9.1 \times 10^9$ L mol^{-1} s^{-1} (44)) reduced the percentage of damaged molecules from 9.7 ± 0.3 to 3.7 ± 0.1% (after correction for unirradiated DNA) following a dose of 2400 Gy. In the presence of ethanol, a hydroxyl radical scavenger ($k_e = 1.9 \times 10^9$ (44)), N_2O induced a further reduction in strand break formation from 1.2 ± 0.3% to 0.4 ± 0.2%. When DNA was irradiated with 300 Gy in the presence of another electron scavenger, O_2 ($k_e = 1.9 \times 10^{10}$ L mol^{-1} s^{-1} (44)), a similar reduction was observed from 3.5 ± 1.2% with N_2 to 0.7 ± 0.8% with O_2. Under these latter conditions, strand break levels were indistinguishable from unirradiated samples. At 300 Gy, addition of ethanol had no effect on radioinduced damage (0.4 ± 0.5% and 0.4 ± 0.7% without or with O_2, respectively). Evidently, the presence of 25 mM EDTA in all samples efficiently reduced hydroxyl radical attack because total degradation of non-substituted DNA was less than 6% following a dose of 2400 Gy, compared to more than 60% in the absence of any hydroxyl radical scavenger (not shown).

DISCUSSION

Hydration and BrdU sensitization

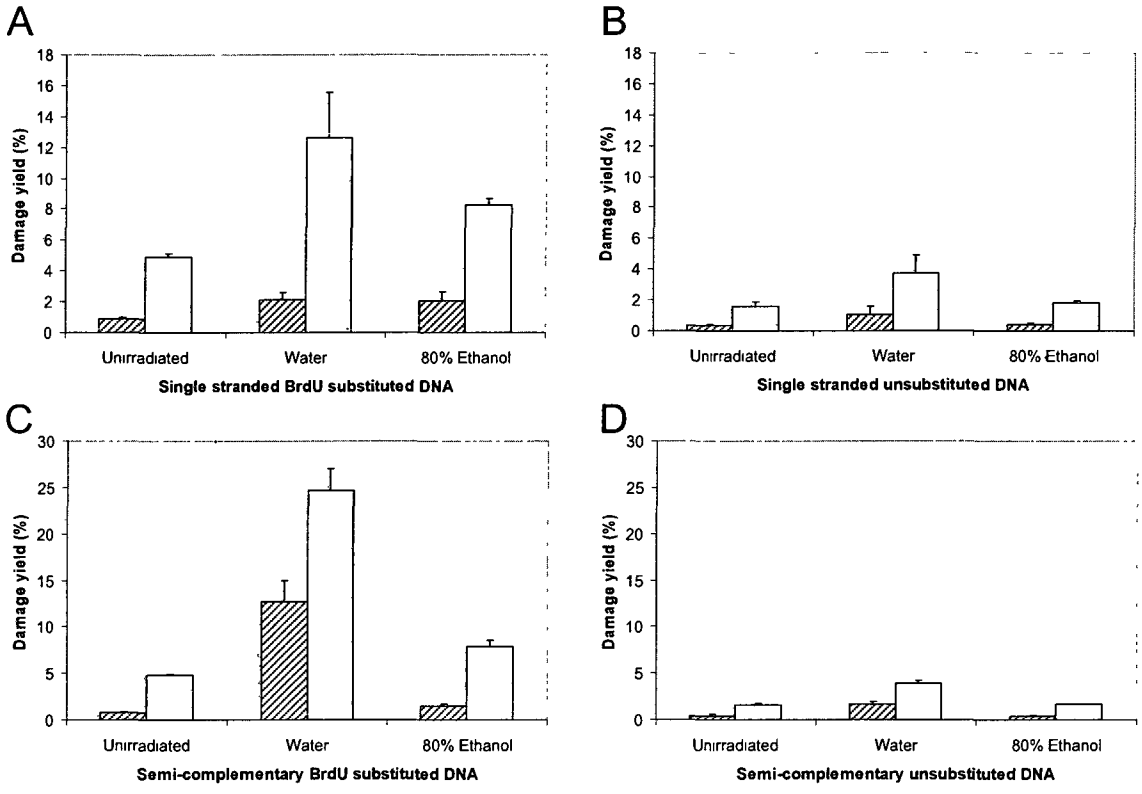

Figure II-5 : Damage yield in the mismatch zone as a function of DNA structure. Strand breaks (hatched) and strand breaks + alkali-labile DNA lesions (plain) were measured in the mismatch zone in the absence or presence of 80 % ethanol for single stranded DNA with (SS AB*, panel A) or without (SS AT*, panel B) BrdU and hybridized, semi-complementary DNA with (DSsc AB*//AT, panel C) or without (DSsc AT*//AT, panel D) BrdU. DNA lesions (base lesions and sugar damage) were revealed using hot piperidine. Double stranded DNA adopts an A conformation in 80% ethanol and a B conformation in aqueous solution.

When a hybridized, semi-complementary oligonucleotide is irradiated at increasing levels of hydration, the frequency of strand breakage in BrdU-substituted DNA is indistinguishable from non-substituted DNA between $\Gamma \approx 6$ and $\Gamma \approx 21$. Specific BrdU sensitization (strand breaks + ICL) is only found at $\Gamma \approx 31$. In contrast, when

irradiated DNA is treated with hot piperidine, alkali-labile DNA lesions that are specific for BrdU-substituted DNA are revealed over the entire hydration range (Figure II-2, lanes 16-19, and Figure II-3). Such evidence of DNA lesions under low hydration conditions is in agreement with previous results obtained under Ultra High Vacuum (UHV) (42-43). However, in these previous studies, no evidence of strand breaks was detected, most probably because irradiation under UHV precluded any hydration of DNA. Our experimental system indicates that a striking change in reactivity occurs between $\Gamma \approx 21$ and $\Gamma \approx 31$, leading to strand breaks as well as alkali-labile DNA lesions when DNA is in a higher hydration state.

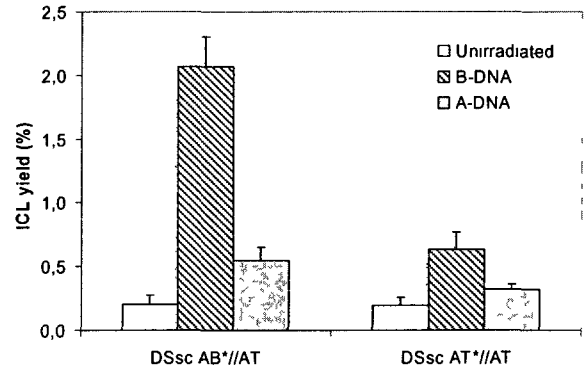

Figure II-6 : Interstrand cross-link yield as a function of DNA structure. Interstrand cross-links (ICLs) were measured in the absence (B-DNA) or presence (A-DNA) of 80% ethanol for mismatched DNA, brominated semi-complementary DNA (DSsc AB*//AT), or non-brominated semi-complementary DNA (DSsc AT*//AT), respectively.

DNA adopts an A-form between 45 ($\Gamma \approx 6$) and 90% ($\Gamma \approx 20$) of relative humidity, which shifts to a B-form when hydration is increased (11-14). Because BrdU sensitization has been shown to be extremely dependent on the hybridization state and on the presence of a mismatched region, we propose that the change in reactivity observed between $\Gamma \approx 21$ and $\Gamma \approx 31$ occurs because of a conversion from A-form to B-form DNA. However, it should be noted that at $\Gamma \approx 21$, the conversion to B-form is already relatively complete and thus one would expect to observe strand breaks at this level of hydration, according to the hypothesis stated above. However, the hydration

levels reported here were not measured by us, but were assumed on the basis of the measurement of relative humidity and the hydration levels published for these values. The use of synthetic oligonucleotides rather than plasmid DNA may produce slightly different levels of hydration. Plasmid or cellular DNA, even when every care is taken to purify it, often contains contaminants (e.g., proteins and Tris buffer) originating from the extraction protocol or the storage conditions. The presence of other molecules could affect the measurement of the hydration levels of plasmid DNA. Therefore, it is possible that the reason no strand breaks are observed at $\Gamma \approx 21$ is because the hydration level is slightly lower than $\Gamma \approx 20$, where DNA would mostly still be in A-form. For this reason, we proceeded to validate our hypothesis by inducing an A-form DNA in solution using ethanol, to verify the effect of conformation on DNA radiosensitization by BrdU.

Damage localization and DNA structure

It is well known that strand breaks occur predominantly at the nucleotide 5′ to the BrdU. This is because the pathway leading to strand breaks involves hydrogen abstraction from the 2′-deoxyribose moiety of the base 5′ to BrdU (45). When we examined strand break location as a function of DNA structure, we observed a similar tendency for both dissolved and hydrated DNA ($\Gamma \approx 31$, Figure II-4, panel A), although damage spread farther in the hydrated sample (Figure II-2, lane 7) than in solution (lane 2). In the former case, the limited availability of water molecules probably allows radicals to migrate farther along the DNA strand before being trapped by H_2O. Remarkably, piperidine-sensitive DNA lesions occurred exclusively at the site of BrdU substitution (Figure II-4, panel B). Several factors other than conformation may explain this observation; a higher hydration level may favor protonation of the uracil-5-yl radical and creation of a radical cation, thus allowing migration of the damage. However, no preference for the G's near the initial damage site was observed, as would be expected if this were the case. In addition, the greater mobility and wobbling afforded by a higher hydration level of DNA may facilitate

charge transfer of radicals to distant bases (46). This could also explain why a Gaussian distribution of DNA damage is observed (Figure II-4, panel B) when all types of damage (strand breaks + alkali-labile DNA lesions) are taken into account. The increase in mobility generated by the formation of a mismatch zone (24) has already been proposed by our group to be responsible for the increase in strand breaks and the production of interstrand cross-links observed in mismatched DNA. Indeed, the mobility of the DNA bases is likely to affect the ability of any radical created in DNA to react and migrate, and higher levels of hydration greatly increase the mobility of DNA compared to that of solid-state DNA. However, hydration also induces a change in conformation that affects the number of potential donors and acceptors near the radical created at the BrdU site. Therefore, it becomes extremely difficult to distinguish between the two factors. Because several factors can indeed affect the chemistry of solid-state DNA and for the reasons cited in the previous section, we proceeded to examine the effect of conformation on the radiosensitization of BrdU-substituted DNA in solution.

The role of DNA structure for BrdU sensitization in solution

It is well established that ethanol will cause a conformational change in DNA from the canonical B-DNA to A-DNA (47). In light of our results with hydrated DNA, we examined the effect of conformation for BrdU-substituted DNA in solution. In this system, 25 mM EDTA ($k_{OH} = 4.0 \times 10^8$ L mol^{-1} s^{-1} (48)) was added to each sample to scavenge hydroxyl radicals and thus to reduce both random breakage of our DNA as well as site-specific strand breakage resulting from hydroxyl radical attack on BrdU. Therefore, the majority of strand breaks produced by irradiation of these samples were assumed to be due to the interaction of solvated electrons with BrdU. With this experimental system, we saw no effect of ethanol on the sensitization of single stranded DNA by BrdU (Figure II-5, panels A and B), whereas mismatched DNA displayed a conformation dependency similar to what was observed with the hydration experiment, regardless of whether strand breaks (Figure II-5, panels C and

D) or ICLs (Figure II-6) were considered. It is possible that the interaction of ethanol with the uracil-5-yl radical could prevent the attack of the latter on the sugar and the subsequent creation of a strand break. However, our observation that ethanol does not influence break formation in single stranded DNA argues against this hypothesis (Figure II-5A). A slight reduction of alkali-labile DNA lesions was observed upon addition of ethanol and could be attributed to a low level of remaining hydroxyl radicals that are scavenged by ethanol. Although solvated electrons are thought to be the primary reactive species to interact with BrdU, hydroxyl radicals can also attack BrdU, leading to debromination and creation of a strand break (37). Because further scavenging of these hydroxyl radicals by ethanol could explain part of the reduction in strand breaks, we performed another experiment where DNA was again irradiated in the presence or absence of ethanol, but also in the presence of dinitrous oxide (N_2O), an efficient electron scavenger. Although we observed a diminution in strand breaks in both A-form and B-form DNA in the presence of N_2O, we were unable to eliminate all strand breaks produced at 2400 Gy. Because it is possible that a substantial amount of N_2O is consumed at this high dose, we lowered the dose to 300 Gy and used oxygen, another well-known electron scavenger. Strand breaks produced in B-form DNA were completely suppressed by irradiation in the presence of oxygen. However, the number of strand breaks produced in A-form DNA at 300 Gy was too low to observe an effect of the presence of oxygen. These results clearly indicate that, although hydroxyl radicals may be responsible for a small portion of strand breaks generated in B-form DNA at 2400 Gy, the majority of damage to BrdU-substituted DNA indeed occurs through a reductive pathway, and that the reduction in strand break formation observed upon addition of ethanol originates from a change in DNA conformation. Thus, the results obtained with ethanol support those from our hydration experiments: the type of DNA damage resulting from BrdU sensitization is dependent on DNA conformation. Irradiation of A-DNA will induce alkali-labile DNA lesions, whereas B-DNA will lead primarily to frank strand breaks and lower levels of alkali-labile DNA lesions.

DNA structure has an important influence on the production of strand breaks. Both simulations (49) and experimental results (50) suggest that hydroxyl radical attack is dependent on DNA structure, both on the DNA form (A, B or Z) and on the hybridization state (single or double stranded). Several papers also report that chromatin structure per se affects the extent of DNA damage by ionizing radiation (51-55). As for brominated DNA, recent evidence by Kimura et al. pointed to enhanced reactivity in Z-DNA compared to that in B-DNA in 8-bromo-2′-deoxyguanosine substituted oligonucleotides (56). Cecchini et al. (25) also presented evidence that hybridization lowered the total yield of single strand breaks induced by γ-rays in BrdU-substituted DNA by up to 20-fold, and that substituted double stranded oligonucleotides containing a mismatch produced ICLs. Previous reports have already shown that DNA structure strongly influences the preference for hydrogen abstraction on C1′ or C2′ by a uracil-5-yl radical generated by photoirradiation of BrdU-substituted DNA (57-58; for a review, see 59). Thus, it is likely that DNA conformation also affects the type of damage resulting from uracil-5-yl radical production. In our case, the A-form that exists between $\Gamma \approx 6$-21 probably prevents both the H-atom abstraction from the 2′-deoxyribose moiety and the migration of damage along the DNA strand, precluding the formation of frank strand breaks. Thus, the uracil-5-yl radical remains localized at the BrdU site, possibly allowing slower, competing pathways to create other types of lesions that can be revealed by piperidine treatment and are located exclusively at the site of BrdU substitution. However, the structural conditions necessary for the generation of strand breaks exist in B-form DNA at $\Gamma \approx 31$ and in solution. Although the nature of the alkali-labile lesions was not determined in these experiments, it is entirely possible that they coincide with the damage leading to the production of the base fragments observed under UHV (42-43). Electron attachment to BrdU leads to the formation of the same uracil-5-yl radical, whether the electron is solvated or not (38-41), although it is also possible that the dissociative electron attachment mechanism that was proposed by Sanche's group (60) could also yield products different from those observed in solution. Because of the high dependence of BrdU sensitization on DNA structure, the experimental system presented here may be a sensitive probe for DNA conformation.

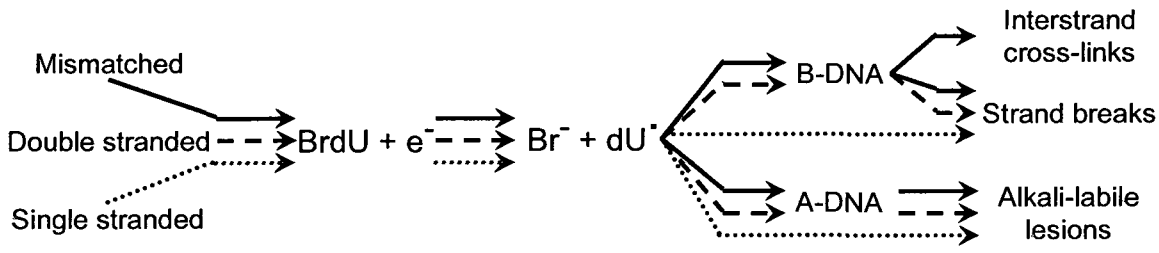

Figure II-7 : Major degradation pathway for BrdU-substituted DNA for single stranded, double stranded, and mismatched DNA for A- and B-DNA. (Note: Only the most important lesions are indicated. Minor levels of alkali-labile lesions are generated in B-DNA.)

Figure II-7 summarizes our conclusions. We propose that, during irradiation, electron attachment to DNA occurs in all oligonucleotides, independent of the hybridization state and structure. However, dehalogenation and creation of the uracil-5-yl, which are dependent on the probability of electron localization on BrdU, are strongly influenced by DNA structure (25). Furthermore, once the uracil-5-yl radical is formed, the type of damage will also depend on DNA conformation. We propose that in A-DNA, the uracil-5-yl radical is unable to abstract a hydrogen atom from the adjacent 2′-deoxyribose, thus localizing the radical at the site of substitution. Further reactions with adjoining donors will lead to the creation of alkali-labile lesions that possibly include the fragmented base that was observed by Sanche's group (42-43). In B-DNA, the structural requirements for hydrogen abstraction exist, and strand breaks will occur, although a certain amount of transient, unfavorable structure must also exist because a fraction of the uracil-5-yl radical population will generate alkali-labile DNA lesions and ICLs. B-DNA also allows the migration of the original radical to distant bases, thereby permitting delocalized induction of strand breaks. Therefore, A-DNA will produce piperidine-sensitive DNA lesions localized on BrdU, whereas B-DNA will also produce strand breaks that can spread on either side of the original radical. ICL production will be limited to mismatched DNA in B-form. Single stranded DNA will not be affected because of its less ordered configuration in

solution. It is interesting to note that the reactivity of the mismatched nucleotides in semi-complementary DNA is affected by DNA conformation. This observation appears to favor the zipper-like structure for mismatches (61-63) rather than the open bubble model (24), which would possibly not be affected by DNA conformation.

BrdU and DNA structure: biological implications

In light of the results presented in this article, we propose that the fate of BrdU upon irradiation is highly dependent on the regional conformation of DNA. BrdU-substituted DNA in A-form will only produce base lesions, as was observed in experiments studying dehydrated DNA. Strand breaks will occur at BrdU sites when DNA adopts a B-form, because the DNA conformation is now favorable to the steps leading to sugar-phosphate backbone cleavage. How this hypothesis would translate into a cellular environment is still unknown: in cells, DNA is mostly in B-form, but is wrapped tightly around nucleosomes to form chromatin. DNA packing and chromatin structure was shown to have an effect on radiation sensitivity (64), and some evidence points to a similar influence on BrdU sensitization (34, 65).

Although clinical studies using BrdU as a radiosensitizer have been disappointing up to this point, a new class of halogenated pyrimidines has been emerging as a potent radiosensitizer (66-67). Sensitization by chlorodeoxycytidine (CldC) relies on the elevated levels of deoxycytidine kinase and dCMP deaminase in human tumors compared to normal tissues. CldC is incorporated in DNA as chlorodeoxyuridine (CldU). Because electron attachment to CldU will yield the same uracil-5-yl radical that is responsible for BrdU sensitization, it is extremely likely that similar sensitization mechanisms will also apply to CldC, including the production of ICLs. Therefore, it is crucial to pursue the study of the chemical processes leading to BrdU- and CldC-induced damage in order to develop and exploit tumor radiosensitizers.

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References

1. Dewey, W. C., and Humphrey, R. M. (1965) Increase in Radiosensitivity to Ionizing Radiation Related to Replacement of Thymidine in Mammalian Cells with 5-Bromodeoxyuridine. *Radiat. Res. 26*, 538-553.

2. Sano, K., Hoshino, T., and Nagai, M. (1968) Radiosensitization of Brain Tumor Cells with a Thymidine Analogue (Bromouridine). *J. Neurosurg. 28*, 530-538.

3. Limoli, C. L., and Ward, J. F. (1993) A New Method for Introducing Double-Strand Breaks into Cellular DNA. *Radiat. Res. 134*, 160-169.

4. Saffhill, R., and Ockey, C. H. (1985) Strand Breaks Arising from the Repair of the 5-Bromodeoxyuridine-Substituted Template and Methyl Methanesulphonate-Induced Lesions can Explain the Formation of Sister Chromatid Exchanges. *Chromosoma. 92*, 218-224.

5. Dewey, W. C., Sedita, B. A., and Humphrey, R. M. (1966) Radiosensitization of X Chromosome of Chinese Hamster Cells Related to Incorporation of 5-Bromodeoxyuridine. *Science. 152*, 519-521.

6. Coleman, C. N., and Mitchell, J. B. (1999) Clinical Radiosensitization: Why it does and does Not Work. *J. Clin. Oncol. 17*, 1-3.

7. Dabaja, B. S., McLaughlin, P., Ha, C. S., Pro, B., Meyers, C. A., Seabrooke, L. F., Wilder, R. B., Kyritsis, A. P., Preti, H. A., Yung, W. K., Levin, V., Cabanillas, F., and Cox, J. D. (2003) Primary Central Nervous System Lymphoma: Phase I Evaluation of Infusional Bromodeoxyuridine with Whole Brain Accelerated Fractionation Radiation Therapy After Chemotherapy. *Cancer. 98*, 1021-1028.

8. Groves, M. D., Maor, M. H., Meyers, C., Kyritsis, A. P., Jaeckle, K. A., Yung, W. K., Sawaya, R. E., Hess, K., Bruner, J. M., Peterson, P., and Levin, V. A. (1999) A Phase II Trial of High-Dose Bromodeoxyuridine with Accelerated Fractionation Radiotherapy Followed by Procarbazine, Lomustine, and Vincristine for Glioblastoma Multiforme. *Int. J. Radiat. Oncol. Biol. Phys. 45*, 127-135.

9. Prados, M. D., Scott, C., Sandler, H., Buckner, J. C., Phillips, T., Schultz, C., Urtasun, R., Davis, R., Gutin, P., Cascino, T. L., Greenberg, H. S., and Curran, W. J.,Jr. (1999) A Phase 3 Randomized Study of Radiotherapy Plus Procarbazine, CCNU, and Vincristine (PCV) with Or without BUdR for the Treatment of Anaplastic Astrocytoma: A Preliminary Report of RTOG 9404. *Int. J. Radiat. Oncol. Biol. Phys. 45*, 1109-1115.

10. Prados, M. D., Seiferheld, W., Sandler, H. M., Buckner, J. C., Phillips, T., Schultz, C., Urtasun, R., Davis, R., Gutin, P., Cascino, T. L., Greenberg, H. S., and Curran, W. J.,Jr. (2004) Phase III Randomized Study of Radiotherapy Plus

Procarbazine, Lomustine, and Vincristine with Or without BUdR for Treatment of Anaplastic Astrocytoma: Final Report of RTOG 9404. *Int. J. Radiat. Oncol. Biol. Phys. 58*, 1147-1152.

11. Goodfellow, J. M., Cruzeiro-Hansson, L., Norberto de Souza, O., Parker, K., Sayle, T., and Umrania, Y. (1994) DNA Structure, Hydration and Dynamics. *Int. J. Radiat. Biol. 66*, 471-478.

12. Saenger, W. (1984) *Principles of Nucleic Acid Structure.* Springer-Verlag, New York.

13. Wolf, B., and Hanlon, S. (1975) Structural Transitions of Deoxyribonucleic Acid in Aqueous Electrolyte Solutions. II. the Role of Hydration. *Biochemistry. 14*, 1661-1670.

14. Milano, M. T., and Bernhard, W. A. (1999) The Effect of Packing and Conformation on Free Radical Yields in Films of Variably Hydrated DNA. *Radiat. Res. 151*, 39-49.

15. Milano, M. T., and Bernhard, W. A. (1999) The Influence of Packing on Free Radical Yields in Solid-State DNA: Film Compared to Lyophilized Frozen Solution. *Radiat. Res. 152*, 196-201.

16. Huttermann, J., Rohrig, M., and Kohnlein, W. (1992) Free Radicals from Irradiated Lyophilized DNA: Influence of Water of Hydration. *Int. J. Radiat. Biol. 61*, 299-313.

17. Wang, W., Becker, D., and Sevilla, M. D. (1993) The Influence of Hydration on the Absolute Yields of Primary Ionic Free Radicals in Gamma-Irradiated DNA at 77 K. I. Total Radical Yields. *Radiat. Res. 135*, 146-154.

18. Mroczka, N., and Bernhard, W. A. (1993) Hydration Effects on Free Radical Yields in DNA X-Irradiated at 4 K. *Radiat. Res. 135*, 155-159.

19. Wang, W., Yan, M., Becker, D., and Sevilla, M. D. (1994) The Influence of Hydration on the Absolute Yields of Primary Free Radicals in Gamma-Irradiated DNA at 77 K. II. Individual Radical Yields. *Radiat. Res. 137*, 2-10.

20. Weiland, B., and Huttermann, J. (1998) Free Radicals from X-Irradiated 'Dry' and Hydrated Lyophilized DNA as Studied by Electron Spin Resonance Spectroscopy: Analysis of Spectral Components between 77K and Room Temperature. *Int. J. Radiat. Biol. 74*, 341-358.

21. Yokoya, A., Cunniffe, S. M., and O'Neill, P. (2002) Effect of Hydration on the Induction of Strand Breaks and Base Lesions in Plasmid DNA Films by Gamma-Radiation. *J. Am. Chem. Soc. 124*, 8859-8866.

22. La Vere, T., Becker, D., and Sevilla, M. D. (1996) Yields of OH in Gamma-Irradiated DNA as a Function of DNA Hydration: Hole Transfer in Competition with OH Formation. *Radiat. Res. 145*, 673-680.

23. Debije, M. G., Strickler, M. D., and Bernhard, W. A. (2000) On the Efficiency of Hole and Electron Transfer from the Hydration Layer to DNA: An EPR Study of Crystalline DNA X-Irradiated at 4 K. *Radiat. Res. 154*, 163-170.

24. Cecchini, S., Girouard, S., Huels, M. A., Sanche, L., and Hunting, D. J. (2005) Interstrand Cross-Links: A New Type of Gamma-Ray Damage in Bromodeoxyuridine-Substituted DNA. *Biochemistry. 44*, 1932-1940.

25. Cecchini, S., Girouard, S., Huels, M. A., Sanche, L., and Hunting, D. J. (2004) Single-Strand-Specific Radiosensitization of DNA by Bromodeoxyuridine. *Radiat. Res. 162*, 604-615.

26. Cecchini, S., Masson, C., La Madeleine, C., Huels, M. A., Sanche, L., Wagner, J. R., and Hunting, D. J. (2005) Interstrand Cross-Link Induction by UV Radiation in Bromodeoxyuridine-Substituted DNA: Dependence on DNA Conformation. *Biochemistry. 44*, 16957-16966.

27. Stokes, R. H., and Robinson, R. A. (1949) Standard Solutions for Humidity Control at 25°C. *Ind. Eng. Chem. 41*, 2013-2013.

28. Cai, Z., Cloutier, P., Hunting, D., and Sanche, L. (2006) Enhanced DNA Damage Induced by Secondary Electron Emission from a Tantalum Surface Exposed to Soft X Rays. *Radiation Research. 165*, 365-371.

29. Maxam, A. M., and Gilbert, W. (1980) Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages. *Methods Enzymol. 65*, 499-560.

30. Zimbrick, J. D., Ward, J. F., and Myers, L. S.,Jr. (1969) Studies on the Chemical Basis of Cellular Radiosensitization by 5-Bromouracil Substitution in DNA. I. Pulse- and Steady-State Radiolysis of 5-Bromouracil and Thymine. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 16*, 505-523.

31. Patterson, L. K., and Bansal, K. M. (1972) Pulse Radiolysis Studies of 5-Halouracils in Aqueous Solutions. *J. Phys. Chem. 76*, 2392-2399.

32. Adams, G. E., and Willson, R. L. (1972) On the Mechanism of BUdR Sensitization: A Pulse Radiolysis Study of One Electron Transfer in Nucleic-Acid Derivatives. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 22*, 589-597.

33. Ling, L. L., and Ward, J. F. (1990) Radiosensitization of Chinese Hamster V79 Cells by Bromodeoxyuridine Substitution of Thymidine: Enhancement of Radiation-Induced Toxicity and DNA Strand Break Production by Monofilar and Bifilar Substitution. *Radiat. Res. 121*, 76-83.

34. Lawrence, T. S., Davis, M. A., and Normolle, D. P. (1995) Effect of Bromodeoxyuridine on Radiation-Induced DNA Damage and Repair Based on DNA Fragment Size using Pulsed-Field Gel Electrophoresis. *Radiat. Res. 144*, 282-287.

35. Webb, C. F., Jones, G. D., Ward, J. F., Moyer, D. J., Aguilera, J. A., and Ling, L. L. (1993) Mechanisms of Radiosensitization in Bromodeoxyuridine-Substituted Cells. *Int. J. Radiat. Biol. 64*, 695-705.

36. Abdoul-Carime, H., Huels, M. A., Brüning, F., Illenberger, E., and Sanche, L. (2000) Dissociative Electron Attachment to Gas-Phase 5-Bromouracil. *J. Chem. Phys. 113*, 2517-2521.

37. Bansal, K. M., Patterson, L. K., and Schuler, R. H. (1972) The Production of Halide Ion in the Radiolysis of Aqueous Solutions of the 5-Halouracils. *J. Phys. Chem. 76*, 2386-2392.

38. Klyachko, D. V., Huels, M. A., and Sanche, L. (1999) Halogen Anion Formation in 5-Halouracil Films: X Rays Compared to Subionization Electrons. *Radiat. Res. 151*, 177-187.

39. Abdoul-Carime, H., Huels, M. A., Illenberger, E., and Sanche, L. (2001) Sensitizing DNA to Secondary Electron Damage: Resonant Formation of Oxidative Radicals from 5-Halouracils. *J. Am. Chem. Soc. 123*, 5354-5355.

40. Graslund, A., Rupprecht, A., Kohnlein, W., and Huttermann, J. (1981) Radiation-Induced Free Radicals in Oriented Bromouracil-Substituted DNA. *Radiat. Res. 88*, 1-10.

41. Sevilla, M. D., Failor, R., and Zorman, G. (1974) Radicals Formed After Electron Attachment to 5-Halouracils in Aqueous Glasses. *J. Phys. Chem. 78*, 696-699.

42. Abdoul-Carime, H., Dugal, P. C., and Sanche, L. (2000) Damage Induced by 1-30 eV Electrons on Thymine- and Bromouracil-Substituted Oligonucleotides. *Radiat. Res. 153*, 23-28.

43. Dugal, P. -., Abdoul-Carime, H., and Sanche, L. (2000) Mechanisms for Low-Energy (0.5-30 eV) Electron-Induced Pyrimidine Ring Fragmentation within Thymine- and Halogen-Substituted Single Strands of DNA. *J. Phys. Chem. B. 104*, 5610-5617.

44. Buxton, G. V., Greenstock, C. L., Helman, W. P., and Ross Alberta, B. (1988) Critical Review of Rate Constants for Reactions of Hydrated Electrons, Hydrogen Atoms and Hydroxyl Radicals in Aqueous Solution. *J. Phys. Chem. Ref. Data. 17*, 513-886.

45. Zimbrick, J. D., Ward, J. F., and Myers, L. S.,Jr. (1969) Studies on the Chemical Basis of Cellular Radiosensitization by 5-Bromouracil Substitution in DNA. II. Pulse- and Steadystate Radiolysis of Bromouracil-Substituted and Unsubstituted DNA. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 16*, 525-534.

46. Valis, L., Wang, Q., Raytchev, M., Buchvarov, I., Wagenknecht, H. A., and Fiebig, T. (2006) Base Pair Motions Control the Rates and Distance Dependencies of

Reductive and Oxidative DNA Charge Transfer. *Proc. Natl. Acad. Sci. U. S. A. 103*, 10192-10195.

47. Ivanov, V. I., Minchenkova, L. E., Minyat, E. E., Frank-Kamenetskii, M. D., and Schyolkina, A. K. (1974) The B to A Transition of DNA in Solution. *Journal of Molecular Biology. 87*, 817-833.

48. Lati, J., and Meyerstein, D. (1978) Oxidation of First-Row Bivalent Transition-Metal Complexes Containing Ethylenediaminetetra-Acetate and Nitrilotriacetate Ligands by Free Radicals: A Pulse-Radiolysis Study. *J. Chem. Soc., Dalton Trans.* , 1105-1118.

49. Michalik, V., Spotheim Maurizot, M., and Charlier, M. (1995) Calculation of Hydroxyl Radical Attack on Different Forms of DNA. *J. Biomol. Struct. Dyn. 13*, 565-575.

50. Tartier, L., Michalik, V., Spotheim-Maurizot, M., Rahmouni, A. R., Sabattier, R., and Charlier, M. (1994) Radiolytic Signature of Z-DNA. *Nucleic Acids Res. 22*, 5565-5570.

51. Warters, R. L., and Lyons, B. W. (1992) Variation in Radiation-Induced Formation of DNA Double-Strand Breaks as a Function of Chromatin Structure. *Radiat. Res. 130*, 309-318.

52. Valota, A., Ballarini, F., Friedland, W., Jacob, P., Ottolenghi, A., and Paretzke, H. G. (2003) Modelling Study on the Protective Role of OH Radical Scavengers and DNA Higher-Order Structures in Induction of Single- and Double-Strand Break by Gamma-Radiation. *Int. J. Radiat. Biol. 79*, 643-653.

53. Olive, P. (1992) DNA Organization Affects Cellular Radiosensitivity and Detection of Initial Strand Breaks. *Int. J. Radiat. Biol. 62*, 389-396.

54. Elmroth, K., Nygren, J., Stenerlow, B., and Hultborn, R. (2003) Chromatin- and Temperature-Dependent Modulation of Radiation-Induced Double-Strand Breaks. *Int. J. Radiat. Biol. 79*, 809-816.

55. Nygren, J., Ljungman, M., and Ahnstrom, G. (1995) Chromatin Structure and Radiation-Induced DNA Strand Breaks in Human Cells: Soluble Scavengers and DNA-Bound Proteins Offer a Better Protection Against Single- than Double-Strand Breaks. *Int. J. Radiat. Biol. 68*, 11-18.

56. Kimura, T., Kawai, K., Tojo, S., and Majima, T. (2004) One-Electron Attachment Reaction of B- and Z-DNA Modified by 8-Bromo-2'-Deoxyguanosine. *J. Org. Chem. 69*, 1169-1173.

57. Sugiyama, H., Fujimoto, K., and Saito, I. (1997) Preferential C1' Hydrogen Abstraction by a Uracilyl Radical in a DNA-RNA Hybrid. *Tetrahedron Lett. 38*, 8057-8060.

58. Kawai, K., Saito, I., and Sugiyama, H. (1999) Conformation-Dependent Photochemistry of 5-Halouracil-Containing DNA: Stereospecific 2'a-Hydroxylation of Deoxyribose in Z-Form DNA. *J. Am. Chem. Soc. 121*, 1391-1392.

59. Xu, Y., and Sugiyama, H. (2006) Photochemical Approach to Probing Different DNA Structures. *Angew. Chem., Int. Ed 45*, 1354-1362.

60. Boudaiffa, B., Cloutier, P., Hunting, D., Huels, M. A., and Sanche, L. (2000) Resonant Formation of DNA Strand Breaks by Low-Energy (3 to 20 eV) Electrons. *Science. 287*, 1658-1660.

61. Chou, S. H., Chin, K. H., and Wang, A. H. (2003) Unusual DNA Duplex and Hairpin Motifs. *Nucleic Acids Res. 31*, 2461-2474.

62. Kondo, J., Umeda, S., Fujita, K., Sunami, T., and Takenaka, A. (2004) X-Ray Analyses of d(GCGAXAGC) Containing G and T at X: The Base-Intercalated Duplex is Still Stable Even in Point Mutants at the Fifth Residue. *J. Synchrotron Radiat. 11*, 117-120.

63. Shepard, W., Cruse, W. B., Fourme, R., de la Fortelle, E., and Prange, T. (1998) A Zipper-Like Duplex in DNA: The Crystal Structure of d(GCGAAAGCT) at 2.1 A Resolution. *Structure. 6*, 849-861.

64. Oleinick, N. L., Balasubramaniam, U., Xue, L., and Chiu, S. (1994) Nuclear Structure and the Microdistribution of Radiation Damage in DNA. *Int. J. Radiat. Biol. 66*, 523-529.

65. Iliakis, G., Kurtzman, S., Pantelias, G., and Okayasu, R. (1989) Mechanism of Radiosensitization by Halogenated Pyrimidines: Effect of BrdU on Radiation Induction of DNA and Chromosome Damage and its Correlation with Cell Killing. *Radiat. Res. 119*, 286-304.

66. Greer, S., Alvarez, M., Mas, M., Wozniak, C., Arnold, D., Knapinska, A., Norris, C., Burk, R., Aller, A., and Dauphinee, M. (2001) Five-Chlorodeoxycytidine, a Tumor-Selective Enzyme-Driven Radiosensitizer, Effectively Controls Five Advanced Human Tumors in Nude Mice. *Int. J. Radiat. Oncol. Biol. Phys. 51*, 791-806.

67. Perez, L. M., Mekras, J. A., Briggle, T. V., and Greer, S. (1984) Marked Radiosensitization of Cells in Culture to X Ray by 5-Chlorodeoxycytidine Coadministered with Tetrahydrouridine, and Inhibitors of Pyrimidine Biosynthesis. *Int. J. Radiat. Oncol. Biol. Phys. 10*, 1453-1458.

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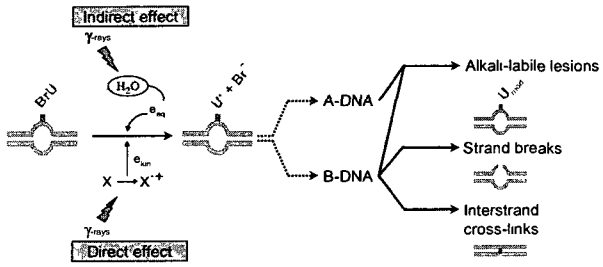

Chapitre III – 2e article

Reaching for the other side: generating sequence-dependent interstrand cross-links with 5-bromodeoxyuridine and γ-rays.

Auteurs de l'article : Marie-Eve Dextraze, Sylvain Cecchini, François Bergeron, Sonia Girouard, Kathleen Turcotte, J. Richard Wagner and Darel J. Hunting.

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Avant-propos :

Contribution à la rédaction et à l'expérimentation :

Sonia Girouard a fait les expériences d'irradiation des différentes séquences d'ADN, sous la supervision de Sylvain Cecchini, alors étudiant au Ph.D. L'irradiation d'ADN simple brin et l'analyse par HPLC a été faite par Kathleen Turcotte (stagiaire) sous ma supervision. J'ai analysé les données brutes et j'ai rédigé la totalité de cet article, sous la supervision de mes co-directeurs, Richard Wagner et Darel Hunting.

Résumé :

Dans cet article, nous présentons l'effet de la séquence sur la production de dommage à l'ADN bromé. Douze séquences ont été étudiées, comprenant trois variations du brin bromé et quatre variations du brin semi-complémentaire. À l'aide de ce modèle expérimental, nous montrons que la séquence d'ADN entourant le BrdU influence

énormément la production de dommage à l'ADN bromé. En effet, autant que le rendement des dommages (production de cassures et de dimères interbrins) que la localisation du dommage est affectée par la séquence. Plus précisément, cet article montre qu'à partir d'un radical initial, plusieurs structures de dimère interbrins peuvent être formées, ce qui signale une chimie complexe où la migration de radical pourrait être impliquée. Cet article vient appuyer notre hypothèse, qui soutient que l'environnement où le radical initial est créé – et plus particulièrement la séquence de l'ADN dans cet article – lors de l'irradiation de l'ADN a un effet important sur la létalité du dommage qui est produit.

Reaching for the Other Side: Generating Sequence-Dependent Interstrand Cross-Links with 5-Bromodeoxyuridine and γ-rays

† *This work was supported by the Cancer Research Society.*

*Marie-Eve Dextraze, Sylvain Cecchini, François Bergeron, Sonia Girouard, Kathleen Turcotte, J. Richard Wagner and Darel J. Hunting**

ABBREVIATIONS A, Adenine; BrdU, 5-bromo-2′-deoxyuridine; ^{Br}U, 5-bromo-2′-deoxyuridine-substituted oligonucleotide; C, cytosine; e_{aq}, solvated electron; EDTA, ethylenediaminetetraacetic acid; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; HPLC, High Performance Liquid Chromatography; G, guanine; ICL, interstrand cross-link; 2-PrOH, 2-propanol; T, thymine; TEAA, triethylammonium acetate.

ABSTRACT Interstrand cross-links impede critical cellular processes such as transcription and replication and are thus considered to be one of the most toxic types of DNA damage. Although several studies now point to the existence of γ-radiation-induced cross-links in cellular DNA, little is known about the characteristics required for their creation. Recently, we reported the formation of interstrand cross-links that were specific for mismatched nucleotides within 5-bromo-2′-deoxyuridine-substituted DNA. Given the structural specificity for interstrand cross-link formation, it is likely that open or mismatched regions of DNA in cells may be particularly favorable for cross-link production. Herein, we investigated the effect of the local DNA sequence on the formation of interstrand cross-links, using 5-bromo-2′-deoxyuridine to generate radicals in a mismatched region of DNA. We investigated a total of 12 variations of bases in the mismatched region. The oligonucleotides were irradiated with γ-rays, and interstrand cross-link formation was analyzed by denaturing gel electrophoresis. We found that the efficiency of cross-link formation was highly dependent on the nature of mismatched bases and, on the basis of electrophoretic mobility, observed several distinctive cross-link structures with specific DNA sequences. This study provides new insights into the reactivity of mismatched DNA and the mechanisms leading to interstrand cross-link formation. The potential application of 5-bromo-2′-deoxyuridine-induced interstrand cross-links to the field of DNA repair is discussed.

Among the plethora of DNA lesions produced by ionizing radiation, double-strand breaks are generally considered to be the most toxic (*1-3*). However, evidence of the formation of another potentially more toxic type of lesion is now emerging. Interstrand cross-links (ICLs) prevent the separation of the DNA strands, thus blocking several key cellular processes (*4, 5*). Traditionally, ionizing radiation has not been regarded as a cross-linking agent; however, several instances of intrastrand cross-link formation have been reported (*6-12*), and recent data from Greenberg and co-workers point to the existence of interstrand cross-links induced by hydroxyl radicals in synthetic DNA (*13, 14*).

Although 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue that can be incorporated into DNA, is widely used as a marker of replicating cells (*15-17*), it was first reported as a photo- and radiosensitizing agent (*18, 19*). When exposed to ionizing radiation, BrdU produces single- and double-strand breaks (*20, 21*), as well as chromosomal aberrations (*22*) that are generally believed to be responsible for its sensitizing properties. Recently, we reported the formation of a new type of BrdU-sensitized damage in synthetic DNA: ICLs that were specific for mismatched nucleotides within BrdU-substituted DNA (*23*). These ICLs were later found to be highly dependent on the presence of B-form DNA, supporting the evidence that the regional structure of DNA is a prerequisite for their formation (*24*) and that these ICLs may only be produced in open or mismatched regions of cellular DNA. Although open regions of DNA transiently exist during transcription and replication and mismatched regions are found in centromeres (*25-27*), there are surprisingly few studies asking how these regions are affected by ionizing radiation (*28, 29*). Given the structural prerequisites for ICL formation observed in our studies, it is possible that open regions of DNA may be particularly favorable for ICL production.

Here, we used BrdU, together with ionizing radiation, as a tool to generate radicals in open regions of DNA and to examine how these regions react. To study the reactivity of radicals with different DNA bases in an open region of DNA, we

modified the nucleotide sequence surrounding the site of BrdU substitution in both the brominated and the opposite semi-complementary strands. We investigated a total of 12 mismatched oligonucleotides, using three variations of the brominated strand and four variations of the semi-complementary strand. The yield of both ICLs and strand breaks was found to be highly dependent on the identity of the neighboring bases. Furthermore, we observed several distinctive ICL structures that were specific for given sequences. This study provides new insights into the reactivity of mismatched DNA and the mechanisms leading to interstrand cross-link formation. Finally, we propose that, given the sequence specificity of BrdU-induced ICLs, this model system may provide a method of producing single ICL structures for use in DNA repair studies.

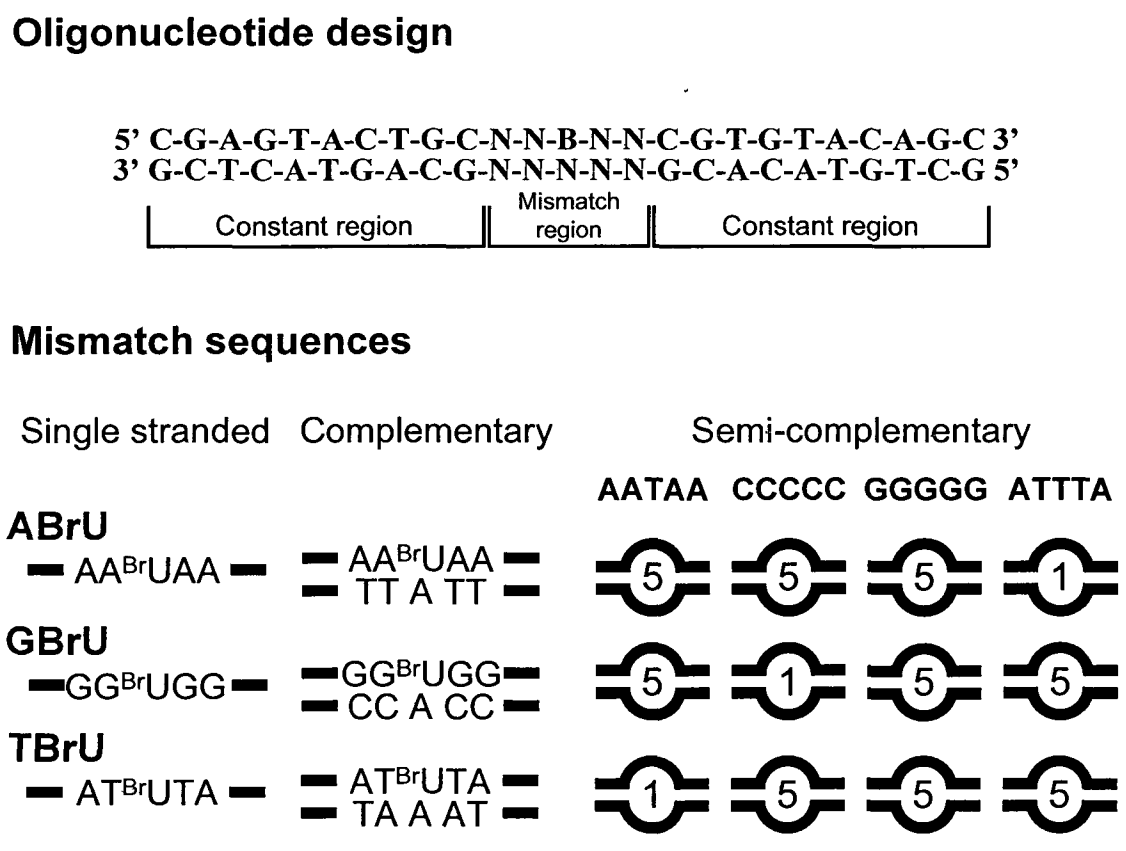


Figure III-1 : Oligonucleotide design. Each 25-mer sequence consisted of a variable central 5 bp region, surrounded by two constant regions. Three variations of the central region of the BrdU-substituted (B) strand were synthesized ($AA^{Br}UAA$, $GG^{Br}UGG$ and $AT^{Br}UTA$) and were either irradiated in single-stranded form or

hybridized with their complementary or one of the four semicomplementary strands to form double-stranded of mismatched oligonucleotides, respectively. The mismatch size is indicated for each combination.

MATERIALS AND METHODS

Oligonucleotide Design

5-Bromo-2'-deoxyuridine-modified and nonmodified oligonucleotides were purchased from the University Core DNA Services (University of Calgary, Calgary, AB). Oligonucleotides were designed on the basis of previous sequences used by our group (*23, 24*). Each sequence included two constant regions, with a variable central 5 bp region described in Figure III-1. Three variations of the brominated sequence were used (AA^{Br}UAA, GG^{Br}UGG, and AT^{Br}UTA) and were hybridized either with their complementary sequence or with one of the semicomplementary sequences (AATAA, CCCCC, GGGGG, ATTTA). The choice of hybridized sequences determined the regional conformation of the DNA (double-stranded or mismatched).

Sequence-dependent interstrand cross-link and strand break formation

Control experiments were performed using single- and double-stranded DNA to verify the hybridization specificity previously observed with AA^{Br}UAA (*30*). For mismatched DNA, each combination of brominated and semicomplementary strands was irradiated and analyzed on the same gel. Also, for each permutation, the production of damage on each DNA strand was investigated by alternatively labeling either the brominated or the semicomplementary strand. A comparison of interstrand cross-link structures was performed using the following guidelines. (1) Oligonucleotides were labeled and irradiated concomitantly and were analyzed together on the same gel by denaturing polyacrylamide gel electrophoresis. (2) For each sequence, an equal amount of radioactivity was loaded on the gel. (3) The

structures presented in Figure III-4 were taken from a single gel in which the original alignment was conserved.

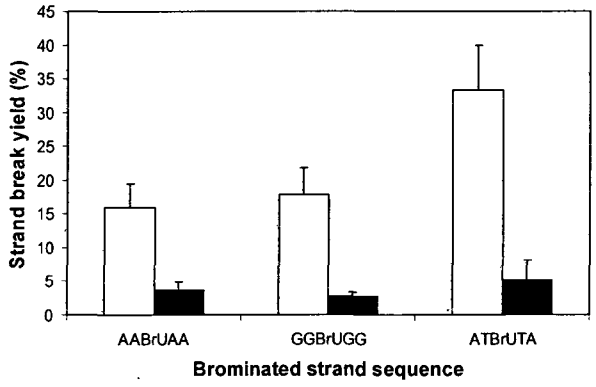


Figure III-2 : Contribution of double strandedness to strand breaks as a function of sequence. The three variations of the brominated strand were irradiated as single-stranded (white) or double stranded DNA (black). The yield of strand breaks in the brominated region was measured by denaturing gel electrophoresis.

Experimental Procedures

In each experiment, the 5′ end of one strand was end-labeled with ^{32}P using T4 polynucleotide kinase (Amersham Pharmacia Biotech). Hybridization was carried out by adding a 2-fold concentration of complementary oligonucleotide, heating to 90 °C, and slowly cooling to room temperature for 2.5 h. The hybridization state was confirmed by nondenaturing gel electrophoresis. Prior to irradiation, DNA was bubbled for 1 min with N_2 to remove excess oxygen and then irradiated with 750 Gy in a Gammacell-220 irradiator (^{60}Co, Nordion Canada, dose rate of 5.08 Gy/min) at a final concentration of 0.3 μM in phosphate buffer (10 mM, pH 7.0), using 25 mM EDTA as a hydroxyl radical scavenger, as described by Cecchini et al. (*23, 30*). Strand breaks and interstrand cross-links were assessed by denaturing gel electrophoresis, followed by quantification of damage by the ImageQuant software, as described by Cecchini et al. (*23*).

Conversion of BrdU to Uracil

Conversion of BrdU to uracil in single-stranded DNA was assessed by high performance liquid chromatography (HPLC). The three BrdU-substituted sequences ($AA^{Br}UAA$, $GG^{Br}UGG$ and $AT^{Br}UTA$) were irradiated with 750 Gy as single-stranded oligonucleotides in 10 mM phosphate buffer (pH 7.0) and 25 mM EDTA (pH 8.0). These conditions were similar to those used in the gel electrophoresis experiments, except that the DNA concentration was 25 μM rather than 0.3 μM. After irradiation, DNA was digested using 3 units each of snake venom phosphodiesterase (phosphodiesterase I, USB) and nuclease P1 (Sigma) and incubated at 37 °C for 120 min. Alkaline phosphatase (3 units, Roche) was then added, and digestion was continued for 120 min at 37 °C to generate nucleosides. Chloroform extraction was performed to remove proteins prior to injection onto the HPLC system. HPLC analysis was performed using an Alliance system (Waters 2795 or 2690) connected to dual-wavelength UV detectors (Waters 2487) and a Millenium workstation (Waters version 4). The analysis of 2′-deoxyuridine formation was carried out as follows: 150 pmol of digested DNA was separated using a reversed phase column (5μm ODS-A. 250 mm × 6.0 mm; YMC). The gradient was as follows: 95% solvent A1 and 5% solvent B for 5 min, changing to 85% solvent A1 and 15% solvent B over 45 min (0.25%/min). Solvent A1 was composed of 25 mM triethylammonium acetate (TEAA; pH 7.0). Solvent B was composed of 95% acetonitrile and 5% H_2O. The flow rate was 1 mL/min, and the column was maintained at a temperature of 20 °C.

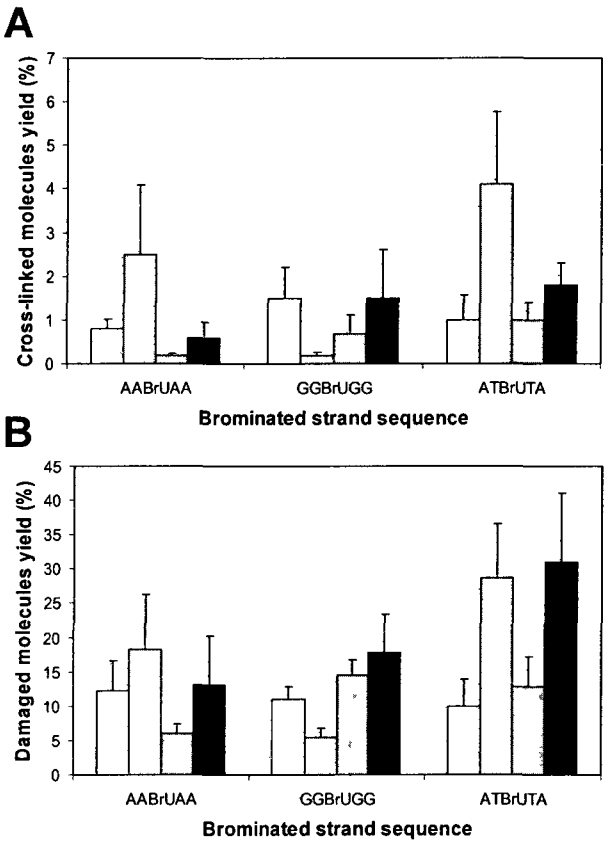


Figure III-3 : Damage yield as a function of sequence in mismatched DNA. Interstrand cross-links and total cross-link plus frank strand break damage (panels A and B, respectively) were measured following irradiation of the 12 combinations. Each variation of the brominated strand was hybridized with each of the four variations of the semicomplementary strand (AATAA, white; CCCCC, light gray; GGGGG, dark gray; and ATTTA, black). Total frank damage corresponds to the sum of interstrand cross-links and strand breaks located on both the brominated and semicomplementary strand.

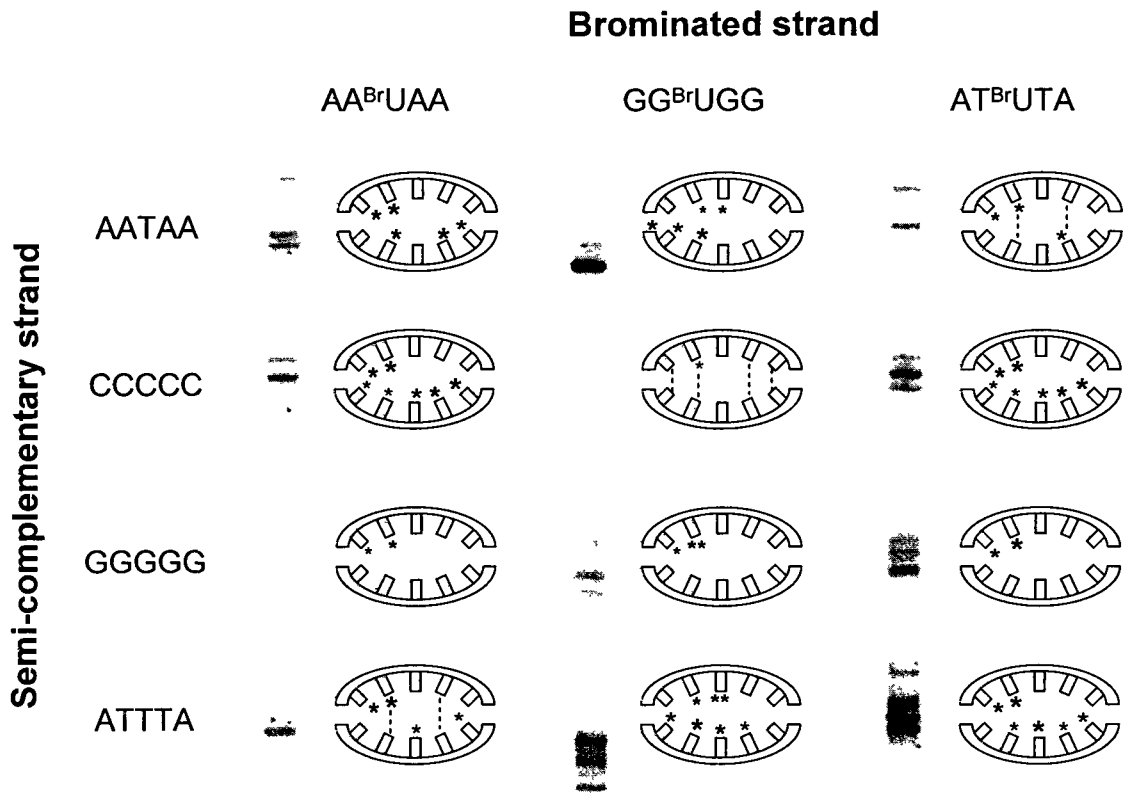


Figure III-4 : Interstrand cross-link structures and strand breaks location as a function of sequence. For each of the mismatched sequences, the cross-link patterns and the location of strand breaks were reported (only cross-link structures are shown on the left). The size of the asterisk indicates the relative yield of strand break for a particular nucleotide, while dashed lines specify the existence of possible canonical Watson-Crick bonds.

RESULTS

Contribution of Secondary Structure to Strand Break Formation as a Function of Sequence

We first investigated the effect of adjacent bases on BrdU-related damage in single- and double-stranded complementary DNA. In agreement with previous results, Figure III-2 shows an increase in the number of strand breaks in single-stranded compared to double-stranded DNA containing BrdU (*30*). The yield of strand breaks on the brominated strand, for both single- and double-stranded DNA, was similar for both

AA^{Br}UAA and GG^{Br}UGG, whereas there was more damage in AT^{Br}UTA in both hybridization states. However, conversion of BrdU to 2'-deoxyuridine by irradiation of single-stranded DNA was not significantly affected by the surrounding nucleotides (AA^{Br}UAA, 25.3 ± 7.0%; GG^{Br}UGG, 24.9 ± 5.9%; AT^{Br}UTA 28.0 ± 7.8%). Interstrand cross-links were not found in either hybridization state, regardless of the sequence (not shown).

Sequence-Dependent Formation of Interstrand Cross-Links and Strand Breaks in Mismatched DNA

Interstrand cross-links specific for BrdU-substituted mismatched DNA were first reported by our group in 2005 (*23*), using the AA^{Br}UAA//CCCCC mismatched oligonucleotide. Using AA^{Br}UAA//AATAA, we also showed that B-form DNA was necessary for the production of ICLs (*24*). Here, we investigated the effect of several sequences on the production of ICLs and strand breaks, to understand the role of DNA sequence in ICL formation. Figure III-3 shows the variation in the yields of ICL and the formation of total frank damage as a function of sequence (panels A and B, respectively). In general, pyrimidines, either the brominated or the semicomplementary strand, tend to produce more ICLs and strand breaks, while guanine typically does not promote cross-linking. For the three combinations that produced a mismatched region smaller than 5 bp or where internal Watson–Crick bonds could occur; namely, AA^{Br}UAA//ATTTA, GG^{Br}UGG//CCCCC, and AT^{Br}UTA//AATAA, production of ICLs and strands breaks was the lowest, with the exception of AA^{Br}UAA//GGGGG. Table 1 shows the strand location of breaks as a function of DNA sequence. Typically, there was no significant difference between strand breaks produced on each strand (i.e., brominated vs. nonbrominated) of a given combination. However, five permutations diverged from this pattern: GG^{Br}UGG//GGGGG and AT^{Br}UTA//ATTTA produced more breaks on the brominated strand, whereas the generation of breaks was more important in the non brominated semicomplementary strands of GG^{Br}UGG//AATAA, GG^{Br}UGG//ATTTA and AA^{Br}UAA//GGGGG. Labeling either the brominated or the semicomplementary

strand did not affect the yield of ICLs (not shown). Figure III-4 shows the detailed location and frequency of these strand breaks for each strand, as well as the corresponding ICL structures. Several distinct, sequence-dependent structures are observed, with only two permutations producing no cross-links at all: AA^{Br}UAA//GGGGG and GG^{Br}UGG//CCCCC.

Tableau III-1 : Localization of Strand Breaks on the Brominated and Semicomplementary Strands

sequence	strand breaks (%)		statistical significance	preferred strand (in bold)
	brominated strand	semicomplementary strand	p^a	
AABrUAA	15.90 ± 3.66			
AABrUAA//AATAA	5.60 ± 0.78	5.77 ± 3.39	0.9137	
AABrUAA//CCCCC	6.28 ± 2.85	9.42 ± 3.64	0.2186	
AABrUAA//GGGGG	1.30 ± 0.30	4.53 ± 0.98	0.0004	AABrUAA//**GGGGG**
AABrUAA//ATTTA	8.05 ± 5.87	4.45 ± 0.97	0.3395	
GGBrUGG	17.85 ± 3.89			
GGBrUGG//AATAA	2.90 ± 1.13	6.55 ± 0.08	0.0450	GGBrUGG//**AATAA**
GGBrUGG//CCCCC	2.25 ± 0.49	2.92 ± 0.80	0.3770	
GGBrUGG//GGGGG	8.90 ± 0.71	4.97 ± 1.02	0.0189	**GGBrUGG**//GGGGG
GGBrUGG//ATTTA	3.47 ± 1.86	12.91 ± 2.52	0.0064	GGBrUGG//**ATTTA**
ATBrUTA	33.30 ± 6.66			
ATBrUTA//AATAA	4.85 ± 1.48	4.14 ± 1.89	0.6891	
ATBrUTA//CCCCC	13.18 ± 4.56	11.48 ± 1.64	0.5725	
ATBrUTA//GGGGG	6.35 ± 2.87	5.51 ± 1.02	0.6550	
ATBrUTA//ATTTA	21.14 ± 5.46	8.01 ± 4.20	0.0063	**ATBrUTA**//ATTTA

[a] p determined by a paired Student's t test

DISCUSSION

Contribution of secondary structure to strand break formation as a function of sequence

The interaction of ionizing radiation with DNA produces radicals through direct and indirect processes that eventually lead to base lesions and strand breaks (*3*). However, with the exception of charge transfer along the double helix, there is little evidence that the nature of the bases surrounding the initial radical influences the type of lesion produced (*31-34*). Even in tandem lesions such as intra- and interstrand cross-links, only the bases immediately adjacent to the initial radical are involved (*3, 8, 35-39*). This study provides the first evidence that the identity of the bases adjacent to the initial radical affects both the type (i.e., strand break vs cross-links) and location of lesions produced. Furthermore, we show that, even in single-stranded DNA, the nature of the bases surrounding the BrdU substitution determines the extent of strand breakage following irradiation. We previously reported the formation of strand breaks that were specific for single-stranded oligonucleotides in BrdU-substituted DNA (*30*). As expected, we observed that the same single-strand specificity first described for strand break generation in $AA^{Br}UAA$ also extended to $GG^{Br}UGG$ and $AT^{Br}UTA$ (Figure III-2). More intriguing was the observation that the identity of neighboring bases affects the production of strand breaks. Replacement of the DNA bases next to BrdU (replacement of A with T) in $AA^{Br}UAA$ increases the degree of formation of strand breaks in single-stranded $AT^{Br}UTA$ 2-fold. Conversely, replacement of A with G, which have oxidation potentials of 1.56 and 1.29 V at pH 7.0, respectively (*3*), did not affect the production of strand breaks in single- or double-stranded DNA. Thus, the degree of formation of single strand breaks is increased by the presence of a base with a relatively high electron affinity (T) but is not affected by a base with a low oxidation potential. Given the fact that T is a preferential site of electron addition in

normal DNA (*40, 41*), it is possible that T acts as an additional "electron antenna" to capture electrons and subsequently transfer them onto BrdU. However, the level of conversion of BrdU to 2′-deoxyuridine is only slightly (but not significantly) higher for AT^{Br}UTA than for AA^{Br}UAA and GG^{Br}UGG, indicating that this step is not limiting the production of strand breaks, and that generation of other products in AA^{Br}UAA and GG^{Br}UGG may explain the reduction observed in strand break yields. Incidentally, formation of intrastrand cross-links between BrdU and adjacent purines has been observed after UV irradiation (*38*). Although the mechanism for UV sensitization of BrdU-substituted DNA involves the production of two adjacent radicals, only one radical is produced in the case of ionizing radiation. However, it is possible that the same products may be produced by alternative pathways, depending on the radiation source. These results eloquently illustrate that not only the secondary structure but also the surrounding sequence affects the ability of BrdU to damage DNA during irradiation, influencing the type and yield of lesions produced.

Sequence-Dependent Formation of Interstrand Cross-Links and Strand Breaks in Mismatched DNA

ICL and Strand Break Yields

Figure III-3A shows that the efficiency of cross-link formation is highly dependent on the nature of the mismatched bases in the brominated as well as the opposite semicomplementary strand. These cross-links are produced with the same yield, whether the brominated or semi-complementary strand is labeled, which indicates that they represent interstrand cross-links, rather than interhelix cross-links. Cross-link preferences increased in the following order: T > A ~ G and C > T > A > G in the brominated and semicomplementary strands, respectively. Our observation that pyrimidines, regardless of the strand where they were located, produced more total frank damage (Figure III-3B) supports the hypothesis that solvated electrons may be attracted to sites containing a large number of electron affinic pyrimidines.

Interestingly, cytosine is present on the opposite strand of the two combinations that produced high yields of ICLs, which points to the 5,6-double bond of cytosine as a preferred site of attack for cross-linking (*6, 42*). The size of the mismatch also influences the production of ICLs and strand breaks; the three combinations that either produced a mismatched region smaller than 5 bp or where internal Watson-Crick bonds are possible ($AA^{Br}UAA$//ATTTA, $GG^{Br}UGG$//CCCCC and $AT^{Br}UTA$//AATAA) resulted in less damage than the other sequences. Interestingly, $AA^{Br}UAA$//GGGGG also produced the least damage of any sequence, possibly because of a particular secondary structure, as discussed below. Incidentally, the yield of strand breaks produced in $GG^{Br}UGG$//CCCCC is similar to that produced in the complementary double-stranded counterpart, with no detectable ICL produced. This indicates that a single mismatch in a run of GC base pairs closely resembles complementary DNA. In contrast, the A-T rich sequences, $AA^{Br}UAA$//ATTTA and $AT^{Br}UTA$//AATAA, in which the Watson-Crick bonds are surrounded by mismatched base pairs, probably produce a structure close to a typical 5 bp mismatched DNA. More intriguing, however, is the fact that $AA^{Br}UAA$//GGGGG, the only other sequence to produce no detectable cross-links and only trace levels of strand breaks, cannot form canonical DNA base pairs. Over the years, several reports have been published indicating that stretches of mismatched GA can form sheared base pairs, provided that certain requirements are satisfied regarding the surrounding nucleobases (*26, 43, 44*). However, these sheared base pairs were only reported for alternating GA//AG sequences, with no indication that an AA//GG mismatch can produce such a structure. Another possibility is that wobble G-A bonds are responsible for this behavior. Certainly, the data presented here provide further evidence that BrdU may serve as an internal sensor for probing DNA structure (*24, 45*). Moreover, our results indicate that the reactivity of open or mismatched regions of cellular DNA to radicals, as well as the type of damage that is produced, is probably highly dependent on the nature of bases surrounding the initial radical.

Strand Break Location and ICL Structures

For BrdU-substituted DNA, the mechanism of single-strand break formation involves attachment of an electron to BrdU, followed by departure of bromide anion, leading to the generation of uracil-5-yl radicals. In turn, these radicals react with DNA bases and sugar moieties to produce ICLs and strand breaks. Our results show that strand breaks are produced on the 5′ side of BrdU and at several positions on the opposite semicomplementary strand, indicating that in mismatched DNA, uracil-5-yl radicals are able to attack multiple sites (Figure III-4). It is unclear whether these strand breaks arise from the direct reaction of uracil-5-yl radicals with sugar moieties within the open duplex structure (as far as several bases away) and/or from a multistep reaction involving the formation of base radicals in the proximity of the initial uracil-5-yl radical, followed by transfer to the site of damage. The presence of a relatively long mismatched region in our experimental system is likely to alter the distance of reactive sites normally found in double-stranded DNA, which may permit a diffusion of damage in the proximity of nascent uracil-5-yl radicals.

Although strong evidence exists that mismatched nucleotides disrupt the normal behavior of DNA, such as charge transfer along the double helix (40, 46), little is known about how consecutive mismatches react following treatment with ionizing radiation. Thus, it is possible that the inherent flexibility of these DNA regions allows reactions that are not typically seen in complementary DNA. Also, given the fact that uracil-5-yl radicals are good hydrogen atom abstractors ($k_{2\text{-PrOH}} = 4.1 \times 10^7$ dm^3 mol^{-1} s^{-1}, (47)), mismatched regions of DNA may be particularly favorable for radical transfer to distant bases, followed by strand breakage. Our observation that fewer strand breaks occur on the brominated strand when BrdU is surrounded by guanines (Table 1, GG^{Br}UGG//AATAA and GG^{Br}UGG//ATTTA) suggests that adjacent bases, particularly guanine, interfere with the pathway leading to frank strand breaks. This hypothesis is supported by the decreased yield of strand breaks that is observed when purines are flanking the site of BrdU substitution, and which may be attributable to competitive reactions such as the formation of intrastrand cross-links. Whether this

interference involves the intermediate formation of guanine radical cations has not been established. Most often, these radicals do not directly lead to frank strand breaks but give 8-oxo-7,8-dihydroguanine, which is a relatively stable lesion, and other compounds, including 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 2,2,4-triamino-(5*H*)-oxazolone, which are alkali labile lesions (*48*) Previous observations by Graslund et al. (*49*) indicated an increase in the level of guanine cation radicals in BrdU-substituted oriented fibers, compared to unsubstituted fibers. However, this experiment was conducted in solid state hydrated DNA in the absence of radical scavengers. Thus, these radicals could also arise from either the direct interaction of radiation or hydroxyl radical attack on BrdU.

If we set aside the hypothesis of radical transfer, then the distributions of strand breaks shown in Figure III-4 must arise from the physical contact of the uracil-5-yl radical with the damaged sugar moiety. Strikingly, in the case of AA^{Br}UAA//CCCCC and AT^{Br}UTA//CCCCC, bases at the 5′ extremities of the mismatch are those with the higher yield of strand breaks, with strand breaks even being produced outside the mismatch in the case of GG^{Br}UGG//AATAA. If these strand breaks truly reflect the proximity of BrdU, this suggests that the structure of mismatched DNA regions is highly dependent on the sequence. In double-stranded complementary DNA, no strand breaks are observed on the strand opposite the BrdU. Undoubtedly, the presence of a mismatch in our experimental system affects the number and location of nucleotides susceptible to react, especially if mismatched regions of DNA adopt a zipper-like structure rather than a "bubble" configuration (*23, 24, 27, 50-52*). In zipper-like DNA structures, the bases interlace with their counterpart on the opposite strand, promoting close base-base contacts that may favor the transfer of a radical to the other strand. One of the best indications in favor of this model is the creation of ICLs, where a radical on one strand attacks a nucleotide on the opposite strand to generate a covalent bond.

Without a doubt, the most striking feature of the results presented in this study is the diversity of ICL structures that are obtained when the bases surrounding the BrdU are modified (Figure III-4). Overall, 10-12 distinct bands are observed, with several of them present in multiple DNA sequences. Of these, the three-band pattern observed in AA^{Br}UAA//CCCCC, AT^{Br}UTA//CCCCC, and AT^{Br}UTA//ATTTA is perhaps the most intriguing. First, it is present in pyrimidines rich sequences. Second, at least one of these bands is observed with a combination of other bands in four other sequences (AA^{Br}UAA//ATTTA, AT^{Br}UTA//AATAA, AT^{Br}UTA//GGGGG and GG^{Br}UGG//GGGGG), which indicates that they must represent structures with a higher likelihood of formation. This is supported by the higher ICL yield found in sequences that produce this three-band pattern. Unfortunately, the chemical structure of these cross-links cannot be inferred from their electrophoretic mobility. Nevertheless, valuable information can be extracted from these data, especially concerning the large number of sites that are involved in ICL formation. If, as discussed above, we dismiss radical transfer, then ICLs can only occur when the radical located at the site of BrdU substitution reacts with one of the five bases on the opposite strand. Because denaturing gel electrophoresis cannot resolve symmetrical structures with the same molecular weight, this predicts that only three different bands should be observed on denaturing gels. Since a greater number of bands are observed, this supports our hypothesis that transfer of a radical to adjacent nucleotides does occur, on both the brominated and the opposite strand, which would greatly increase the number of sites susceptible to being part of an ICL and consequently the number of bands found on the gel. Although radical transfer has not traditionally been considered to be part of the sensitization mechanism, the data presented here certainly raise the question of whether transfer to distant bases is an important part of BrdU sensitization in mismatched DNA. Undoubtedly, the identity of the surrounding bases influences the location of strand breaks, as well as the ICL structures that are produced in open or mismatched regions of DNA. Given the specificity we reported for ICL formation, it is entirely possible that ICL "hot spots" exist in regions of cellular DNA that possess the appropriate sequence and structural characteristics.

5-Bromodeoxyuridine: a Tool for the Study of ICL Repair?

In this study, we found that BrdU-substituted DNA, when exposed to ionizing radiation, produces highly sequence-dependent ICLs. These ICLs are so dependent on the nucleotide sequence that 10-12 structures result from a single initial radical. More importantly, we were able to produce a single major ICL structure with $GG^{Br}UGG//AATAA$. This is of particular interest to the field of ICL repair, which requires the production of single structures to study how these lesions are processed by DNA repair and replication machinery. Furthermore, because most ICL-inducing agents studied to date produce bulky lesions as well as mono-adducts, which can influence repair mechanisms, it would be advantageous to investigate how cells deal with non-bulky DNA cross-link lesions (*53, 54*). However, several of the agents developed over the years to form these types of cross-links cannot be incorporated into cellular DNA (*55-57*), and thus they may not adequately represent repair mechanisms in cells. Finally, as indirect evidence of the existence of BrdU-specific ICLs was recently found in cells (*58, 59*), information regarding its repair in cancer cells would be of high interest in view of future preclinical and clinical studies (*60*).

References

1. Jeggo, P. A., and Lobrich, M. (2007) DNA Double-Strand Breaks: Their Cellular and Clinical Impact? *Oncogene. 26*, 7717-7719.

2. Jeggo, P., and Lobrich, M. (2006) Radiation-Induced DNA Damage Responses. *Radiat. Prot. Dosimetry. 122*, 124-127.

3. von Sonntag, C. (2006) *Free-Radical-Induced DNA Damage and its Repair.* Springer-Verlag, Berlin.

4. Noll, D. M., Mason, T. M., and Miller, P. S. (2006) Formation and Repair of Interstrand Cross-Links in DNA. *Chem. Rev. 106*, 277-301.

5. Grillari, J., Katinger, H., and Voglauer, R. (2007) Contributions of DNA Interstrand Cross-Links to Aging of Cells and Organisms. *Nucleic Acids Res. 35*, 7566-7576.

6. Hong, H., Cao, H., and Wang, Y. (2007) Formation and Genotoxicity of a Guanine-Cytosine Intrastrand Cross-Link Lesion in Vivo. *Nucleic Acids Res. 35*, 7118-7127.

7. Regulus, P., Duroux, B., Bayle, P. A., Favier, A., Cadet, J., and Ravanat, J. L. (2007) Oxidation of the Sugar Moiety of DNA by Ionizing Radiation Or Bleomycin could Induce the Formation of a Cluster DNA Lesion. *Proc. Natl. Acad. Sci. U. S. A. 104*, 14032-14037.

8. Bellon, S., Ravanat, J. L., Gasparutto, D., and Cadet, J. (2002) Cross-Linked Thymine-Purine Base Tandem Lesions: Synthesis, Characterization, and Measurement in Gamma-Irradiated Isolated DNA. *Chem. Res. Toxicol. 15*, 598-606.

9. Jiang, Y., Hong, H., Cao, H., and Wang, Y. (2007) In Vivo Formation and in Vitro Replication of a Guanine−Thymine Intrastrand Cross-Link Lesion†. *Biochemistry (N. Y.). 46*, 12757-12763.

10. Zhang, Q., and Wang, Y. (2003) Independent Generation of 5-(2'-Deoxycytidinyl)Methyl Radical and the Formation of a Novel Cross-Link Lesion between 5-Methylcytosine and Guanine. *J. Am. Chem. Soc. 125*, 12795-12802.

11. Zhang, Q., and Wang, Y. (2005) Generation of 5-(2'-Deoxycytidyl)Methyl Radical and the Formation of Intrastrand Cross-Link Lesions in Oligodeoxyribonucleotides. *Nucl. Acids Res. 33*, 1593-1603.

12. Gu, C., and Wang, Y. (2004) LC-MS/MS Identification and Yeast Polymerase η Bypass of a Novel γ-Irradiation-Induced Intrastrand Cross-Link Lesion G[8−5]C†. *Biochemistry (N. Y.). 43*, 6745-6750.

13. Ding, H., and Greenberg, M. M. (2007) Gamma-Radiolysis and Hydroxyl Radical Produce Interstrand Cross-Links in DNA Involving Thymidine. *Chem. Res. Toxicol. 20*, 1623-1628.

14. Hong, I. S., and Greenberg, M. M. (2005) Efficient DNA Interstrand Cross-Link Formation from a Nucleotide Radical. *J. Am. Chem. Soc. 127*, 3692-3693.

15. Leif, R. C., Stein, J. H., and Zucker, R. M. (2004) A Short History of the Initial Application of Anti-5-BrdU to the Detection and Measurement of S Phase. *Cytometry A. 58*, 45-52.

16. Quinones-Hinojosa, A., Sanai, N., Smith, J. S., and McDermott, M. W. (2005) Techniques to Assess the Proliferative Potential of Brain Tumors. *J. Neurooncol. 74*, 19-30.

17. Eisch, A. J., and Mandyam, C. D. (2007) Adult Neurogenesis: Can Analysis of Cell Cycle Proteins Move Us "Beyond BrdU"? *Curr. Pharm. Biotechnol. 8*, 147-165.

18. Dewey, W. C., and Humphrey, R. M. (1965) Increase in Radiosensitivity to Ionizing Radiation Related to Replacement of Thymidine in Mammalian Cells with 5-Bromodeoxyuridine. *Radiat. Res. 26*, 538-553.

19. Sano, K., Hoshino, T., and Nagai, M. (1968) Radiosensitization of Brain Tumor Cells with a Thymidine Analogue (Bromouridine). *J. Neurosurg. 28*, 530-538.

20. Limoli, C. L., and Ward, J. F. (1993) A New Method for Introducing Double-Strand Breaks into Cellular DNA. *Radiat. Res. 134*, 160-169.

21. Saffhill, R., and Ockey, C. H. (1985) Strand Breaks Arising from the Repair of the 5-Bromodeoxyuridine-Substituted Template and Methyl Methanesulphonate-Induced Lesions can Explain the Formation of Sister Chromatid Exchanges. *Chromosoma. 92*, 218-224.

22. Dewey, W. C., Sedita, B. A., and Humphrey, R. M. (1966) Radiosensitization of X Chromosome of Chinese Hamster Cells Related to Incorporation of 5-Bromodeoxyuridine. *Science. 152*, 519-521.

23. Cecchini, S., Girouard, S., Huels, M. A., Sanche, L., and Hunting, D. J. (2005) Interstrand Cross-Links: A New Type of Gamma-Ray Damage in Bromodeoxyuridine-Substituted DNA. *Biochemistry. 44*, 1932-1940.

24. Dextraze, M. -., Wagner, J. R., and Hunting, D. J. (2007) 5-Bromodeoxyuridine Radiosensitization: Conformation-Dependent DNA Damage. *Biochemistry. 46*, 9089-9097.

25. Ferrer, N., Azorin, F., Villasante, A., Gutierrez, C., and Abad, J. P. (1995) Centromeric Dodeca-Satellite DNA Sequences Form Fold-Back Structures. *J. Mol. Biol. 245*, 8-21.

26. Huertas, D., and Azorin, F. (1996) Structural Polymorphism of Homopurine DNA Sequences. d(GGA)n and d(GGGA)n Repeats Form Intramolecular Hairpins Stabilized by Different Base-Pairing Interactions. *Biochemistry. 35*, 13125-13135.

27. Shepard, W., Cruse, W. B., Fourme, R., de la Fortelle, E., and Prangé, T. (1998) A Zipper-Like Duplex in DNA: The Crystal Structure of d(GCGAAAGCT) at 2.1 å Resolution. *Structure. 6*, 849-861.

28. Elmroth, K., Nygren, J., Stenerlow, B., and Hultborn, R. (2003) Chromatin- and Temperature-Dependent Modulation of Radiation-Induced Double-Strand Breaks. *Int. J. Radiat. Biol. 79*, 809-816.

29. Warters, R. L., and Lyons, B. W. (1992) Variation in Radiation-Induced Formation of DNA Double-Strand Breaks as a Function of Chromatin Structure. *Radiat. Res. 130*, 309-318.

30. Cecchini, S., Girouard, S., Huels, M. A., Sanche, L., and Hunting, D. J. (2004) Single-Strand-Specific Radiosensitization of DNA by Bromodeoxyuridine. *Radiat. Res. 162*, 604-615.

31. Edfeldt, N. B., Harwood, E. A., Sigurdsson, S. T., Hopkins, P. B., and Reid, B. R. (2004) Sequence Context Effect on the Structure of Nitrous Acid Induced DNA Interstrand Cross-Links. *Nucleic Acids Res. 32*, 2795-2801.

32. Giese, B. (2002) Electron Transfer in DNA. *Curr. Opin. Chem. Biol. 6*, 612-618.

33. Giese, B., Amaudrut, J., Kohler, A. K., Spormann, M., and Wessely, S. (2001) Direct Observation of Hole Transfer through DNA by Hopping between Adenine Bases and by Tunnelling. *Nature. 412*, 318-320.

34. Wagenknecht, H. A. (2006) Electron Transfer Processes in DNA: Mechanisms, Biological Relevance and Applications in DNA Analytics. *Nat. Prod. Rep. 23*, 973-1006.

35. Box, H. C., Dawidzik, J. B., and Budzinski, E. E. (2001) Free Radical-Induced Double Lesions in DNA. *Free Radic. Biol. Med. 31*, 856-868.

36. Kovanda, J., Kejnovsky, E., Arnold, L., and Kypr, J. (1996) UV Light-Induced Crosslinking of Short DNA Duplex Strands: Nucleotide Sequence Preferences and a Prominent Role of the Duplex Ends. *J. Biomol. Struct. Dyn. 14*, 57-65.

37. Nejedly, K., Kittner, R., Pospisilova, S., and Kypr, J. (2001) Crosslinking of the Complementary Strands of DNA by UV Light: Dependence on the Oligonucleotide Composition of the UV Irradiated DNA. *Biochim. Biophys. Acta. 1517*, 365-375.

38. Zeng, Y., and Wang, Y. (2006) Sequence-Dependent Formation of Intrastrand Crosslink Products from the UVB Irradiation of Duplex DNA Containing a 5-Bromo-2'-Deoxyuridine Or 5-Bromo-2'-Deoxycytidine. *Nucleic Acids Res. 34*, 6521-6529.

39. Zeng, Y., and Wang, Y. (2007) UVB-Induced Formation of Intrastrand Cross-Link Products of DNA in MCF-7 Cells Treated with 5-Bromo-2'-Deoxyuridine. *Biochemistry. 46*, 8189-8195.

40. Wagenknecht, H. A. (2003) Reductive Electron Transfer and Transport of Excess Electrons in DNA. *Angew. Chem. Int. Ed Engl. 42*, 2454-2460.

41. Fuciarelli, A. F., Sisk, E. C., and Zimbrick, J. D. (1994) Electron Migration in Oligonucleotides upon Gamma-Irradiation in Solution. *Int. J. Radiat. Biol. 65*, 409-418.

42. Zhang, Q., and Wang, Y. (2004) Independent Generation of the 5-Hydroxy-5,6-Dihydrothymidin-6-Yl Radical and its Reactivity in Dinucleoside Monophosphates. *J. Am. Chem. Soc. 126*, 13287-13297.

43. Heus, H. A., Wijmenga, S. S., Hoppe, H., and Hilbers, C. W. (1997) The Detailed Structure of Tandem G.A Mismatched Base-Pair Motifs in RNA Duplexes is Context Dependent. *J. Mol. Biol. 271*, 147-158.

44. Li, Y., Zon, G., and Wilson, W. D. (1991) Thermodynamics of DNA Duplexes with Adjacent G.A Mismatches. *Biochemistry. 30*, 7566-7572.

45. Xu, Y., Tashiro, R., and Sugiyama, H. (2007) Photochemical Determination of Different DNA Structures. *Nat. Protoc. 2*, 78-87.

46. Boon, E. M., and Barton, J. K. (2003) DNA Electrochemistry as a Probe of Base Pair Stacking in A-, B-, and Z-Form DNA. *Bioconjugate Chem. 14*, 1140-1147..

47. Mertens, R., and Sonntag, C. v. (1994) Determination of the Kinetics of Vinyl Radical Reactions by the Characteristic Visible Absorption Spectra of Vinylperoxyl Radicals. *Angewandte Chemie International Edition in English. 33*, 1262-1264.

48. Cadet, J., Douki, T., Gasparutto, D., and Ravanat, J. L. (2003) Oxidative Damage to DNA: Formation, Measurement and Biochemical Features. *Mutat. Res. 531*, 5-23.

49. Graslund, A., Rupprecht, A., Kohnlein, W., and Huttermann, J. (1981) Radiation-Induced Free Radicals in Oriented Bromouracil-Substituted DNA. *Radiat. Res. 88*, 1-10.

50. Chou, S. H., Chin, K. H., and Wang, A. H. (2003) Unusual DNA Duplex and Hairpin Motifs. *Nucleic Acids Res. 31*, 2461-2474.

51. Chou, S. H., and Chin, K. H. (2001) Zipper-Like Watson-Crick Base-Pairs. *J. Mol. Biol. 312*, 753-768.

52. Chou, S. H., and Chin, K. H. (2001) Solution Structure of a DNA Double Helix Incorporating Four Consecutive Non-Watson-Crick Base-Pairs. *J. Mol. Biol. 312*, 769-781.

53. Berardini, M., Mackay, W., and Loechler, E. L. (1997) Evidence for a Recombination-Independent Pathway for the Repair of DNA Interstrand Cross-Links Based on a Site-Specific Study with Nitrogen Mustard. *Biochemistry. 36*, 3506-3513.

54. McHugh, P. J., Spanswick, V. J., and Hartley, J. A. (2001) Repair of DNA Interstrand Crosslinks: Molecular Mechanisms and Clinical Relevance. *Lancet Oncol. 2*, 483-490.

55. Alzeer, J., and Scharer, O. D. (2006) A Modified Thymine for the Synthesis of Site-Specific Thymine-Guanine DNA Interstrand Crosslinks. *Nucleic Acids Res. 34*, 4458-4466.

56. Noll, D. M., Noronha, A. M., Wilds, C. J., and Miller, P. S. (2004) Preparation of Interstrand Cross-Linked DNA Oligonucleotide Duplexes. *Front. Biosci. 9*, 421-437.

57. Wilds, C. J., Noronha, A. M., Robidoux, S., and Miller, P. S. (2005) Synthesis and Characterization of DNA Duplexes Containing an N3T-Ethyl-N3T Interstrand Crosslink in Opposite Orientations. *Nucleosides Nucleotides Nucleic Acids. 24*, 965-969.

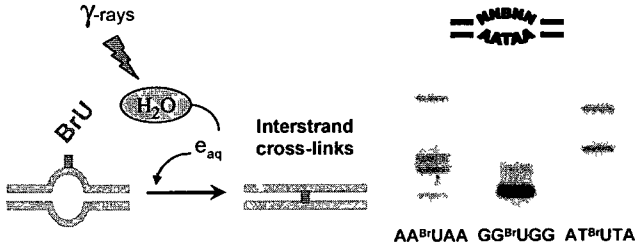
58. Wojcik, A., Bochenek, A., Lankoff, A., Lisowska, H., Padjas, A., Szumiel, I., von Sonntag, C., and Obe, G. (2006) DNA Interstrand Crosslinks are Induced in Cells Prelabelled with 5-Bromo-2'-Deoxyuridine and Exposed to UVC Radiation. *J. Photochem. Photobiol. B. 84*, 15-20.

59. Bruckmann, E., Wojcik, A., and Obe, G. (1999) X-Irradiation of G1 CHO Cells Induces SCE which are both True and False in BrdU-Substituted Cells but Only False in Biotin-dUTP-Substituted Cells. *Chromosome Res. 7*, 277-288.

60. Wardman, P. (2007) Chemical Radiosensitizers for use in Radiotherapy. *Clin. Oncol. (R. Coll. Radiol). 19*, 397-417.

For Table of Contents Use Only

Marie-Eve Dextraze, Sylvain Cecchini, François Bergeron, Sonia Girouard, Kathleen Turcotte, J. Richard Wagner and Darel J. Hunting. Reaching for the other side: generating sequence-dependent interstrand cross-links with 5-bromodeoxyuridine and γ-rays.



Chapitre IV – 3e article

Mapping interstrand cross-links induced by gamma and UVB radiation in bromodeoxyuridine-substituted DNA.

Auteurs de l'article : Marie-Eve Dextraze, J. Richard Wagner and Darel J. Hunting

Statut de l'article : En préparation.

Avant-propos

Contribution à la rédaction et à l'expérimentation :

J'ai effectué la totalité des expériences présentées dans cet article. J'ai aussi rédigé cet article, sous la supervision de mes co-directeurs, Richard Wagner et Darel Hunting.

Résumé :

Cet article présente une méthode visant à identifier l'emplacement des dimères interbrins formés lorsque l'ADN bromé est soumis aux radiations. En effet, nos expériences précédentes ont montré que près d'une douzaine de bandes différentes étaient observées lorsque l'ADN bromé irradié était analysé par électrophorèse, ce qui représente au moins une douzaine de structures distinctes potentielles. Grâce à cette méthode, les dimères sont transformés en cassures, ce qui permet de déterminer leur emplacement dans l'ADN. Ainsi, nous avons établi que les dimères formés étaient hautement dépendants de la séquence entourant le BrdU et la source de radiation

utilisée pour produire les dimères. Les résultats montrent clairement que le transfert de radicaux est possible dans un ADN mésapparié. Les résultats de cette étude suggèrent aussi, pour la première fois, que le radical cation formé lors de l'irradiation de l'ADN bromé avec des rayons UV est impliqué dans la formation du dimère.

Mapping interstrand cross-links induced by gamma and UVB radiation in bromodeoxyuridine-substituted DNA

† *This work was supported by the Natural Sciences and Engineering Research Council*

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ABSTRACT Recently, we reported the formation of interstrand cross-links that were specific for mismatched nucleotides within bromodeoxyuridine-substituted DNA that had been exposed to ionizing and UV radiation. Given that several bands were observed when the cross-links were analyzed by denaturing gel electrophoresis, we proposed that these bands represented distinct structures formed by a single initial radical. To further study the formation of interstrand cross-links in mismatched DNA, we submitted the purified cross-links to formic acid and piperidine treatment, thus transforming the cross-linked bases into strand breaks, so that their location could then be mapped using denaturing gel electrophoresis. Using this method, we observed that bases adjacent to the site of the initial radical were involved in the cross-links, which supports the hypothesis that radical transfer occurs in mismatched DNA, and is highly dependent on sequence.

Double-strand breaks are generally considered to be one of the most toxic types of DNA lesions produced by ionizing radiation (1-3). However, evidence of the formation of another potentially more toxic type of lesion, interstrand cross-links, is now emerging. Interstrand cross-links (ICLs), where the two DNA strands are covalently linked, prevent the separation of the DNA strands, thus blocking several key cellular processes (4-6). Traditionally, ionizing radiation has not been regarded as a cross-linking agent; however, several instances of intrastrand cross-link formation have been reported and recent data points to the existence of interstrand cross-links induced by hydroxyl radicals in synthetic DNA and in cells (7, 8). Nevertheless, there is yet very little information regarding ICL formation and repair in cells that have been subjected to ionizing radiation.

5-bromo-2′-deoxyuridine (BrdU) is a thymidine analogue that can be incorporated into cellular DNA. Although it is now widely used as a marker of replicating cells (9, 10), it was first reported as a photo- and radiosensitizing agent (11, 12). When exposed to ionizing radiation, BrdU produces single- and double-strand breaks, as well as chromosomal aberrations that are generally believed to be responsible for its sensitizing properties (13-15). The mechanism for single strand break formation involves electron attachment to BrdU, followed by the departure of a bromide anion and generation of an uridin-yl radical that further reacts to create strand breaks (16). Recently, we reported the formation of a new type of BrdU-sensitized damage in synthetic DNA: ICLs that were specific for mismatched nucleotides within BrdU-substituted DNA (17). These ICLs were later found to be highly dependent on the presence regional DNA structure and sequence (18, 19), supporting the evidence that a precise regional environment was a prerequisite for their formation and that these ICLs may only be produced in open or mismatched regions of cellular DNA. Furthermore, when the effect of the surrounding sequence was studied, several bands with distinct electrophoretic mobility were observed in the region corresponding to the ICL, outlining the possibility that several ICL structures were formed by a single initial radical.

In the present study, we modified a method developed by Millard et al. (20) to determine the location of ICLs in BrdU-substituted DNA exposed to ionizing radiation. The ICLs were purified by preparative denaturing gel electrophoresis, and then were treated with cold formic acid and hot piperidine, thus transforming the cross-linked sites into strand breaks. This protocol allows us to map the precise location of the ICLs. To validate the protocol, we applied it to Mitomycin-induced cross-links already described in the study by Millard, and confirmed that the observed location of the cross-link with our method was the same as originally reported. Moreover, the sequence preference was in good agreement with the literature. Finally, we applied this method to 3 of the 12 BrdU-substituted sequences originally studied. Furthermore, we compared the ICLs produced under gamma and UV radiation as a function of sequence. We found that the sites involved in the cross-linking process are highly dependent on the surrounding sequence, as well as the type of radiation used. Furthermore, our results substantiate the hypothesis that radical transfer does occur in consecutive mismatches, further supporting our proposition that mismatched or open regions of cellular DNA are hotspots for ICL formation.

MATERIALS AND METHODS

Oligonucleotide design

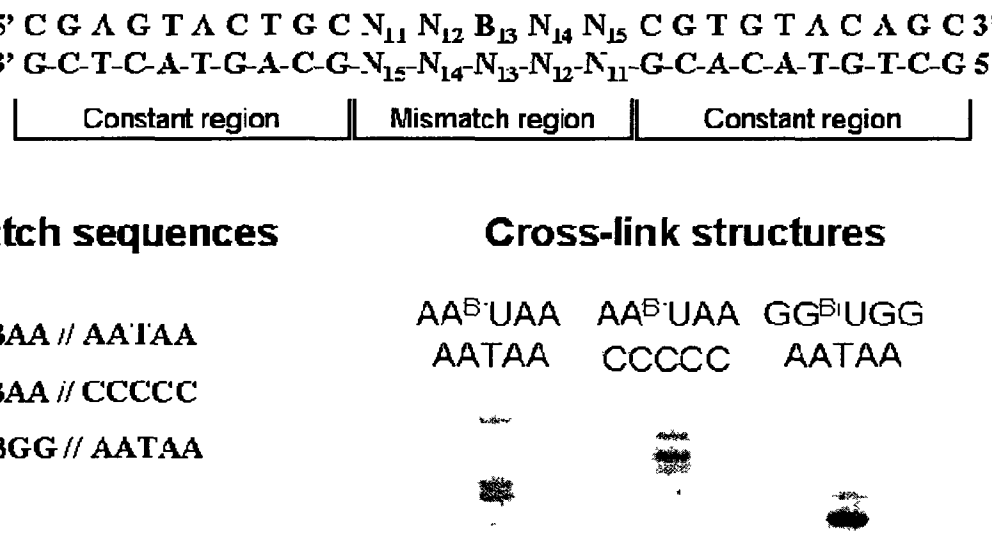

Figure IV-1: Oligonucleotide design. Each 25-mer sequence consisted of a variable central 5 bp region, surrounded by two constant regions. The ICLs formed following irradiation of three mismatch sequences were investigated, with the ICL bands observed by denaturing gel electrophoresis shown right.

Oligonucleotides 5-Bromodeoxyuridine modified and nonmodified oligonucleotides were purchased from the University Core DNA Services (University of Calgary, AB, Canada). Oligonucleotides were designed on the basis of previous sequences used by our group. Each sequence included two constant regions, with a variable central 5 bp region described in Figure IV-1. DNA was end-labeled either with ^{32}P [γ-ATP] using T4 polynucleotide kinase (Amersham Pharmacia Biotech) for 5′ labeling, or ^{32}P [α-ATP] using terminal transferase (Roche) for 3′ labeling. Labeling was carried at an initial oligonucleotide concentration of 2 μM for 45min at 37 °C with 10 U of enzyme. The enzyme was inactivated by heating for 10 min at 75 °C. Oligonucleotides were then purified on a G50 Sephadex microcolumn and the

labeling efficiency was assessed by counting 1µl of the labeling reaction with a RackBeta 1219 liquid scintillation counter (LKB Wallac). Hybridization was carried out at a final concentration of 1.4 µM of the labeled strand and a 2-fold excess of the unlabeled strand in a volume of 56µl. Samples were heated to 82 °C for 5 min, then cooled slowly for 2.5h. Deionized, sterile water was used in all experimental protocols.

Preparation of mitomycin C-cross-linked DNA Cross-links were prepared as described in details by Millard et al (20). Briefly, 100 pmol of DNA was radiolabeled, as described above. Afterwards, 76 pmol of labeled DNA was hybridized with 76 pmol of complementary DNA and hybridized by heating to 82 °C for 5 min, then cooled slowly for 2.5h and lyophilized. The double stranded DNA was then resuspended in 100 µL of 15 mM Tris (pH 7.5) and 10 µL of mitomycin C (20 mM in 33% aqueous methanol) and incubated for 1 h at 37 °C. The samples were then placed in a septa covered tube and deoxygenated by bubbling through with nitrogen for 15 min, before being put on ice. Activation of mitomycin C was carried out by 3 additions of 6 µL of fresh sodium hydrosulfite at 15 minutes intervals. The sodium hydrosulfite solution was prepared by flushing 0.0087 g of sodium hydrosulfite (Sigma) in a septa-covered tube with nitrogen, then by adding 1.5 mL of deoxygenated water (bubbled for 25 min with argon) with a syringe through the septum, for a final concentration of 33 mM. The sodium hydrosulfite solution was rapidly vortexed and used within 1 min. Fifteen minutes after the last addition, 3µl of glycerol was added and the cross-linked DNA was loaded onto the preparative gel.

Irradiation For gamma-irradiation, 50µl of 5′ labeled, hybridized DNA, 3µl of EDTA 0.5M (used as a hydroxyl radical scavenger) and 3µl of 100 mM phosphate buffer, pH 7.5, was bubbled for 2 min with N_2 to remove excess oxygen. Oligonucleotides were given 2400 Gy, either with a Gammacell 220 (^{60}Co, Nordion Canada, 2.6 Gy/min) or with a Gammacell 3000 Elan (^{137}Cs, Best Theratronics, 12.1 Gy/min). For UV irradiation, 50µl of hybridized DNA and 3µl of 100 mM phosphate

buffer (pH 7.5) was irradiated with 10 J/cm^2 for 90 min using a 1000 W Hg-Xe arc lamp (Oriel Corp.) fitted with an infrared filter and monochromator set at 313 ±2 nm (Spectral Energy). The results for each experiment were confirmed by performing the experiment with the 3′ ends labeled.

Cross-link purification by preparative gel electrophoresis Interstrand cross-links and undamaged DNA were separated by preparative denaturing gel electrophoresis. Briefly, irradiated or mitomycin-treated samples were loaded onto a 19.5 cm × 20.5 cm denaturing gel (1X TBE buffer, 20% acrylamide and 7M urea), where a reversed comb had been used to create a single well. Migration was carried out at 20 W for ~1h15, or until bromophenol blue migrated to 10cm of the bottom of the gel. The gel was then exposed for at least 2h in a Phosphor Screen cassette (Molecular Dynamics, Inc.), and was scanned with a fluorescence scanner (Storm, Molecular Dynamics, Inc.) The bands corresponding to the ICLs and parental strand were excised and recuperated in 1.5 mL tubes. The gel plugs were then flash-frozen in liquid nitrogen and crushed with a pestle. Sterile water (1 mL) was added to each tube, and samples were vortexed twice (4 hours and overnight, respectively) in order to extract the DNA. Each time, the mixture of eluted DNA and acrylamide was centrifuged for 5 min before the DNA supernatant was recuperated. Samples were desalted using either two consecutive homemade Sephadex G-50 columns or a single Illustra NAP-25 column (GE Healthcare), and then dried using a Speedvac evaporator.

Mild hydrolysis treatment and gel analysis The purified DNA was resuspended in 100µl sterile water and the recuperation efficiency was assessed by counting 1µl with a LKB scintillation counter. Unirradiated single stranded DNA, purified ICLs, as well as the irradiated parental strand was submitted to the treatment. The mild hydrolysis treatment was as follow: for each reaction, 20 000 cpm was diluted in a final volume of 20µl. Afterwards, 50µl of formic acid (J.T. Baker, 88%) was added to the chilled tubes, with a 5 min incubation period on ice. Sample were then lyophilized with a Speedvac evaporator and washed once with 30µl distilled

water to remove the remaining formic acid. Subsequently, 20µl of 10% piperidine was added and the DNA was incubated for 30min at 90°C. Samples were then lyophilized and three water chases were performed by adding sequentially 30µl, 30µl and 20µl distilled water and evaporating to dryness. Control experiments using only the cold formic acid and hot piperidine treatments were also loaded on the same gel. Following the hydrolysis treatment, the analysis was performed by classical denaturing gel electrophoresis (1X TBE buffer, 20% acrylamide, 7M urea, 35 × 43 cm) using a DNA ladder generated by chemical sequencing (G + A reaction).

RESULTS

Method validation: Mitomycin C

The increased reactivity found in an open region of DNA following irradiation was first reported by our group in 2009, where we showed that a single radical created in a 5-base mismatched region created up to twelve distinct bands when ICLs production was examined by denaturing gel electrophoresis (19). We studied twelve sequence combinations, and found that in a given sequence, the initial radical produced several bands, some of which were also observed in other sequences. At that time, we proposed that these bands represented different structures, and that this was evidence that radical migration occurred between the site of the initial radical and surrounding nucleotides, which then lead to ICL production. However, it was impossible to confirm this by gel electrophoresis. Given the large number of possible structures, we decided to characterize the cross-links by gel electrophoresis using their sensitivity to mild acidic and basic conditions as criteria for locating their position in oligonucleotides. To this end, the analysis of cross-links induced by BrdU was accomplished using a method first developed by Hopkins in 1991.

A

5′ AACCTATAA [TTCG$_{13}$ATATTG$_{19}$CG$_{21}$CT] ATAATTA 3′
3′ GATATT [AAGC$_{13}$TATAAC$_{19}$GC$_{21}$GA] TATTAATTAA 5′

B

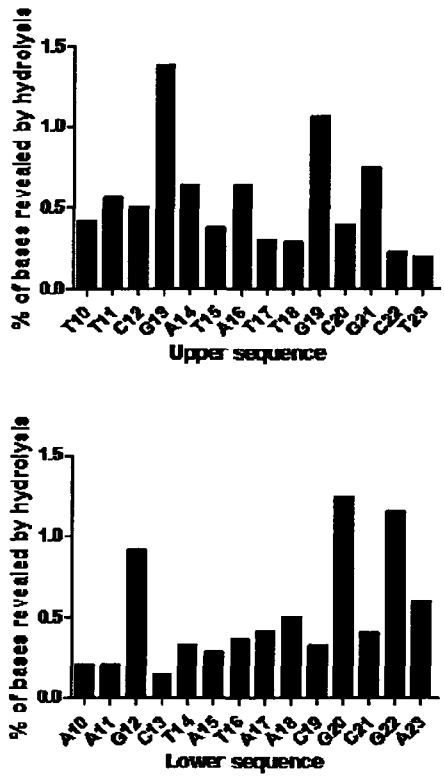

Figure IV-2: Method validation. The use of the mild hydrolysis protocol to locate the cross-linked sites was validated with cross-link with a well-known structure. Three cross-linking sites were present at G_{13}, G_{19}, and G_{21} (panel A, on the upper strand). When the mild hydrolysis protocol was applied, these sites were further processes as strand breaks, and appeared as shorter fragments on a denaturing gel electrophoresis. The relative yield is shown for both on the upper strand and lower strand (panel B, upper and lower panel, respectively).

To validate the method proposed in this paper, we applied it to an ICL with a chemistry and sequence preference that was well-known and confirmed by both gel electrophoresis and mass spectrometry. Mitomycin C is a chemotherapeutic agent that reacts with two adjacent guanines upon reductive activation (21-23), and is one of the cross-linking agents initially investigated by Millard et al. Thus, we applied our mild hydrolysis protocol to cross-links that had been induced by mitomycin C within one of the sequences that had been used originally.

Figure IV-2 (panel A) shows the sequence used to validate our method using mitomycin C-induced cross-links. Three 5′-CpG-3′ sites are included (at positions 13, 19 and 21 on the upper strand, respectively), which represent three separate possible interstrand cross-linking sites. Panel B shows the bases that were revealed by the mild hydrolysis treatment, which reflect the bases that are involved in the ICLs. As expected, the three potential cross-linking sites show increased reactivity, independent of the strand that is labeled, although the reactivity of the G21 site was closer to the background level when the upper strand was investigated. Furthermore, in good agreement with the results presented by Hopkins, we obtained ratios of 1.3:1 and 2.6:1 for the reactivity between the 5′-GCGC and the 5′-TCGA sites (for the upper and lower strands, respectively), compared to 2:1 in the literature (24). Thus, the mitomycin-induced ICL responds to the mild hydrolysis treatment, revealing the sites of the cross-linked guanines, as well as the existence of a preferred cross-linking sequence.

Ionizing irradiation

To study the reactions involved in the cross-linking process, three of the twelve sequences we had previously studied were chosen, each with a distinctive band pattern. AA^{Br}UAA//AATAA was chosen because of the distinctive pattern of bands on gel, as well as the fact that these bands were for the most part unique to this sequence. AA^{Br}UAA//CCCCC was chosen because of the three-band ICL pattern, whose bands were most commonly found among the other sequences and which possibly represented structures with a high probability of formation. GG^{Br}UGG//AATAA was chosen because of its single major band, since it would be a likely target for future structure determination. Furthermore, these sequences allowed us to examine the effect of variations in the brominated and opposite strand on the chemistry of ICL formation.

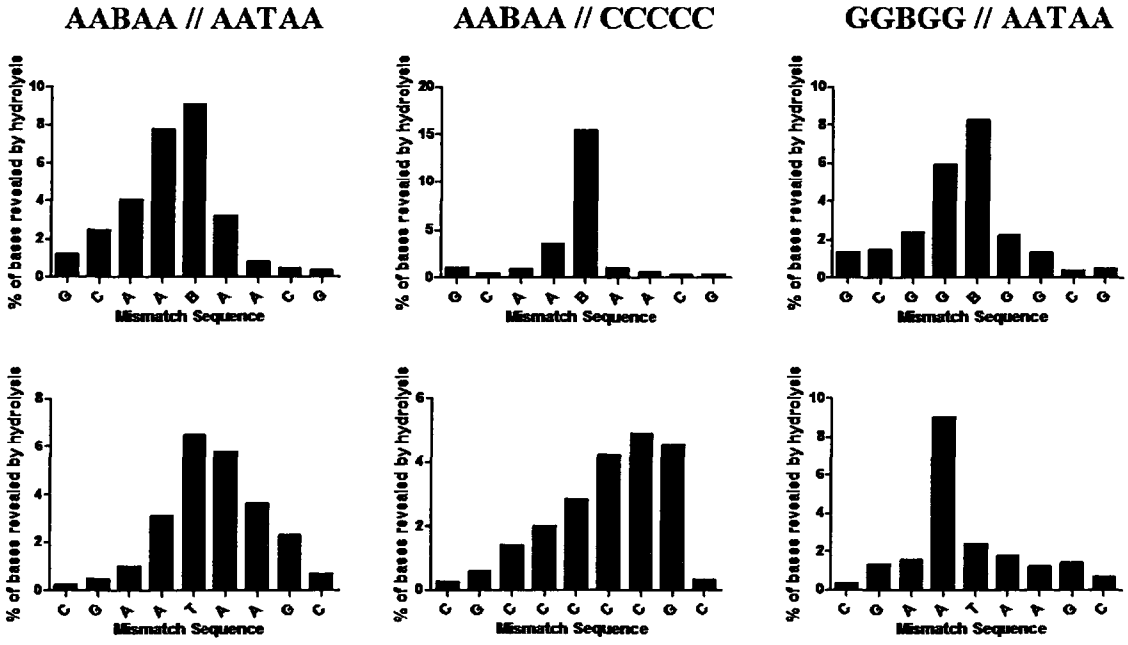

Figure IV-3: Cross-linked sites as a function of sequence (ionizing radiation). The mild hydrolysis protocol was applied to purified ICLs formed after exposition of the three mismatched sequences (left, AA^{Br}UAA//AATAA; center, AA^{Br}UAA//CCCCC; right, GG^{Br}UGG//AATAA) to ionizing irradiation. The degradation profile is shown for the brominated strand (upper line) and the opposite strand (lower line) for 5′ labeled oligos.

Figure IV-3 shows the location of cross-linking sites in each of the three chosen sequences when ionizing radiation was used. While several sites on the brominated strand are involved in the cross-linking process in the case of AA^{Br}UAA//AATAA, only one major site is observed for AA^{Br}UAA//CCCCC. As for GGBrUGG//AATAA, while two notable sites are observed on the brominated strand, in both cases the base react with the 3′ (A_{14}) adenine on the opposite strand. This allows the identification of the major band observed on gel as a cross-link between B_{13}-A_{14} and the minor band as G_{12}-A_{14}. Furthermore, the G_{12}-A_{14} ICL was found to lose one of its four arms, thus converting it from a X to a Y type structure (see supporting information).

When the reactivity of the different ICLs to the hydrolysis treatment was investigated, two different profiles were observed. For all but one of the sequence, no damage modification was observed on gels when DNA was treated with formic acid only, i.e.

no additional cross-link unhooking or phosphodiester bond cleavage was found (in the form of parental strand or strand break increase, respectively). Only in the case of AA^{Br}UAA//AATAA treated with γ-rays did formic acid lead to an increase in the proportion of the parental strand (figure IV-4). Formic acid led to the unhooking of the ICLs, which then created further strand breaks when the piperidine treatment was applied. However, when this sequence was irradiated with UV rays, the behavior was similar to the other sequences, with no discernable reaction to formic acid.

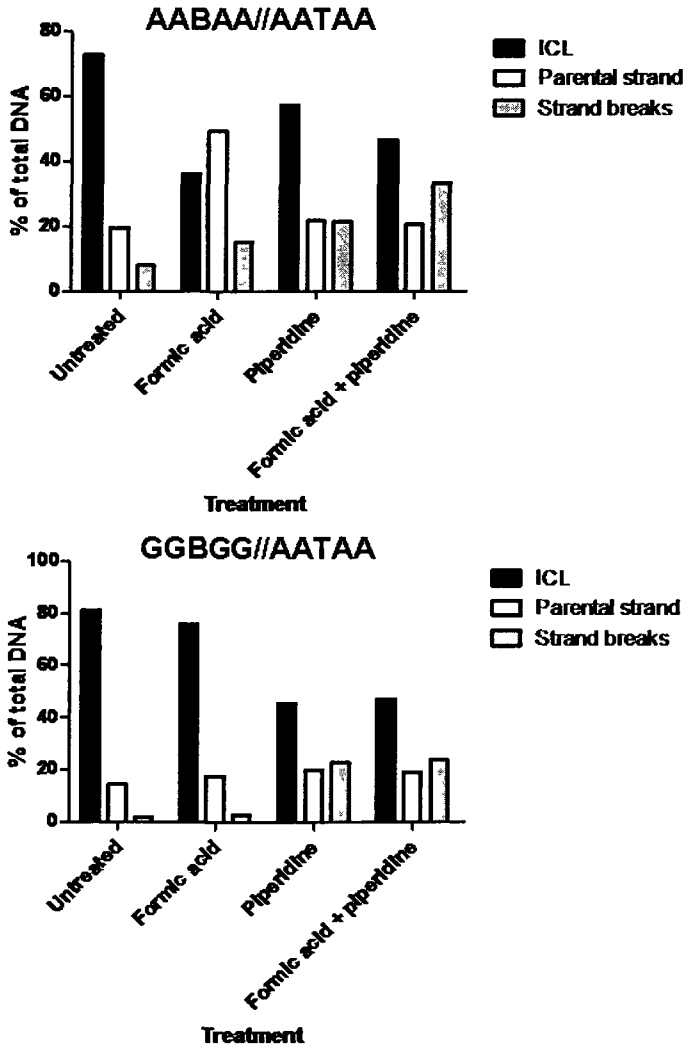

Figure IV-4: Overall treatment profile for AABrUAA//AATAA and GGBrUGG//AATAA. The relative yield of each form of DNA (ICL, parental strand, DNA fragments) is shown after each step of the mild hydrolysis treatment was applied to purified ICLs. Only in the case of AA^{Br}UAA//AATAA does the ICLs react to the

formic acid treatment, which appears as additional parental strand. Neither AA^{Br}UAA//CCCCC nor GG^{Br}UGG//AATAA showed any increase in damage upon exposure to formic acid (only GG^{Br}UGG//AATAA is shown).

UV irradiation

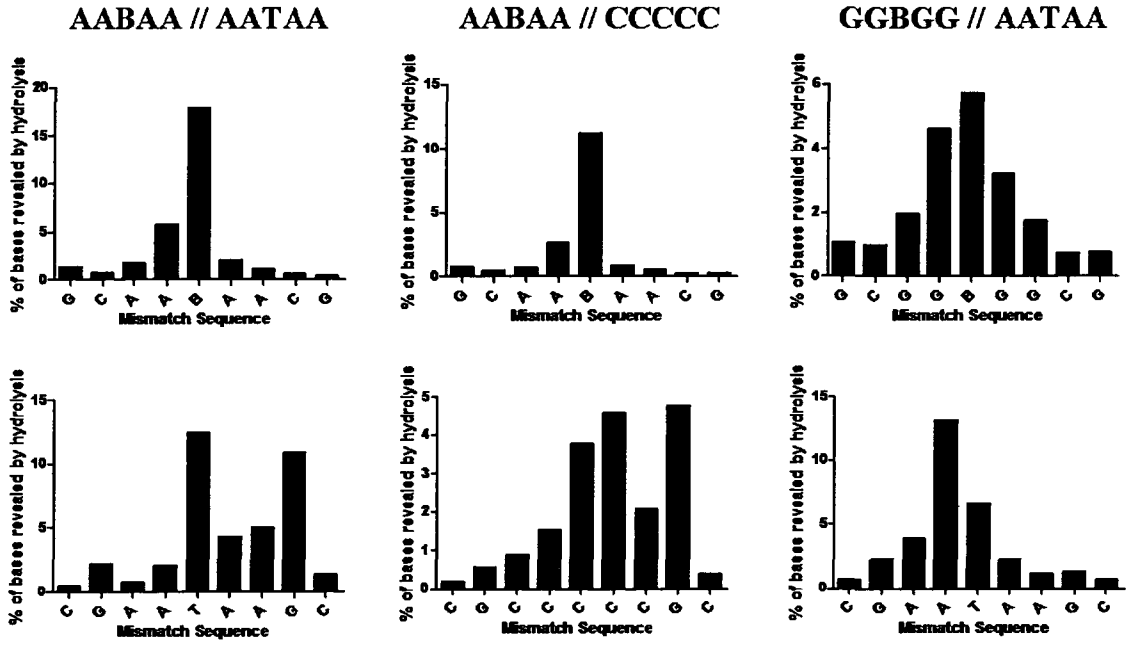

Figure IV-5: Cross-linked sites as a function of sequence (UV radiation). The mild hydrolysis protocol was applied to purified ICLs formed after exposition of the three mismatched sequences (left, AA^{Br}UAA//AATAA; center, AA^{Br}UAA//CCCCC; right, GG^{Br}UGG//AATAA) to UV rays. The degradation profile is shown for the brominated strand (upper row) and the opposite strand (lower row) for 5′ labeled oligos.

Although UV radiation also induces strand breaks and ICLs in BrdU-substituted DNA, the reaction mechanism is different from that of ionizing radiation. For example, in the case of ionizing radiation, fewer strand breaks are found in perfectly complementary double stranded DNA compared to single stranded DNA, whereas the opposite is found with UV rays. Thus, we proceeded to examine how the type of radiation changed the ICL chemistry. Figure IV-5 shows the location of cross-linking

sites in each of the three chosen sequence when UV radiation was used. Both in the case of AA^{Br}UAA//AATAA and AA^{Br}UAA//CCCCC, the site of the BrdU substitution was the major site of cross-linking on the brominated strand. Although the cross-linking sites on the opposite strand varied, in both cases the guanine at the 5′ extremity of the bubble (G_{10}) was extremely reactive. On the other hand, when 4 guanines were present on the brominated strand in GG^{Br}UGG//AATAA, the G_{10} site disappeared and reactivity was increased in the brominated strand, with a primary site at A_{14} on the opposite strand, similar to what was observed for γ-rays.

DISCUSSION

Method validation: Mitomycin C

In order to analyze products formed by radiation or other types of damaging agents, the typical procedure is to identify the damage by mass spectrometry, and in the case of unknown damage, subsequently confirm the structure with larger amounts of damage by nuclear magnetic resonance (NMR) (25). However, this approach may be difficult because of the number and low yield of potential products in model systems such as oligonucleotides containing BrdU. In addition, it is necessary to digest modified oligonucleotides to single components in order to efficiently separate and detect them by mass spectrometry, and thus, the position of the damage in the oligonucleotide is sacrificed during analysis. In 1991, Millard et al. first reported a method to identify the sites of cross-linking in mitomycin C-induced ICLs. The heterogeneous mixture of cross-links was studied by first purifying ICLs on a preparative gel and then submitting each one to random cleavage by hydroxyl radicals. Thus, the sites where a decrease in strand breakage was observed corresponded to the cross-linked sites. However, this method only allowed for indirect identification of the cross-linking sites, and could not be applied if several sites were

in close proximity. For example, the cross-links in the two nearby guanines of the 5′-GCGC site could not be easily resolved. Thus, we modified this method to reveal only the bases on each strand that had been damaged in the cross-linking process.

As expected, figure IV-2 shows that the three cross-link sites respond to the mild hydrolysis treatment, revealing cross-links at each guanine on the upper and lower strands. In this case, the two cross-links of the 5′-GCGC region become easily distinguishable, indicating that this method is suitable even when the potential cross-linking sites are in close proximity. However, it is important to note that the mild hydrolysis protocol may not be applicable to every type of ICL. For example, treatment of an ICL formed when DNA is exposed to high doses of ionizing radiation may reveal damage that was not initially exposed, such as base lesions. Furthermore, given that the mild hydrolysis response of the G21 site on the upper strand was relatively close to the background level, it was crucial to validate these results by labeling either the opposite strand or by labeling the 3′ end as well as the 5′ end. Nevertheless, we believe that the method proposed in this paper has the potential to serve as a powerful probe to determine the site of cross-links and give initial information about their acid and base lability.

Ionizing radiation

When our group first observed that an impressive number of bands were produced from a single initial radical in a mismatched DNA, we proposed that these bands corresponded to distinct structures, and that they were evidence that radical migration occurred between the site of the BrdU substitution and the surrounding nucleotides. Strikingly, AA^{Br}UAA//CCCCC represents what was expected if no radical transfer occurred between the bases; a major cross-linking site at the site of the initial radical creation on the brominated strand, which reacts with each of the bases on the opposite strand (figure IV-3). The fact that bases on the 5′ side of the mismatch are favored

probably reflects close structural proximity to the BrdU site, as the strand break pattern is similar (19).

In the case of AA^{Br}UAA//AATAA and GG^{Br}UGG//AATAA, however, BrdU is not the only cross-linking site on the brominated strand. The 5′ guanine of GG^{Br}UGG//AATAA is strongly involved in the cross-linking process, as are several nucleotides in AA^{Br}UAA//AATAA. As the mild hydrolysis protocol reveals only the bases that were modified by the cross-linking process, this provides clear evidence that the initial radical is able to migrate to surrounding nucleotides before it reacts with the other strand to create an ICL. It is important at this point to clarify that the cross-linking sites discussed here do not represent *intrastrand* cross-links, as the purification step separated them from the ICLs using denaturing gel electrophoresis. However, when the hydrolysis protocol was applied to the parental strand, (i.e. DNA that had the same molecular weight as the undamaged DNA) a strong response was always found for the BrdU site and the 5′ base (not shown). Whether these represent intrastrand cross-links or damage that had not completely gone through the strand breakage process remains unclear. Although intrastrand cross-links were already reported by Wang in BrdU-DNA irradiated with UV (26), it has yet to be established clearly if it the case for ionizing radiation. However, our observation that radical transfer occurs between the bases on the brominated strand certainly substantiates this possibility.

In addition to revealing the nucleotides involved in the cross-links, the mild hydrolysis protocol presented here reveals additional information concerning the ICLs that were formed for each sequence. For example, the G_{12}-A_{14} ICL in GG^{Br}UGG//AATAA transforms from a four-armed "X" structure to a three armed "Y" structure, where a strand break is created but the two strands remain covalently linked. In contrast, the major ICL of GG^{Br}UGG//AATAA, the B_{13}-A_{14} ICL, results in the degradation of the cross-link and the disconnection of the two strands. This implies that two drastically different ICL structures are formed, and that the ICL

formed between G_{12} and A_{14} in GG^{Br}UGG//AATAA is different from the ones formed in AA^{Br}UAA//AATAA. This may result from either a reactivity specific to guanine rather than purines, or a difference in the structural environment. Incidentally, the mild hydrolysis protocol also revealed that the ICLs formed in AA^{Br}UAA//AATAA also possessed distinct characteristics, as they were the only ones observed to react upon treatment with formic acid (figure IV-4). Whether this indicates that the ICLs are reverting under acid conditions or that base release occurs will have to be investigated. Certainly, the information obtained by this experiment sheds light not only on the location, but also on the chemical nature of the cross-links, which will aid in their identification.

UV irradiation

The major difference between irradiation of BrdU-substituted DNA by ionizing or UV radiation is the source of the electron that reacts with BrdU to create the uracil-5-yl radical. Whereas with gamma rays the electron is a product of water radiolysis, in the case of UV radiation, it is the base 5′ to the BrdU that provides the electron (16, 27). This means that, in addition to the uracil-5-yl radical, a radical cation is created. This difference is responsible for the drastic change in reactivity between the two types of radiation for BrdU-substituted DNA that we already reported (17, 28, 29). This evidence is also highlighted here by the contrasts in the results presented in figures IV-3 and IV-5.

Strikingly, both in AA^{Br}UAA//AATAA and AA^{Br}UAA//CCCCC, the guanine just outside the bubble at the 5′ extremity (G_{10}) was extremely reactive. Given the fact that guanine has a low oxidation potential, it is entirely possible that the radical cation created on A_{12} transfers to the G_{10} guanine on the other strand. Whether the preference for the 5′ guanine reflects a preferred transfer route or close structural proximity

remains unclear. However, the hypothesis that radical cation transfer to guanine occurs is reinforced by the results obtained from irradiation of $GG^{Br}UGG//AATAA$, where guanine sinks are present on the brominated strand. In this case, the reactivity on the G_{10} base disappears; instead, the guanines surrounding the BrdU become highly reactive. This implies not only that the radical cation produced by irradiation of BrdU-substituted DNA is able to transfer to distant bases within a mismatch, but also that this transfer is involved in the cross-linking process. Given that, in a similar experimental setup, only intrastrand cross-links were found when complementary BrdU-substituted DNA was studied (26), this represents further evidence that mismatched regions of DNA display increased reactivity (30).

Conclusion

In this study, we mapped the bases involved in ICL formation of BrdU-substituted DNA exposed to ionizing radiation. We observed that the identity of the nucleotides surrounding the initial radical had a dramatic effect on the end products. The mild hydrolysis ICL mapping treatment allowed us to probe further into the cross-linking mechanism observed in BrdU-substituted DNA, and provides clear evidence of radical transfer in a mismatched DNA for the first time. In addition, it also helped identify prospective targets among a number of structures for a subsequent structure study. This protocol, which requires only minute amounts of purified material ($\leq$10 ng), can be used to probe the structure of a mixture of ICLs that are located in close proximity, or to examine sequence preference. We believe that this method will be a useful tool to gather further knowledge regarding the formation of interstrand cross-links.

Supporting information:

Identification of the ICL degrading into a Y-cross-link upon hydrolysis treatment

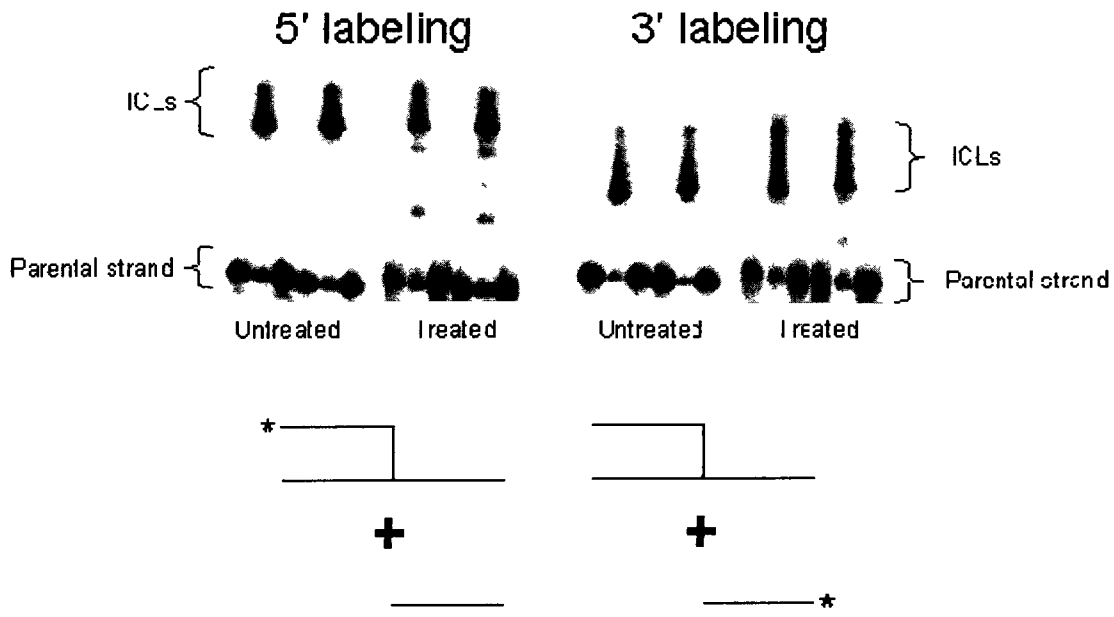

Figure IV-S1: Formation of Y cross-links following mild hydrolysis treatment. The damaged species that appeared following treatment of purified ICLs migrated between the ICLs (upper region on gel) and the parental strand (lower region). These bands where present when the ICL was labeled at the 5′ end, as well as when the opposite strand was labeled on the 3′ end, but not when the brominated strand was labeled at the 3′ end, and was identified as a Y cross-link.

When the purified ICLs produced from irradiation of GG^{Br}UGG//AATAA were treated and analyzed by gel electrophoresis, formation of an additional band was observed between the ICLs and the parental strand. This band was present when the ICL was labeled at the 5′ end, as well as when the opposite strand was labeled on the 3′ end, but not when the brominated strand was labeled at the 3′ end (figure IV-S1). Because of this, the band was identified as a Y cross-link, with the 5' arm of the

brominated strand still linked to the opposite strand, while the 3′ arm had been severed by the treatment.

This implied that the site of the break could be identified by the length of the 3′ fragment. The strand break profiles following treatment of the purified ICL labeled at the 5′ (left) and at the 3′ (right) end is shown in figure IV-S2, panel A.

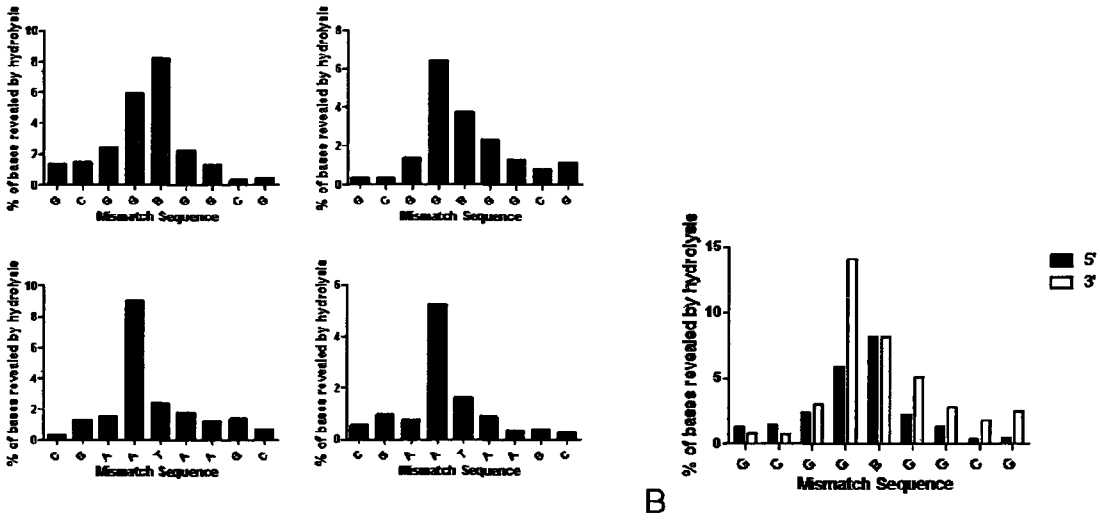

Figure IV-S2: Identification of Y cross-link. The strand break profile is shown in panel A for the 5′ (left) and 3′ (right) labeled brominated (upper row) or opposite strand (lower row). The normalized profile is shown in panel B for the brominated strand labeled at the 5′ (left) and 3′ end (right).

Given the shape of the Y cross-link observed following treatment, we deduced that the additional fragment is only observed when the 3′ arm of the brominated strand is analyzed (upper right quadrant of figure IV-S2, panel A), with the results from the 5′ labeled DNA showing only the degradation profile. In order to facilitate the comparison between the 5′ and the 3′ labeled DNA, we normalized the profiles using the yield observed at the BrdU substitution (shown in figure IV-S2B). From this, we see that the guanine 5′ of the BrdU is the most likely candidate as the site of breakage leading to the formation of the Y cross-link.

References

1. Jeggo,P.A. and Lobrich,M. (2007) DNA double-strand breaks: Their cellular and clinical impact? *Oncogene,* **26,** 7717-7719.
2. Jeggo,P. and Lobrich,M. (2006) Radiation-induced DNA damage responses. *Radiat. Prot. Dosimetry,* **122,** 124-127.
3. von Sonntag,C. (2006) Free-radical-induced DNA damage and its repair.
4. Grillari,J., Katinger,H. and Voglauer,R. (2007) Contributions of DNA interstrand cross-links to aging of cells and organisms. *Nucleic Acids Res.,* **35,** 7566-7576.
5. Rothfuss,A. and Grompe,M. (2004) Repair kinetics of genomic interstrand DNA cross-links: Evidence for DNA double-strand break-dependent activation of the fanconi anemia/BRCA pathway. *Mol. Cell. Biol.,* **24,** 123-134.
6. Sczepanski,J.T., Jacobs,A.C., Van Houten,B. and Greenberg,M.M. (2009) Double-strand break formation during nucleotide excision repair of a DNA interstrand cross-link. *Biochemistry,* **48,** 7565-7567.
7. Ding,H. and Greenberg,M.M. (2007) Gamma-radiolysis and hydroxyl radical produce interstrand cross-links in DNA involving thymidine. *Chem. Res. Toxicol.,* **20,** 1623-1628.
8. Hong,I.S. and Greenberg,M.M. (2005) Efficient DNA interstrand cross-link formation from a nucleotide radical. *J. Am. Chem. Soc.,* **127,** 3692-3693.
9. Leif,R.C., Stein,J.H. and Zucker,R.M. (2004) A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase. *Cytometry A.,* **58,** 45-52.
10. Quinones-Hinojosa,A., Sanai,N., Smith,J.S. and McDermott,M.W. (2005) Techniques to assess the proliferative potential of brain tumors. *J. Neurooncol.,* **74,** 19-30.
11. Dewey,W.C. and Humphrey,R.M. (1965) Increase in radiosensitivity to ionizing radiation related to replacement of thymidine in mammalian cells with 5-bromodeoxyuridine. *Radiat. Res.,* **26,** 538-553.
12. Sano,K., Hoshino,T. and Nagai,M. (1968) Radiosensitization of brain tumor cells with a thymidine analogue (bromouridine). *J. Neurosurg.,* **28,** 530-538.
13. Limoli,C.L. and Ward,J.F. (1993) A new method for introducing double-strand breaks into cellular DNA. *Radiat. Res.,* **134,** 160-169.
14. Saffhill,R. and Ockey,C.H. (1985) Strand breaks arising from the repair of the 5-bromodeoxyuridine-substituted template and methyl methanesulphonate-induced lesions can explain the formation of sister chromatid exchanges. *Chromosoma,* **92,** 218-224.
15. Dewey,W.C., Sedita,B.A. and Humphrey,R.M. (1966) Radiosensitization of X chromosome of chinese hamster cells related to incorporation of 5-bromodeoxyuridine. *Science,* **152,** 519-521.
16. Schyman,P., Zhang,R.B., Eriksson,L.A. and Laaksonen,A. (2007) Hydrogen abstraction from deoxyribose by a neighbouring uracil-5-yl radical. *Phys. Chem. Chem. Phys.,* **9,** 5975-5979.

17. Cecchini,S., Girouard,S., Huels,M.A., Sanche,L. and Hunting,D.J. (2005) Interstrand cross-links: A new type of gamma-ray damage in bromodeoxyuridine-substituted DNA. *Biochemistry,* **44,** 1932-1940.
18. Dextraze,M.-., Wagner,J.R. and Hunting,D.J. (2007) 5-bromodeoxyuridine radiosensitization: Conformation-dependent DNA damage. *Biochemistry,* **46,** 9089-9097.
19. Dextraze,M.E., Cecchini,S., Bergeron,F., Girouard,S., Turcotte,K., Wagner,J.R. and Hunting,D.J. (2009) Reaching for the other side: Generating sequence-dependent interstrand cross-links with 5-bromodeoxyuridine and gamma-rays. *Biochemistry,* **48,** 2005-2011.
20. Millard,J.T., Weidner,M.F., Kirchner,J.J., Ribeiro,S. and Hopkins,P.B. (1991) Sequence preferences of DNA interstrand crosslinking agents: Quantitation of interstrand crosslink locations in DNA duplex fragments containing multiple crosslinkable sites. *Nucleic Acids Res.,* **19,** 1885-1891.
21. Schärer,O.D. (2005) DNA interstrand crosslinks: Natural and drug-induced DNA adducts that induce unique cellular responses. *ChemBioChem,* **6,** 27-32.
22. Noll,D.M., Mason,T.M. and Miller,P.S. (2006) Formation and repair of interstrand cross-links in DNA. *Chem. Rev.,* **106,** 277-301.
23. Noll,D.M., Noronha,A.M., Wilds,C.J. and Miller,P.S. (2004) Preparation of interstrand cross-linked DNA oligonucleotide duplexes. *Front. Biosci.,* **9,** 421-437.
24. Borowy-Borowski,H., Lipman,R. and Tomasz,M. (1990) Recognition between mitomycin C and specific DNA sequences for cross-link formation. *Biochemistry,* **29,** 2999-3006.
25. Wang,Y. and Wang,Y. (2003) Structure elucidation of DNA interstrand cross-link by a combination of nuclease P1 digestion with mass spectrometry. *Anal. Chem.,* **75,** 6306-6313.
26. Zeng,Y. and Wang,Y. (2006) Sequence-dependent formation of intrastrand crosslink products from the UVB irradiation of duplex DNA containing a 5-bromo-2'-deoxyuridine or 5-bromo-2'-deoxycytidine. *Nucleic Acids Res.,* **34,** 6521-6529.
27. Cook,G.P. and Greenberg,M.M. (1996) A novel mechanism for the formation of direct strand breaks upon anaerobic photolysis of duplex DNA containing 5-bromodeoxyuridine. *J. Am. Chem. Soc.,* **118,** 10025-10030.
28. Cecchini,S., Girouard,S., Huels,M.A., Sanche,L. and Hunting,D.J. (2004) Single-strand-specific radiosensitization of DNA by bromodeoxyuridine. *Radiat. Res.,* **162,** 604-615.
29. Cecchini,S., Masson,C., La Madeleine,C., Huels,M.A., Sanche,L., Wagner,J.R. and Hunting,D.J. (2005) Interstrand cross-link induction by UV radiation in bromodeoxyuridine-substituted DNA: Dependence on DNA conformation. *Biochemistry,* **44,** 16957-16966.
30. Dextraze,M., Gantchev,T., Girouard,S. and Hunting,D. (2010) DNA interstrand cross-links induced by ionizing radiation: An unsung lesion. *Mutation Research/Reviews in Mutation Research,* **704,** 101-107.

Chapitre V – 4e article

DNA interstrand cross-links induced by ionizing radiation: an unsung lesion.

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Avant-propos :

Contribution à la rédaction et à l'expérimentation :

J'ai rédigé 95 % de cet article sous la supervision de mon directeur de recherche. Tsvetan Gantchev a rédigé une partie de la section 1.2 qui porte sur la réparation des dimères interbrins, en plus de la section 4.2, qui décrit ses travaux de recherche. J'ai aussi été responsable de la préparation des figures et de la mise en page, en plus d'avoir révisé l'article en fonction des commentaires des arbitres.

Résumé :

Cet article est une revue de littérature présentant les résultats des derniers travaux de recherche dans notre laboratoire, incluant les résultats obtenus durant ma thèse. De plus, nous présentons une revue de littérature concernant ce qui est connu sur la formation de dimères interbrins par les radiations ionisantes dans l'ADN cellulaire. Nous présentons aussi notre hypothèse selon laquelle la structure secondaire et

tertiaire de l'ADN cellulaire est susceptible d'induire des dommages inattendus, dont les dimères interbrins. Cette hypothèse est reprise plus en détail dans les sections discussion et conclusion de ma thèse.

DNA interstrand cross-links induced by ionizing radiation: An unsung lesion

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Abstract

The induction of DNA interstrand cross-links by ionizing radiation has been largely ignored in favour of studies on double-strand break formation and repair. At least part of the problem is technical; it is difficult to detect and quantify interstrand cross-links when the same agent forms both cross-links and single strand breaks because the detection of interstrand cross-links generally involves a denaturation step. Our group has studied the induction of interstrand cross-links following irradiation of DNA containing bromouracil at specific sites. We found that the formation of interstrand cross-links requires the presence of a few (3-5) mismatched bases, comprising the bromouracil. In the absence of mismatched bases, no radiation-induced cross-linking was observed; however, even in the absence of bromouracil, cross-linking still occurred, albeit at a lower efficiency. Our molecular modelling studies demonstrate that the mobility of the bases in the mismatched region is essential for the cross-linking process. Thus, our hypothesis is that ionizing radiation induces DNA interstrand cross-links in non-hybridized regions of DNA. Some obvious examples of such DNA regions are replication forks, transcription bubbles and the D-loop of telomeres. However, an abundance of studies have made it clear that there must be many single stranded regions in the genome, such as hairpins and cruciforms. For example, alpha satellite DNA, in centromere regions of human chromosomes, forms hairpins. Thus, a variety of non-B DNA structures (hairpins, slipped DNA and tetrahelical structures) exist in the genome and should be susceptible to the formation of radiation induced interstrand cross-links. Although interstrand cross-links have thus far been virtually ignored in radiation biology, it will be worthwhile to develop methods to detect their presence following exposure of cells to biologically relevant levels of ionizing radiation, since, on a per lesions basis, they are probably more toxic than double strand breaks.

Contents

1. *Interstrand cross-links vs. double strand breaks*

1.1 Double strand breaks induced by ionizing radiation

Among the numerous types of DNA lesions presently attributed to ionizing radiation, complex lesions, including double strand breaks (DSBs[1]), are generally considered to be the most toxic [1-3]. Double strand breaks arise either from a single radical leading to the formation of lesions on both strands or, more probably, from two separate strand breaks in close proximity [4]. Although the yield of double strand breaks in cells that are submitted to low LET radiation is much lower than single strand breaks and base lesions (40/Gy vs. ≥2000/Gy, respectively, [5], most of the toxicity of ionizing radiation is currently attributed to DSBs. The repair of double strand breaks require a complex repair mechanism that is not yet fully understood, despite continuous effort and decades of experiments [5,6]. Moreover, defective or incomplete repair of DSBs leads to deletions, insertions, as well as chromosome rearrangements, which can trigger cell death, or lead to oncogenesis [7]. Thus, it is not so surprising that DSBs are usually the focal point of DNA damage studies. However, strong evidence for the formation of another potentially more toxic type of lesion by ionizing radiation is now emerging.

1.2 Interstrand cross-links induced by ionizing radiation

Agents that induce interstrand cross-links (ICLs), where the two DNA strand are covalently linked, have been used to treat cancer for many years. Indeed, 60 % of the most potent genotoxic anticancer agents are bifunctional alkylating agents that are capable of forming ICLs [8]. One of the first ICLs to be identified was formed by mustard gas (1,5-dichloro-3-thiapentane), which was developed prior to the First

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[1] Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; CldC, 5-Chlorodeoxycytidine; CldU, 5-Chlorodeoxyuridine; DSBs, Double strands breaks; ET, Electron transfer; Gy, Gray; HMG, High mobility group proteins; ICLs, Interstrand cross-links; PCV, Procarbazine, lomustine and vincristine; UVB, Ultraviolet B.

World War. Another example of an ICL-inducing agent used in clinic is mitomycin C, which is extracted from *Streptomyces caespitosis*, and is used to treat solid tumors containing hypoxic regions [8,9]. Because interstrand cross-links prevent DNA strand separation, their presence impedes DNA replication and transcription [10]. Furthermore, there is growing evidence that ICL repair involves the generation of a DSB during the unhooking process [11,12]. These lesions are therefore extremely cytotoxic and mutagenic. In addition, over the past decade there is a growing appreciation that ICLs induced by chemical or oxidative agents present a challenge to the cellular DNA repair systems. Most of the information regarding cross-link repair was gathered using psoralens and UVA radiation. Although the majority of ICLs appear to be substrates for the nucleotide excision repair (NER) pathway [13], which specializes in the removal of bulky lesions in locally distorted DNA, often the repair is blocked or of low efficiency [14-16]. The different ICL repair efficiencies have been found to depend on various factors [8,17,18], including the individual ICL chemical structure (e.g. the spacer length in (bis)cis-Pt and nitrogen mustard) [19], DNA sequence [20] and DNA local dynamic structure and flexibility [21]..There is very little information about repair of gamma-radiation induced ICL *per se*, since the only relevant studies concern a tandem lesion comprised of a single strand break and a DNA cross-link, but it is not yet clear if the cross-link is mostly intra- or interstrand [22]. A recent study from the group of Greenberg showed that this same ICL can be transformed into a DSB by UvrABC, but no detailed information exists regarding repair in eukaryotes [12].

Many of the studies on repair of interstrand cross-links used cells from Fanconi anemia patients. Fanconi anemia is a rare, autosomal, recessive human disease that is characterized by a hypersensitivity to ICL-inducing agents such as mitomycin C [11,23-25]. Several genes have been identified that are involved in the initial unhooking step, including XPF-ERCC1 [26], however a global picture of the repair of ICLs remains elusive, perhaps due to the number of ICLs structures that are studied. Several approaches have been devised to study ICLs, one of which is the use of chemically synthesized ICLs for use in repair studies [17,27]. Traditionally, ionizing

radiation has not been regarded as a cross-linking agent; however, several instances of intrastrand cross-link formation have now been reported [22,28-33], and recent data from various groups points to the existence of interstrand cross-links induced by hydroxyl radicals in synthetic DNA [34,35]. This is most intriguing, considering that Fanconi anemia patients sometimes suffer from ionizing radiation hypersensitivity [36,37].

1.3 Technical difficulties

There are several technical hurdles associated with the study of ICL formation by an agent that also produce high yields of strand breaks, as is the case of ionizing radiation. Typical methods used in ICL detection, such as the alkaline comet assay and alkaline elution [38,39], are not sensitive when the same agent produces both cross-links and strand breaks. These methods most often rely on the inability of the DNA strands to separate under alkaline conditions due to the presence of the cross-links, resulting in a decrease of electrophoretic mobility, for example. However, the strand breaks that are concomitantly produced by the radiation also decrease the length of the DNA fragments, thus increasing their electrophoretic mobility and masking the effect of the ICLs. Moreover, the conditions used to separate the strands also expose alkali-labile lesions, which are transformed into strand breaks, further increasing this effect. Other techniques, such as high performance liquid chromatography and mass spectrometry, have proven most useful in the identification of several types of lesions [40-42], including ICLs formed by ionizing radiation [22,35]. These methods are highly sensitive, and can be used to study extracts of cellular DNA that has been exposed to ionizing radiation. However, two technical difficulties are associated with these techniques. First, the extraction protocol may modify the irradiation products, leading to the uncoupling of the cross-linked nucleotides, which will then appear as two modified bases. This issue, which is now well known in the case of other radiation products such as 8-oxoguanine, can certainly be minimized by careful control of the extraction and digestion conditions [43]. The other difficulty is that is it somewhat complex to confirm, after a potential lesion has

been found, whether the lesion observed is in fact an interstrand cross-link rather than simply an intrastrand cross-link [22].

For these reasons, our current knowledge of the biological significance of radiation-induced cross-links as well as the underlying mechanisms is very limited in comparison to the panoply of mechanisms actually occurring in cells that are submitted to radiation injury. Thus, the need is pressing to better understand how this type of lesion is produced and repaired in normal and tumor cells, and how this knowledge can be used to gain an advantage in radiotherapy. For this, new methods will have to be developed in order to study ICLs that are formed concomitantly with other types of lesions, such as strand breaks and alkali-labile lesions.

2. *BrdU: a good idea which failed*

5-bromo-2′-deoxyuridine (BrdU) is a thymidine analog that can be incorporated into DNA by cells. It is now widely used as a marker of replicating cells [44-46], although it was first reported as a photo- and radiosensitizing agent [47,48]. When exposed to ionizing radiation, BrdU produces DNA single and double strand breaks [49,50], as well as chromosomal aberrations [51] that are generally believed to be responsible for its sensitizing properties. The mechanism for single strand break formation involves electron attachment to BrdU, followed by the departure of a bromide anion and generation of an uridin-yl radical that further reacts to create strand breaks [52]. Originally, the appealing aspect of BrdU sensitization was that the thymidine analog was incorporated during DNA replication, and incorporation into DNA was a prerequisite for sensitization [53]. Thus, theoretically, rapidly cycling tumor cells would incorporate more BrdU and be more sensitized than the surrounding, mostly quiescent, normal cells. However, clinical studies have given disappointing results, failing to show a survival advantage for patients with a range of tumor types [54-57]. More precisely, a phase III study conducted in 2004 on patients diagnosed with

anaplastic astrocytoma using BrdU in conjunction with procarbazine, lomustine and vincristine (PCV) showed that addition of BrdU showed no survival advantage [58]. Thus, clinical studies using BrdU as a radiosensitizer are not currently a high priority. BrdU has become, as Szybalski presciently described it in 1974 [59]: "a good idea which failed". Nevertheless, the relatively low toxicity of BrdU as well as its rare ability to directly radiosensitize DNA makes it worthy of further studies.

Furthermore, a new class of halogenated pyrimidines, 5-chlorodeoxycytidine (CldC) has been found to be a potent radiosensitizer but an understanding of the sensitization mechanisms remains critical to the success of any radiosensitizer in the clinic. Radiosensitization by CldC relies on the elevated levels of deoxycytidine kinase and dCMP deaminase found in tumor cells rather than simply on increased incorporation in rapidly cycling cells [60,61], thus circumventing problems associated with the possible incorporation of BrdU in rapidly cycling normal cells surrounding the tumor, which results in loss of specificity. However, CldC is incorporated into DNA as 5-chlorodeoxyuridine (CldU), and will almost certainly yield the same uridin-yl radical responsible for DNA damage in BrdU.

3. Generating interstrand cross-links with BrdU and γ-rays

Thus, it is not so surprising that a number of groups have maintained interest in BrdU as a radiosensitizer. Recent studies have shown that our understanding of BrdU sensitization at the time of the clinical trials was incomplete. For example, in the case of BrdU-substituted DNA that had been irradiated with UV-rays, the formation of intrastrand cross-links was observed in synthetic DNA and in cells [62,63], and generation of interstrand cross-links was inferred from the increase in sister chromatid exchanges in cells where BrdU partially replaced thymidine [64,65]. In the case of ionizing radiation, when the effect of DNA strandedness on BrdU radiosensitization was examined, it was observed that in synthetic DNA, the presence of a double

stranded DNA greatly decreased strand breaks compared to a DNA where a 5-base mismatch region had been introduced [66].

More importantly, the formation of a new type of BrdU-sensitized damage was observed: ICLs that were specific for mismatched nucleotides within BrdU-substituted DNA [67]. These ICLs were later found to be highly dependent on the regional DNA structure and sequence [68,69], supporting the evidence that a precise regional environment of DNA was necessary for their formation, such as open or mismatched regions of cellular DNA.

These results, while they are not yet all confirmed in cells, certainly underline the fact that our knowledge of the radiosensitizing properties of BrdU is yet imperfect. More importantly, they offer a potential explanation as to the failure of the phase III clinical trial, given that the PCV treatment already includes a cross-linking agent, which may explain why no survival advantage was observed with the addition of BrdU. This illustrates the importance of comprehensive fundamental testing of the sensitizing mechanisms prior to the introduction of new therapeutic agents.

Another intriguing observation was that, when BrdU was present in a mismatched region of DNA, there was a marked increase in both reactivity and ICL formation. Furthermore, an impressive dependence on sequence was found when the mismatch sequence surrounding the BrdU site was modified, which was not observed in perfectly double-stranded DNA [68]. Finally, evidence of radical transfer from the initial site of radical formation to distant bases on both strands was observed, a feature that is generally believed to be hindered by the presence of a mismatch [70-72].

Taken together, these results indicate that mismatched (and presumably open) regions of synthetic DNA exhibit a distinct chemical reactivity, which may be maintained in the cellular environment. Although open regions of DNA transiently exist during transcription and replication and mismatched regions are found in centromeres [73-75], there are surprisingly few studies asking how these regions are affected by

ionizing radiation. Given the structural prerequisites for ICL formation observed in our studies, it is possible that these regions of DNA may be particularly favorable for ICL production.

4. *Reaching for the other side: a case for flexibility?*

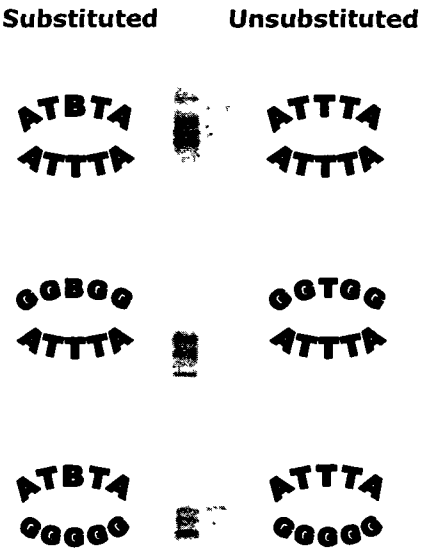

Figure V-1 : Comparison of interstrand cross-links (ICLs) formed in BrdU-substituted and unsubstituted DNA. Three DNA sequences containing a 5-base mismatch where the central thymidine was substituted with BrdU (left) or unsubstituted (right) were irradiated with ^{60}Co. The irradiated DNA was analyzed by denaturing gel electrophoresis. Only the ICL region is shown here. The experimental conditions were as described in [68]. All the band patterns shown here were observed on the same gel.

4.1 ICL formation in BrdU-substituted and unsubstituted DNA

It would be easy to dismiss the reactivity found in mismatched DNA that is substituted with BrdU as the exception rather than the rule; the uracil-5-yl radical that is generated abstracts hydrogen atoms from surrounding radicals at an efficient rate ($k_{2\text{-PrOH}} = 4.1 \times 10^7$ dm^3 mol^{-1} s^{-1}, [76]). However, of the 12 BrdU-substituted

mismatch combinations that were examined in the sequence study, ICLs were observed upon irradiation of three of these same sequences in the absence of BrdU substitution (Figure V-1). In contrast, no ICL was observed when these sequences were irradiated within perfectly complementary double stranded DNA. Thus, ICL formation by ionizing radiation in mismatched DNA is not confined uniquely to BrdU-substituted DNA, but is also possible in unsubstituted DNA that possesses the appropriate sequence and structure. Interestingly, the same is true for mismatched DNA that is irradiated with UVB rays [77], although both the chemical pathways leading to DNA damage as well as the end products are substantially different.

At the time of the first observations of ICL formation in mismatched DNA, it was proposed that the added flexibility introduced by the presence of the 5-base mismatch was responsible for the increased reactivity, conferring single stranded properties to the bases within the mismatch [66].

4.2 Mismatches facilitate electron addition to BrdU and charge transfer along the DNA

In the context of the efficiency of BrdU mediated DNA damage, two primary electron-transfer (ET) processes are of outmost importance: i) primary capture of the hydrated electron by DNA, which depends on the base electron affinity and susceptibility for hydrated electrons and; ii) the intrahelical DNA excess electron density transfer. The accommodation of mismatches within DNA locally perturbs the double helix dynamic structure. The wobble conformations (non-Watson-Crick base pairing) are dynamically unstable and are characterized by transient flipping out of the unpaired bases. It has been long speculated that ET efficiency along DNA could be sensitive to such structural perturbations. Indeed, recently a more effective ET has been reported for mismatched duplexes than for complementary DNA [78]. Mechanistic substantiation of the favorable effect of mismatches on ET efficiency was obtained by our molecular dynamics simulations with DNA duplexes containing single pyrimidine-pyrimidine (dT•dT or BrdU•dT) mismatches [79]. This study

revealed that apart from the mismatched bases flipping, the penultimate base pairs are also affected and, depending on the neighboring sequence, can form a network of relatively stable cross-strand contacts, *i.e.* an alternative to the Watson-Crick H-bonding pattern (Figure V-2A). The later is deemed to present a specific path for a more effective electron injection or hopping beyond the mismatched site and also to facilitate secondary oxido-reductive processes. In addition, the close proximity of the nucleotides on opposite strands provides favorable geometric and dynamic conditions for interstrand interactions of damaged bases, eventually resulting in the formation of cross-links or strand breaks on the opposite strand (Figure V-2B) [68]. In another series of molecular dynamics simulations, we probed the interactions of the hydrated electrons with complementary and mismatched BrdU-substitited DNA duplexes [80,81]. Rather unexpectedly, it was found that the hydrated electron tends to localize longer or to visit more frequently the BrdU site of the mismatched duplex, than that of the complementary one, *i.e.* BrdU when incorporated in mismatched DNA is more accessible for a direct reduction by solvated electrons. Altogether, the above structure-mechanistic studies emphasize the favorable effect of mismatches on the BrdU-dependent DNA damage and subsequent ICL formation.

4.3 Single mismatches and γ-radiation

The reaction of mismatched DNA to radiation injury has mainly been studied as single mismatches, with two non-pairing bases on the opposite sides of the helix, as well as single bulges, where a base is usually flipped out of the helix. These mismatches may occur in a number of circumstances, such as deamination or slipping of the DNA polymerase during replication [82,83]. These disruptions of the helix structure are known to hinder charge transfer along the DNA strand [70-72,84], and are recognized by the nucleotide excision repair, homologous recombination and translesion replication pathways [8]. However, very little is known about how consecutive mismatches react to ionizing radiation. There are several examples of consecutive mismatches in cells [74,85]. For example, DNA hairpins are believed to be involved in the regulation of transcription. Proteins such as the high mobility group

(HMG) chromosomal proteins are known to play a role in chromosomal structure, function and transcription. The HMG proteins bind to regions containing inverted repeats in plasmid DNA, and are able to remove a transcription block in a region of DNA containing a cruciform structure [86,87]. Transcription of other genes also appear to be regulated by the binding of secondary structures in DNA; β-casein, epidermal growth factor and c-myc [88-90].

4.4 Consecutive mismatches and γ-radiation

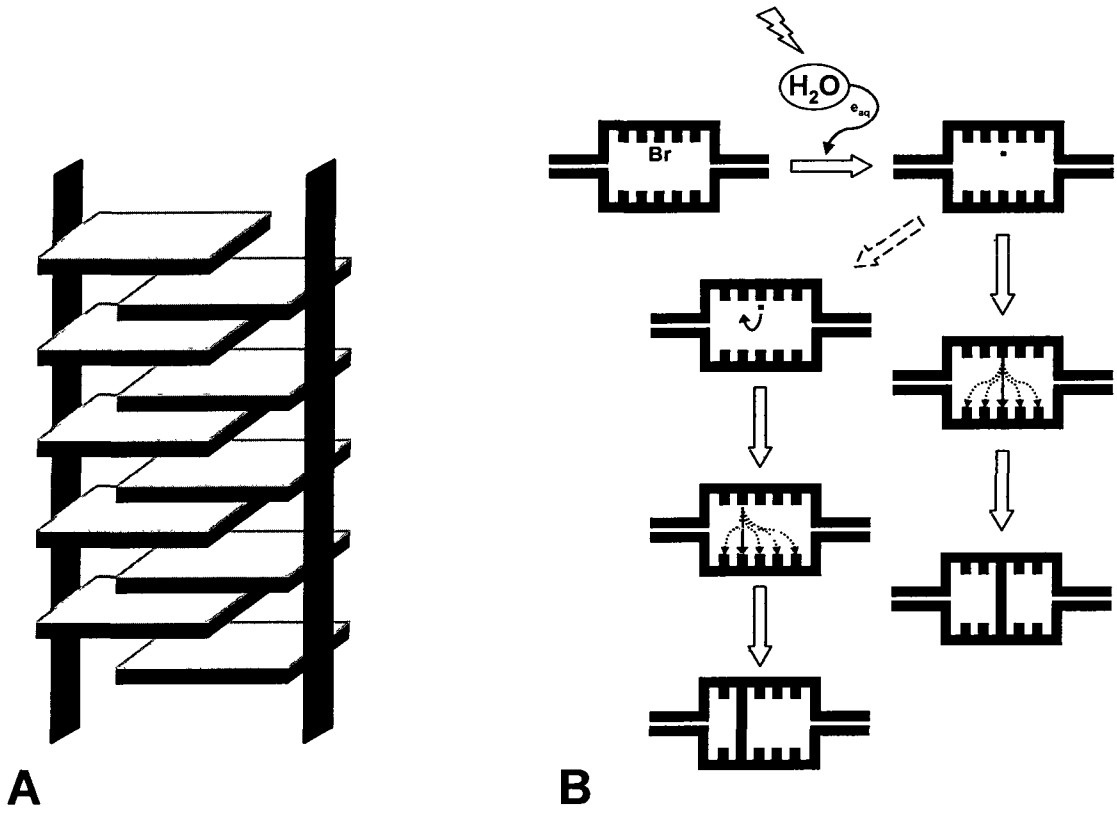

Figure V-2 : Proposed mechanism for ICL formation in mismatched DNA. The presence of consecutive mismatches induces the formation of a zipper-like DNA structure, which increases interstrand contacts between bases (panel A). Upon exposure to ionizing radiation, the radicals that are generated are able to react with bases on the other strand to form ICLs. The dashed arrow shows that additional ICL structures can be formed if radical transfer occur along the DNA strand, as proposed in [68].

Consecutive mismatches are observed in human centromeres [73,91] and *in vitro* studies show that these sequences adopt a zipper-like structure (Figure V-2A) with surprisingly few structural adjustments – even when internal canonical base pairs are present – while keeping the general structure of the double helix relatively intact [92]. Thus, various types of secondary structures exist in cellular DNA, and play a crucial role in the regulation of cellular processes. Given that these structures often display a slightly modified helix structure in order to accommodate the mismatches, with regions of highly reactive single-stranded DNA, it would be of great interest to know how these regions are affected by radiation. Additionally, given the zipper-like structures that are often adopted by consecutives mismatches, as well as the

predisposition of this type of sequences to form ICLs in synthetic DNA upon irradiation, it would also be interesting to know if this is mirrored in cellular DNA.

5. *Higher order chromatin structure and DNA damage*

Of course, mismatched DNA does not represent the perfect model system to study all types of DNA damage induced by radiation injury. However, it is certainly more likely than double stranded DNA to approach the inherent dynamics of cellular DNA, such as breathing, as well as the various processes involving DNA, such as transcription and replication. These processes are already known to greatly influence the response to radiation [93,94], even though wide gaps remain in our understanding of this effect. For example, replication and transcription disrupts chromatin structure, and DNA that is organized into nucleosomes is less sensitive to radiation than naked DNA [95], while transcriptionnally active DNA is sensitized. On a higher level of complexity, the toxicity of radiation through the cell cycle is also an indication of this phenomenon. In a classic experiment from the laboratory of Charlier, it was observed that upon binding of the lac repressor, strand breaks induced by radiation decreased or increased, depending on the specific regional changes in the DNA conformation [96].

In the case of alternative structures of DNA such as hairpins and cruciforms, no direct evidence exists for an increase or decrease in the damage yield upon exposition to ionizing radiation. However, inverted repeats, as well as micro- and minisatellites, are capable of adopting these configurations, and display an increased susceptibility to strand breaks and chromosomal rearrangements [97-99]. This fragility was proposed to occur at replication forks that were blocked by the presence of the hairpin or by attack of the single stranded region of the hairpin loop by nucleases [100]. In the case of ionizing radiation, the latter scenario is most interesting, because single stranded DNA is also more sensitive to radical attack than double stranded DNA. Thus, the

susceptibility of these fragile sites to strand breakage could very well be found also upon exposure to ionizing radiation.

These results, combined with those reviewed previously, underline the possibility that other types of lesions may be produced by ionizing radiation in active cellular DNA that were not previously observed in classical experiments using naked double stranded DNA. They also suggest that distinct DNA structures are likely to react differently to radicals produced by ionizing radiation. Dynamic DNA structures such as hairpins, holiday junctions and highly transcribed regions of DNA may produce unexpected lesions. These lesions may also be more toxic than similar lesions produced in other regions, given the importance of these DNA structures for the regulation of critical cellular processes. In the case where a particular DNA structure is required for a particular function, such as initiation of transcription or DNA repair, if the structure is damaged, the function may very well be lost also. Thus, although much of the work regarding the consequences of injury to cellular DNA has concentrated on lesion characterization and quantification, the existing data certainly raises the question as to whether location is an equally important factor. For this, the study of radiation injury to alternative DNA structures, such as hairpins and mismatches, may prove invaluable, acting as a model for dynamic cellular DNA.

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Conflict of Interest statement:

The authors declare that there are no conflicts of interest.

References

[1] P.A. Jeggo, M. Lobrich. DNA double-strand breaks: Their cellular and clinical impact? Oncogene. 26 (2007) 7717-7719.
[2] P. Jeggo, M. Lobrich. Radiation-induced DNA damage responses, Radiat. Prot. Dosimetry. 122 (2006) 124-127.
[3] C. von Sonntag. Free-radical-induced DNA damage and its repair, (2006) .
[4] J.R. Milligan, J.Y. Ng, C.C. Wu, J.A. Aguilera, R.C. Fahey, J.F. Ward. DNA repair by thiols in air shows two radicals make a double-strand break, Radiat. Res. 143 (1995) 273-280.
[5] S. Lehnert. Biomolecular Action of Ionizing Radiation, Taylor & Francis, New York, 2007.
[6] B. Pardo, B. Gomez-Gonzalez, A. Aguilera. DNA repair in mammalian cells: DNA double-strand break repair: How to fix a broken relationship, Cell Mol. Life Sci. 66 (2009) 1039-1056.
[7] K.K. Khanna, S.P. Jackson. DNA double-strand breaks: Signaling, repair and the cancer connection, Nat. Genet. 27 (2001) 247-254.
[8] D.M. Noll, T.M. Mason, P.S. Miller. Formation and repair of interstrand cross-links in DNA, Chem. Rev. 106 (2006) 277-301.
[9] O.D. Scharer. DNA interstrand crosslinks: Natural and drug-induced DNA adducts that induce unique cellular responses, Chembiochem. 6 (2005) 27-32.
[10] J. Grillari, H. Katinger, R. Voglauer. Contributions of DNA interstrand cross-links to aging of cells and organisms, Nucleic Acids Res. 35 (2007) 7566-7576.
[11] A. Rothfuss, M. Grompe. Repair kinetics of genomic interstrand DNA cross-links: Evidence for DNA double-strand break-dependent activation of the fanconi anemia/BRCA pathway, Mol. Cell. Biol. 24 (2004) 123-134.
[12] J.T. Sczepanski, A.C. Jacobs, B. Van Houten, M.M. Greenberg. Double-strand break formation during nucleotide excision repair of a DNA interstrand cross-link, Biochemistry. 48 (2009) 7565-7567.
[13] T. Lindahl, P. Karran, R.D. Wood. DNA excision repair pathways, Curr. Opin. Genet. Dev. 7 (1997) 158-169.
[14] J.J. Truglio, D.L. Croteau, B. Van Houten, C. Kisker. Prokaryotic nucleotide excision repair: The UvrABC system, Chem. Rev. 106 (2006) 233-252.
[15] G. Duval-Valentin, M. Takasugi, C. Helene, E. Sage. Triple helix-directed psoralen crosslinks are recognized by uvr(A)BC excinuclease, J. Mol. Biol. 278 (1998) 815-825.
[16] F.X. Barre, U. Asseline, A. Harel-Bellan. Asymmetric recognition of psoralen interstrand crosslinks by the nucleotide excision repair and the error-prone repair pathways, J. Mol. Biol. 286 (1999) 1379-1387.
[17] M.B. Smeaton, E.M. Hlavin, T. McGregor Mason, A.M. Noronha, C.J. Wilds, P.S. Miller. Distortion-dependent unhooking of interstrand cross-links in mammalian cell extracts, Biochemistry. 47 (2008) 9920-9930.
[18] M.B. Smeaton, E.M. Hlavin, A.M. Noronha, S.P. Murphy, C.J. Wilds, P.S. Miller. Effect of cross-link structure on DNA interstrand cross-link repair synthesis, Chem. Res. Toxicol. 22 (2009) 1285-1297.
[19] J.A. Brandsma, M. de Ruijter, R. Visse, D. van Meerten, M. van der Kaaden, J.G. Moggs, P. van de Putte. The in vitro more efficiently repaired cisplatin adduct cis-

pt.GG is in vivo a more mutagenic lesion than the relative slowly repaired cis-pt.GCG adduct, Mutat. Res. 362 (1996) 29-40.
[20] M. Ramaswamy, A.T. Yeung. Sequence-specific interactions of UvrABC endonuclease with psoralen interstrand cross-links, J. Biol. Chem. 269 (1994) 485-492.
[21] Y. Cai, D.J. Patel, N.E. Geacintov, S. Broyde. Dynamics of a benzo[a]pyrene-derived guanine DNA lesion in TGT and CGC sequence contexts: Enhanced mobility in TGT explains conformational heterogeneity, flexible bending, and greater susceptibility to nucleotide excision repair, J. Mol. Biol. 374 (2007) 292-305.
[22] P. Regulus, B. Duroux, P.A. Bayle, A. Favier, J. Cadet, J.L. Ravanat. Oxidation of the sugar moiety of DNA by ionizing radiation or bleomycin could induce the formation of a cluster DNA lesion, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14032-14037.
[23] K.J. Patel, H. Joenje. Fanconi anemia and DNA replication repair, DNA Repair (Amst). 6 (2007) 885-890.
[24] F. Rosselli, D. Briot, P. Pichierri. The fanconi anemia pathway and the DNA interstrand cross-links repair, Biochimie. 85 (2003) 1175-1184.
[25] M.L. Dronkert, R. Kanaar. Repair of DNA interstrand cross-links, Mutat. Res. 486 (2001) 217-247.
[26] L.J. Niedernhofer, H. Odijk, M. Budzowska, E. van Drunen, A. Maas, A.F. Theil, J. de Wit, N.G. Jaspers, H.B. Beverloo, J.H. Hoeijmakers, R. Kanaar. The structure-specific endonuclease Ercc1-xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks, Mol. Cell. Biol. 24 (2004) 5776-5787.
[27] D.M. Noll, A.M. Noronha, C.J. Wilds, P.S. Miller. Preparation of interstrand cross-linked DNA oligonucleotide duplexes, Front. Biosci. 9 (2004) 421-437.
[28] H. Hong, H. Cao, Y. Wang. Formation and genotoxicity of a guanine-cytosine intrastrand cross-link lesion in vivo, Nucleic Acids Res. 35 (2007) 7118-7127.
[29] S. Bellon, J.L. Ravanat, D. Gasparutto, J. Cadet. Cross-linked thymine-purine base tandem lesions: Synthesis, characterization, and measurement in gamma-irradiated isolated DNA, Chem. Res. Toxicol. 15 (2002) 598-606.
[30] Y. Jiang, H. Hong, H. Cao, Y. Wang. In vivo formation and in vitro replication of a Guanine−Thymine intrastrand cross-link lesion†, Biochemistry (N. Y.). 46 (2007) 12757-12763.
[31] Q. Zhang, Y. Wang. Independent generation of 5-(2‘-deoxycytidinyl)methyl radical and the formation of a novel cross-link lesion between 5-methylcytosine and guanine, J. Am. Chem. Soc. 125 (2003) 12795-12802.
[32] Q. Zhang, Y. Wang. Generation of 5-(2'-deoxycytidyl)methyl radical and the formation of intrastrand cross-link lesions in oligodeoxyribonucleotides, Nucl. Acids Res. 33 (2005) 1593-1603.
[33] C. Gu, Y. Wang. LC-MS/MS identification and yeast polymerase η bypass of a novel γ-irradiation-induced intrastrand cross-link lesion G[8−5]C†, Biochemistry (N. Y.). 43 (2004) 6745-6750.
[34] H. Ding, M.M. Greenberg. Gamma-radiolysis and hydroxyl radical produce interstrand cross-links in DNA involving thymidine, Chem. Res. Toxicol. 20 (2007) 1623-1628.

[35] I.S. Hong, M.M. Greenberg. Efficient DNA interstrand cross-link formation from a nucleotide radical, J. Am. Chem. Soc. 127 (2005) 3692-3693.
[36] V.M. Kuhnert, L.A. Kachnic, L. Li, M. Purschke, L. Gheorghiu, R. Lee, K.D. Held, H. Willers. FANCD2-deficient human fibroblasts are hypersensitive to ionising radiation at oxygen concentrations of 0% and 3% but not under normoxic conditions, Int. J. Radiat. Biol. 85 (2009) 523-531.
[37] B.P. Alter. Radiosensitivity in fanconi's anemia patients, Radiother. Oncol. 62 (2002) 345-347.
[38] J.A. Hartley, R.L. Souhami, M.D. Berardini. Electrophoretic and chromatographic separation methods used to reveal interstrand crosslinking of nucleic acids, J. Chromatogr. 618 (1993) 277-288.
[39] J.H. Wu, J.B. Wilson, A.M. Wolfreys, A. Scott, N.J. Jones. Optimization of the comet assay for the sensitive detection of PUVA-induced DNA interstrand cross-links, Mutagenesis. 24 (2009) 173-181.
[40] Y. Wang, Q. Zhang, Y. Wang. Tandem mass spectrometry for the determination of the sites of DNA interstrand cross-link, J. Am. Soc. Mass Spectrom. 15 (2004) 1565-1571.
[41] Y. Wang, Y. Wang. Structure elucidation of DNA interstrand cross-link by a combination of nuclease P1 digestion with mass spectrometry, Anal. Chem. 75 (2003) 6306-6313.
[42] F. Bergeron, K. Klarskov, D.J. Hunting, J.R. Wagner. Near-UV photolysis of 2-methyl-1,4-naphthoquinone-DNA duplexes: Characterization of reversible and stable interstrand cross-links between quinone and adenine moieties, Chem. Res. Toxicol. 20 (2007) 745-756.
[43] A.R. Collins, J. Cadet, L. Moller, H.E. Poulsen, J. Vina. Are we sure we know how to measure 8-oxo-7,8-dihydroguanine in DNA from human cells? Arch. Biochem. Biophys. 423 (2004) 57-65.
[44] R.C. Leif, J.H. Stein, R.M. Zucker. A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase, Cytometry A. 58 (2004) 45-52.
[45] A. Quinones-Hinojosa, N. Sanai, J.S. Smith, M.W. McDermott. Techniques to assess the proliferative potential of brain tumors, J. Neurooncol. 74 (2005) 19-30.
[46] A.J. Eisch, C.D. Mandyam. Adult neurogenesis: Can analysis of cell cycle proteins move us "Beyond BrdU"? Curr. Pharm. Biotechnol. 8 (2007) 147-165.
[47] W.C. Dewey, R.M. Humphrey. Increase in radiosensitivity to ionizing radiation related to replacement of thymidine in mammalian cells with 5-bromodeoxyuridine, Radiat. Res. 26 (1965) 538-553.
[48] K. Sano, T. Hoshino, M. Nagai. Radiosensitization of brain tumor cells with a thymidine analogue (bromouridine), J. Neurosurg. 28 (1968) 530-538.
[49] C.L. Limoli, J.F. Ward. A new method for introducing double-strand breaks into cellular DNA, Radiat. Res. 134 (1993) 160-169.
[50] R. Saffhill, C.H. Ockey. Strand breaks arising from the repair of the 5-bromodeoxyuridine-substituted template and methyl methanesulphonate-induced lesions can explain the formation of sister chromatid exchanges, Chromosoma. 92 (1985) 218-224.

[51] W.C. Dewey, B.A. Sedita, R.M. Humphrey. Radiosensitization of X chromosome of chinese hamster cells related to incorporation of 5-bromodeoxyuridine, Science. 152 (1966) 519-521.
[52] P. Schyman, R.B. Zhang, L.A. Eriksson, A. Laaksonen. Hydrogen abstraction from deoxyribose by a neighbouring uracil-5-yl radical, Phys. Chem. Chem. Phys. 9 (2007) 5975-5979.
[53] T.J. Kinsella, J.B. Mitchell, A. Russo, G. Morstyn, E. Glatstein. The use of halogenated thymidine analogs as clinical radiosensitizers: Rationale, current status, and future prospects: Non-hypoxic cell sensitizers, Int. J. Radiat. Oncol. Biol. Phys. 10 (1984) 1399-1406.
[54] C.N. Coleman, J.B. Mitchell. Clinical radiosensitization: Why it does and does not work, J. Clin. Oncol. 17 (1999) 1-3.
[55] B.S. Dabaja, P. McLaughlin, C.S. Ha, B. Pro, C.A. Meyers, L.F. Seabrooke, R.B. Wilder, A.P. Kyritsis, H.A. Preti, W.K. Yung, V. Levin, F. Cabanillas, J.D. Cox. Primary central nervous system lymphoma: Phase I evaluation of infusional bromodeoxyuridine with whole brain accelerated fractionation radiation therapy after chemotherapy, Cancer. 98 (2003) 1021-1028.
[56] M.D. Groves, M.H. Maor, C. Meyers, A.P. Kyritsis, K.A. Jaeckle, W.K. Yung, R.E. Sawaya, K. Hess, J.M. Bruner, P. Peterson, V.A. Levin. A phase II trial of high-dose bromodeoxyuridine with accelerated fractionation radiotherapy followed by procarbazine, lomustine, and vincristine for glioblastoma multiforme, Int. J. Radiat. Oncol. Biol. Phys. 45 (1999) 127-135.
[57] M.D. Prados, C. Scott, H. Sandler, J.C. Buckner, T. Phillips, C. Schultz, R. Urtasun, R. Davis, P. Gutin, T.L. Cascino, H.S. Greenberg, W.J. Curran Jr. A phase 3 randomized study of radiotherapy plus procarbazine, CCNU, and vincristine (PCV) with or without BUdR for the treatment of anaplastic astrocytoma: A preliminary report of RTOG 9404, Int. J. Radiat. Oncol. Biol. Phys. 45 (1999) 1109-1115.
[58] M.D. Prados, W. Seiferheld, H.M. Sandler, J.C. Buckner, T. Phillips, C. Schultz, R. Urtasun, R. Davis, P. Gutin, T.L. Cascino, H.S. Greenberg, W.J. Curran Jr. Phase III randomized study of radiotherapy plus procarbazine, lomustine, and vincristine with or without BUdR for treatment of anaplastic astrocytoma: Final report of RTOG 9404, Int. J. Radiat. Oncol. Biol. Phys. 58 (2004) 1147-1152.
[59] W. Szybalski. X-ray sensitization by halopyrimidines, Cancer Chemother. Rep. 58 (1974) 539-557.
[60] S. Greer, M. Alvarez, M. Mas, C. Wozniak, D. Arnold, A. Knapinska, C. Norris, R. Burk, A. Aller, M. Dauphinee. Five-chlorodeoxycytidine, a tumor-selective enzyme-driven radiosensitizer, effectively controls five advanced human tumors in nude mice, Int. J. Radiat. Oncol. Biol. Phys. 51 (2001) 791-806.
[61] L.M. Perez, J.A. Mekras, T.V. Briggle, S. Greer. Marked radiosensitization of cells in culture to X ray by 5-chlorodeoxycytidine coadministered with tetrahydrouridine, and inhibitors of pyrimidine biosynthesis, Int. J. Radiat. Oncol. Biol. Phys. 10 (1984) 1453-1458.
[62] Y. Zeng, Y. Wang. UVB-induced formation of intrastrand cross-link products of DNA in MCF-7 cells treated with 5-bromo-2'-deoxyuridine, Biochemistry. 46 (2007) 8189-8195.

[63] Y. Zeng, Y. Wang. Sequence-dependent formation of intrastrand crosslink products from the UVB irradiation of duplex DNA containing a 5-bromo-2'-deoxyuridine or 5-bromo-2'-deoxycytidine, Nucleic Acids Res. 34 (2006) 6521-6529.
[64] A. Wojcik, A. Bochenek, A. Lankoff, H. Lisowska, A. Padjas, I. Szumiel, C. von Sonntag, G. Obe. DNA interstrand crosslinks are induced in cells prelabelled with 5-bromo-2'-deoxyuridine and exposed to UVC radiation, J. Photochem. Photobiol. B. 84 (2006) 15-20.
[65] E. Bruckmann, A. Wojcik, G. Obe. X-irradiation of G1 CHO cells induces SCE which are both true and false in BrdU-substituted cells but only false in biotin-dUTP-substituted cells, Chromosome Res. 7 (1999) 277-288.
[66] S. Cecchini, S. Girouard, M.A. Huels, L. Sanche, D.J. Hunting. Single-strand-specific radiosensitization of DNA by bromodeoxyuridine, Radiat. Res. 162 (2004) 604-615.
[67] S. Cecchini, S. Girouard, M.A. Huels, L. Sanche, D.J. Hunting. Interstrand cross-links: A new type of gamma-ray damage in bromodeoxyuridine-substituted DNA, Biochemistry. 44 (2005) 1932-1940.
[68] M.E. Dextraze, S. Cecchini, F. Bergeron, S. Girouard, K. Turcotte, J.R. Wagner, D.J. Hunting. Reaching for the other side: Generating sequence-dependent interstrand cross-links with 5-bromodeoxyuridine and gamma-rays, Biochemistry. 48 (2009) 2005-2011.
[69] M.-. Dextraze, J.R. Wagner, D.J. Hunting. 5-bromodeoxyuridine radiosensitization: Conformation-dependent DNA damage, Biochemistry. 46 (2007) 9089-9097.
[70] N.B. Edfeldt, E.A. Harwood, S.T. Sigurdsson, P.B. Hopkins, B.R. Reid. Sequence context effect on the structure of nitrous acid induced DNA interstrand cross-links, Nucleic Acids Res. 32 (2004) 2795-2801.
[71] B. Giese. Electron transfer in DNA, Curr. Opin. Chem. Biol. 6 (2002) 612-618.
[72] B. Giese, J. Amaudrut, A.K. Kohler, M. Spormann, S. Wessely. Direct observation of hole transfer through DNA by hopping between adenine bases and by tunnelling, Nature. 412 (2001) 318-320.
[73] N. Ferrer, F. Azorin, A. Villasante, C. Gutierrez, J.P. Abad. Centromeric dodeca-satellite DNA sequences form fold-back structures, J. Mol. Biol. 245 (1995) 8-21.
[74] D. Huertas, F. Azorin. Structural polymorphism of homopurine DNA sequences. d(GGA)n and d(GGGA)n repeats form intramolecular hairpins stabilized by different base-pairing interactions, Biochemistry. 35 (1996) 13125-13135.
[75] W. Shepard, W.B. Cruse, R. Fourme, E. de la Fortelle, T. Prangé. A zipper-like duplex in DNA: The crystal structure of d(GCGAAAGCT) at 2.1 å resolution, Structure. 6 (1998) 849-861.
[76] R. Mertens, C.v. Sonntag. Determination of the kinetics of vinyl radical reactions by the characteristic visible absorption spectra of vinylperoxyl radicals, Angewandte Chemie International Edition in English. 33 (1994) 1262-1264.
[77] S. Cecchini, C. Masson, C. La Madeleine, M.A. Huels, L. Sanche, J.R. Wagner, D.J. Hunting. Interstrand cross-link induction by UV radiation in bromodeoxyuridine-substituted DNA: Dependence on DNA conformation, Biochemistry. 44 (2005) 16957-16966.

[78] T. Ito, A. Kondo, T. Kamashita, K. Tanabe, H. Yamada, S. Nishimoto. Pathways of excess electron transfer in phenothiazine-tethered DNA containing single-base mismatches, Org. Biomol. Chem. 7 (2009) 2077-2081.
[79] T.G. Gantchev, S. Cecchini, D.J. Hunting. Dynamic conformational states of DNA containing T.T or BrdU.T mispaired bases: Wobble H-bond pairing versus cross-strand inter-atomic contacts, J. Mol. Model. 11 (2005) 141-159.
[80] T.G. Gantchev, D.J. Hunting. Probing the interactions of the solvated electron with DNA by molecular dynamics simulations: Bromodeoxyuridine substituted DNA, J. Mol. Model. 14 (2008) 451-464.
[81] T.G. Gantchev, D.J. Hunting. Probing the interactions of the solvated electron with DNA by molecular dynamics simulations: II. bromodeoxyuridine-thymidine mismatched DNA, J. Mol. Model. 15 (2009) 9-23.
[82] A. Bhattacharyya, D.M. Lilley. The contrasting structures of mismatched DNA sequences containing looped-out bases (bulges) and multiple mismatches (bubbles), Nucleic Acids Res. 17 (1989) 6821-6840.
[83] Y.H. Wang, J. Griffith. Effects of bulge composition and flanking sequence on the kinking of DNA by bulged bases, Biochemistry. 30 (1991) 1358-1363.
[84] H.A. Wagenknecht. Electron transfer processes in DNA: Mechanisms, biological relevance and applications in DNA analytics, Nat. Prod. Rep. 23 (2006) 973-1006.
[85] Y. Li, G. Zon, W.D. Wilson. Thermodynamics of DNA duplexes with adjacent G.A mismatches, Biochemistry. 30 (1991) 7566-7572.
[86] R.M. Wadkins. Targeting DNA secondary structures, Curr. Med. Chem. 7 (2000) 1-15.
[87] S. Waga, H. Shirakawa, S. Mizuno, M. Yoshida. The selective binding of HMG1 to the cruciform DNA structure and the subsequent resumption of transcription, Nucleic Acids Symp. Ser. (22) (1990) 81-82.
[88] S. Altiok, B. Groner. Beta-casein mRNA sequesters a single-stranded nucleic acid-binding protein which negatively regulates the beta-casein gene promoter, Mol. Cell. Biol. 14 (1994) 6004-6012.
[89] L.L. Chen, M.L. Clawson, S. Bilgrami, G. Carmichael. A sequence-specific single-stranded DNA-binding protein that is responsive to epidermal growth factor recognizes an S1 nuclease-sensitive region in the epidermal growth factor receptor promoter, Cell Growth Differ. 4 (1993) 975-983.
[90] R. Duncan, L. Bazar, G. Michelotti, T. Tomonaga, H. Krutzsch, M. Avigan, D. Levens. A sequence-specific, single-strand binding protein activates the far upstream element of c-myc and defines a new DNA-binding motif, Genes Dev. 8 (1994) 465-480.
[91] S.H. Chou, K.H. Chin. Solution structure of a DNA double helix incorporating four consecutive non-watson-crick base-pairs, J. Mol. Biol. 312 (2001) 769-781.
[92] S.H. Chou, K.H. Chin. Zipper-like watson-crick base-pairs, J. Mol. Biol. 312 (2001) 753-768.
[93] L. Tartier, V. Michalik, M. Spotheim-Maurizot, A.R. Rahmouni, R. Sabattier, M. Charlier. Radiolytic signature of Z-DNA, Nucleic Acids Res. 22 (1994) 5565-5570.
[94] V. Michalik, M. Spotheim Maurizot, M. Charlier. Calculation of hydroxyl radical attack on different forms of DNA, J. Biomol. Struct. Dyn. 13 (1995) 565-575.

[95] R.L. Warters, B.W. Lyons. Variation in radiation-induced formation of DNA double-strand breaks as a function of chromatin structure, Radiat. Res. 130 (1992) 309-318.
[96] J. Franchet-Beuzit, M. Spotheim-Maurizot, R. Sabattier, B. Blazy-Baudras, M. Charlier. Radiolytic footprinting. beta rays, gamma photons, and fast neutrons probe DNA-protein interactions, Biochemistry. 32 (1993) 2104-2110.
[97] C.H. Freudenreich. Chromosome fragility: Molecular mechanisms and cellular consequences, Front. Biosci. 12 (2007) 4911-4924.
[98] A.T. Jonstrup, T. Thomsen, Y. Wang, B.R. Knudsen, J. Koch, A.H. Andersen. Hairpin structures formed by alpha satellite DNA of human centromeres are cleaved by human topoisomerase IIalpha, Nucleic Acids Res. 36 (2008) 6165-6174.
[99] T. Lukusa, J.P. Fryns. Human chromosome fragility, Biochim. Biophys. Acta. 1779 (2008) 3-16.
[100] K.S. Lobachev, A. Rattray, V. Narayanan. Hairpin- and cruciform-mediated chromosome breakage: Causes and consequences in eukaryotic cells, Front. Biosci. 12 (2007) 4208-4220.

Chapitre VI – Résultats supplémentaires

VI.1 – EDTA : un capteur efficace de radicaux hydroxyles

La radiolyse de l'eau génère plusieurs types de radicaux qui, ensemble, sont à l'origine d'une chimie complexe qui n'a pas encore été totalement élucidée. Pour cette raison, il est souvent utile de pouvoir sélectionner une ou plusieurs espèces à étudier en inactivant les espèces radicalaires non désirées. Par exemple, dans le cas du BrdU, les électrons aqueux sont responsables de la majorité des dommages spécifiques à l'ADN bromé. Cependant, aux doses utilisées pour étudier ces dommages, les bris causés à l'ADN par les radicaux hydroxyles sont si importants qu'ils masquent ceux qui sont causés par le BrdU. Pour cette raison, nous utilisons des capteurs de radicaux hydroxyles pour voir les dommages qui sont spécifiques au BrdU. Typiquement, ce sont des alcools comme l'isobutanol et le tert-butanol (k_{OH} = 3.3 x 10^9 et 6.0 x 10^8 L mol^{-1} s^{-1}, Buxton *et al.*, 1988) qui sont utilisés en tant que capteurs de radicaux hydroxyles dans ce type d'expérience. Cependant, il est nécessaire de barboter les échantillons avec de l'azote afin d'enlever l'oxygène présent en solution, qui est un excellent capteur d'électrons. La présence d'alcool en solution affecte la densité de la solution et rend le barbotage inefficace et peu reproductible. Pour cette raison, nous avons choisi un autre capteur de radicaux hydroxyles, moins connu mais tout aussi efficace; l'EDTA (acide ethylènediaminetétraacétique, k_{OH} = 4.0 × 10^8 L mol^{-1} s^{-1}, Mertens et Sonntag, 1994).

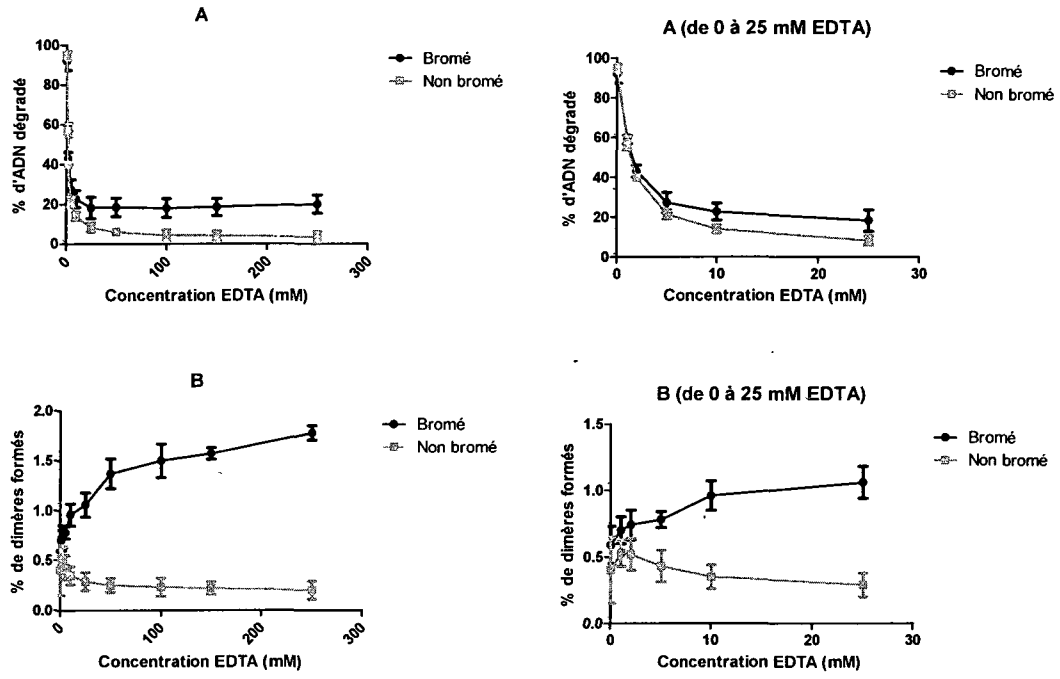

Figure VI-1 : Captation des radicaux hydroxyles par l'EDTA. Un ADN bromé semi-complémentaire a été irradié avec 2400 Gy en présence de concentrations croissantes d'EDTA. La formation de cassures (A) et de dimères interbrins (B) a été mesurée par gel d'électrophorèse. Un agrandissement de chaque figure est montré à droite afin de mettre en évidence la production de dommages à faible concentration d'EDTA.

Afin de valider l'utilisation de l'EDTA en tant que capteur des radicaux hydroxyles dans notre système expérimental, nous avons déterminé sa capacité à capter les radicaux hydroxyles formés dans nos conditions, de même que sa réactivité en présence d'ADN bromé. La figure VI-1A montre que pour l'ADN non substitué, un maximum de radicaux $^{\bullet}OH$ sont captés à partir de 25 mM, avec une pente initiale très marquée jusqu'à 5 mM. On note la formation d'une faible quantité de dimères interbrins formés par les radicaux $^{\bullet}OH$, qui disparaissent en présence de 2 mM EDTA. Ces dimères interbrins, formés par les $^{\bullet}OH$, pourraient s'apparenter à ceux observés par le groupe de Cadet et de Greenberg (Regulus *et al.*, 2007, Sczepanski *et al.*, 2009a), bien que ceux qui aient été observés soient formés dans une région parfaitement complémentaire de l'ADN, plutôt que dans une zone désappariée.

La réactivité de l'ADN bromé est peu ou pas affectée par la présence d'EDTA lors de l'irradiation, comme le montrent les figures VI-1A et B, ainsi que dans la figure VI-2.

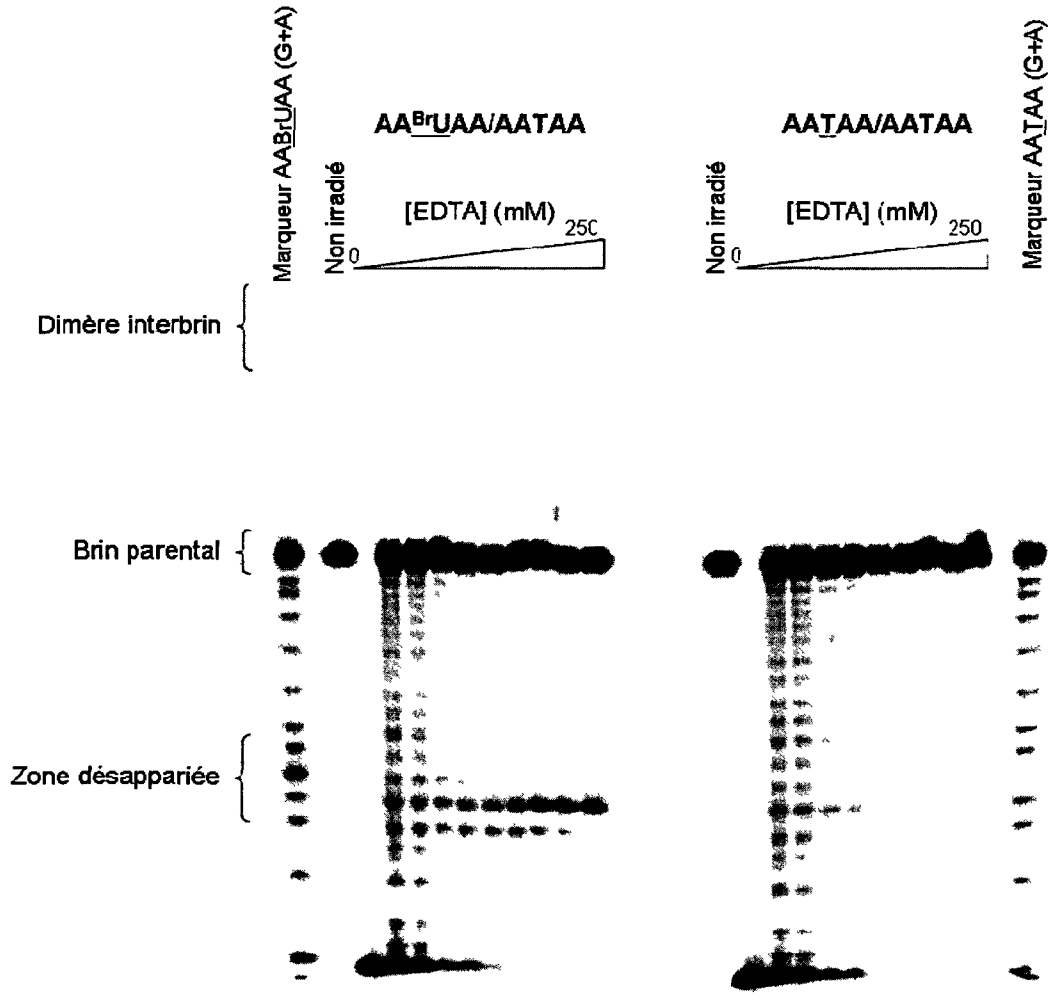

Figure VI-2 : Gel de la formation des cassures à l'ADN bromé et non bromé en fonction de la quantité d'EDTA. Il s'agit d'un des gels à partir duquel les données de la figure VI1 ont été tirées.

Dans la figure A, on assiste aussi à une diminution marquée des cassures alors que la concentration d'EDTA augmente, comme c'était le cas avec l'ADN non bromé. Cependant, le plateau se situe à un niveau plus élevé, qui correspond aux cassures spécifiques au BrdU qui demeurent constantes même en présence de grandes quantités d'EDTA. Le phénomène est encore plus évident lorsque la formation de dimères interbrins est mesurée (figure VI-1B); il y a augmentation de la formation de dimères

en fonction de la concentration d'EDTA, au fur et à mesure que les •OH sont captés. Ceci indique que l'EDTA, contrairement à des capteurs comme le dithiothréitol, n'est pas un agent de réparation chimique.

Ces résultats nous permettent de confirmer que l'EDTA est un capteur de •OH efficace et facile d'utilisation, et que son utilisation n'affecte pas la réactivité de l'ADN bromé.

VI.2 – BrdU en tant que capteur d'électrons aqueux

Bien que les •OH réagissent aussi avec le BrdU, les électrons aqueux sont considérés comme l'espèce réactive la plus importante pour la radiosensibilisation de l'ADN par le BrdU. En effet, les •OH peuvent entraîner la formation de bris sur toutes les bases de l'ADN, tandis que les électrons solvatés créent peu ou pas de cassures. Dans le cas de l'ADN bromé, cependant, les électrons solvatés sont en mesure de produire un radical susceptible de produire des cassures. Ainsi, en modifiant une base de l'ADN, on augmente la production de dommages en amplifiant le pouvoir d'agression d'une espèce réactive qui, autrement, semble très peu contribuer à la toxicité des radiations.

La figure VI-2 montre la conversion du BrU en uracile par les radiations ionisantes. En réalité, le BrU s'avère être un excellent dosimètre, spécialement pour la mesure de la production d'électrons aqueux. Lorsque les radicaux •OH sont captés par l'isobutanol et que seuls les électrons aqueux sont disponibles pour réagir avec le BrU, la production d'uracile est linéaire en fonction de la dose, et cela, au-delà de la dose testée, soit 2400 Gy (figure VI-2, courbe noire). La conversion en uracile est aussi linéaire en absence d'isobutanol (figure VI-2, courbe grise), bien que l'efficacité soit plus faible. Ce résultat s'explique facilement avec les chromatogrammes HPLC de la figure VI-3. Lorsque le BrU est irradié en présence d'isobutanol (courbe gris clair), 100 % du dommage est converti en uracile, alors qu'en absence d'isobutanol

(courbe gris foncé), la présence de $^{\bullet}OH$ entraîne la production d'autres types de dommages, ce qui diminue la quantité d'uracile formée.

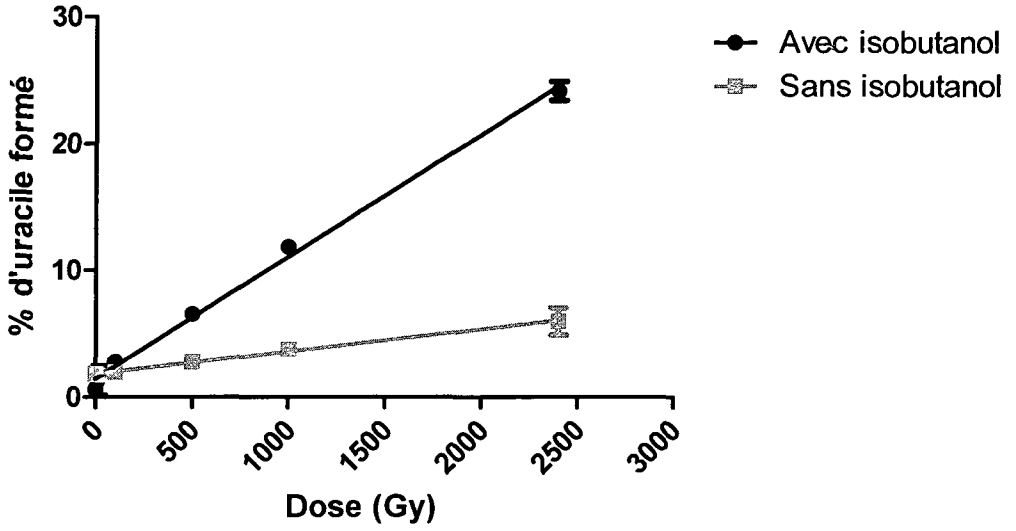

Figure VI-3 : Conversion du BrU en uracile en fonction de la dose. Le BrU (concentration finale de 5 mM BrU, 10 mM tampon phosphate pH 7.0) a été irradié dans des conditions hypoxiques en présence ou en absence de 2% isobutanol. La conversion du BrU en uracile a été mesurée par HPLC sur une colonne en phase inversée couplée à un détecteur UV (260 nm). Conditions d'élution : 0-25 min; 10 mM tampon phosphate pH 5.5, 25-45 min; +2% acétonitrile / min, 45-70 min; 0 % acétonitrile.

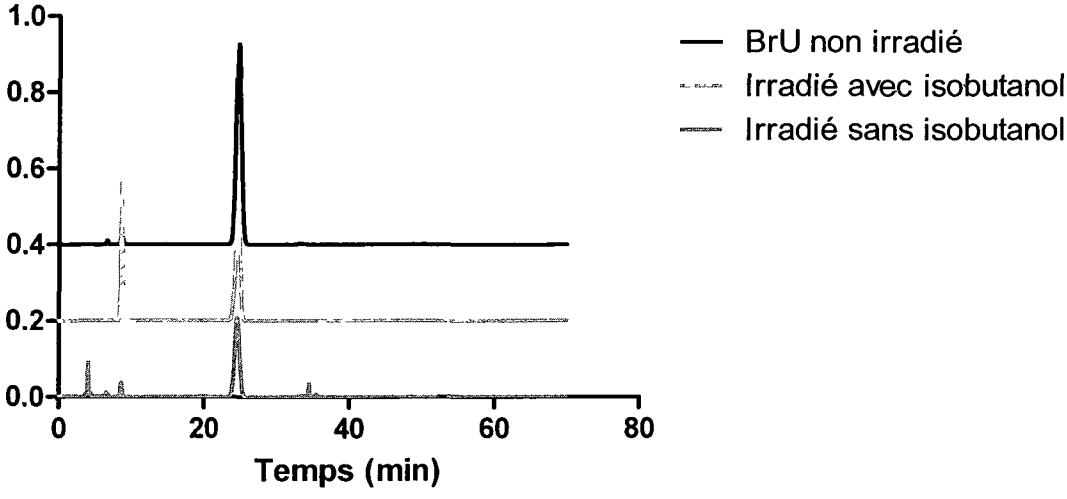

Figure VI-4 : Dégradation du BrU selon les conditions d'irradiation. Le BrU a été irradié avec 2400 Gy en présence ou en absence de 2% isobutanol. Les électrons solvatés issus de la radiolyse de l'eau convertissent le BrU (25 min) en uracile (9 min).

Cette capacité du BrU de capter les électrons et de les piéger rapidement en produisant de l'uracile, notre laboratoire l'a utilisée pour étendre l'utilisation du BrU et du BrdU à la captation des électrons solvatés. La figure VI-4 montre que la production de cassures à l'ADN bromé, qui dépend des électrons aqueux, est radicalement diminuée en présence de concentrations croissantes de BrU. D'autres capteurs d'électrons aqueux sont couramment utilisés dans le domaine de la chimie des radiations, notamment l'oxygène et l'oxyde nitreux (N_2O), ou encore l'acétone. Cependant, les premiers sont des gaz, dont il peut être difficile de contrôler précisément la concentration dans des conditions expérimentales, plus particulièrement dans les cellules. De plus, dans le cas de composés comme l'acétone, ces produits sont susceptibles de déshydrater l'ADN et de modifier sa structure, ou encore de dénaturer certaines protéines. Dans le cas où ces effets seraient problématiques, l'utilisation du BrU ou du BrdU comme capteur d'électrons pourrait s'avérer très intéressante.

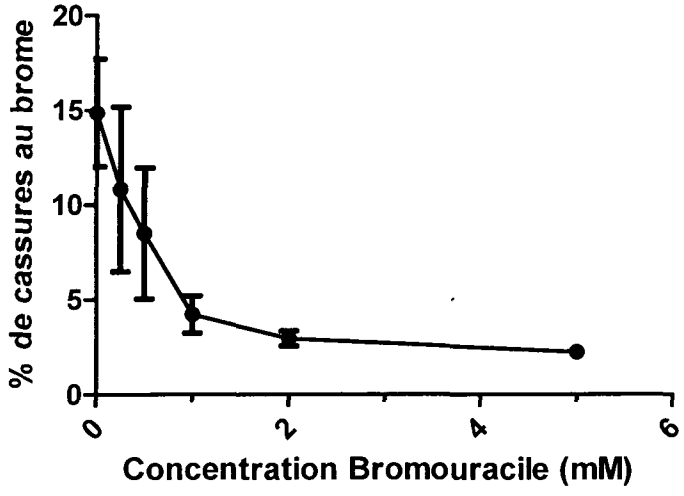

Figure VI-5 : 5-BrU en tant que capteur d'électrons solvatés.

Cependant, il est important de mentionner que d'autres tests pourraient être faits pour valider le 5-BrU en tant que capteur d'électrons solvatés. En effet, la captation de l'électron entraîne la formation d'un radical uracil-5-yl très réactif, qui est susceptible de causer la production d'autres dommages à l'ADN – c'est entre autres ainsi que se forment les cassures lorsque le BrdU est exposé aux radiations. Cependant, nous n'avons observé aucune augmentation dans la formation de cassures franches en fonction de la concentration de BrU présente en solution au moment de l'irradiation, mais la production d'autres types de dommage (p. ex. la formation de lésions aux bases) n'a pas été étudiée. Ainsi, le pouvoir d'agression du radical uracil-5-yl à proximité de l'ADN devra être davantage étudié. Dans le cas d'une application potentielle dans les cellules, il est aussi important de noter qu'une concentration de 10μM BrU dans le milieu de culture est généralement utilisée. Puisque la concentration de BrU qui parvient au noyau risque d'être encore plus faible, cela signifie que pour des doses élevées, le BrU pourrait ne pas capter tous les électrons produits lors de l'irradiation (environ 0.3 μM d'électrons produits pour 1 Gy). Malgré cette limitation, nous croyons que le BrU montre un potentiel intéressant en tant que capteur d'électrons solvatés.

VI.3 – Le BrdU et l'effet direct des radiations

La conclusion du premier article, où la formation de dommage par les radiations ionisantes à l'ADN bromé à différents niveaux d'hydratation est étudiée, est que la présence d'une forme B de l'ADN est nécessaire à la production de cassures et de dimères. Cependant, ces résultats peuvent aussi être étudiés du point de vue de l'effet direct/indirect des radiations. En effet, à bas niveau d'hydratation ($0 < \Gamma < 6$, où Γ représente le nombre de molécules d'eau par nucléotides), la radiation peut ioniser les molécules d'eau, mais cette ionisation se transfère immédiatement à l'ADN. Ce transfert est décrit comme l'effet quasi-direct des radiations (Debije *et al.*, 2000, La Vere *et al.*, 1996). De fait, les radicaux $^{\bullet}OH$ ne sont pas observés avant $\Gamma \approx 9$, dans ce qui est décrit comme la couche primaire interne d'hydratation, mais sont plutôt

détectés dans la couche primaire externe ($10 < \Gamma < 20$). La couche secondaire d'hydratation ($\Gamma > 20$) ne peut être différenciée de l'eau en fonction de la réactivité avec l'ADN. Donc, $\Gamma \approx 6$ représente l'effet quasi direct, tandis que $\Gamma \approx 14$ et 21 montrent l'effet indirect des radiations sur un ADN de forme A. Enfin, un niveau d'hydratation de $\Gamma \approx 31$, quant à lui, montre l'effet indirect des radiations dans un ADN de forme B. Ainsi, les résultats présentés dans le premier article permettent de confirmer l'existence d'un effet direct et spécifique des radiations pour le BrdU, effet qui avait déjà été montré en phase gazeuse et solide sur des nucléotides et de courts oligonucléotides, principalement sous la forme de dommages aux bases suite à une exposition à des électrons de basse énergie (Abdoul-Carime *et al.*, 2000a, Abdoul-Carime *et al.*, 2000b, Dugal *et al.*, 2000). Cependant, l'ADN est dans une forme A à $\Gamma \approx 6$, ce qui fait que seules des lésions aux bases sont observées. Cependant, une question demeure : est-ce que l'effet direct des radiations sur l'ADN bromé produit seulement des lésions aux bases, ou d'autres types de dommages sont aussi produits quand une conformation appropriée de l'ADN est introduite? Des expériences récentes montrent que les électrons de basse énergie peuvent mener à la production de cassures et à la formation d'uracile dans un ADN simple brin bromé (Li *et al.*, en préparation). Cependant, le mécanisme exact de sensibilisation n'est pas encore élucidé et il est possible que la présence d'un ADN double brin affecte les produits de réaction finaux.

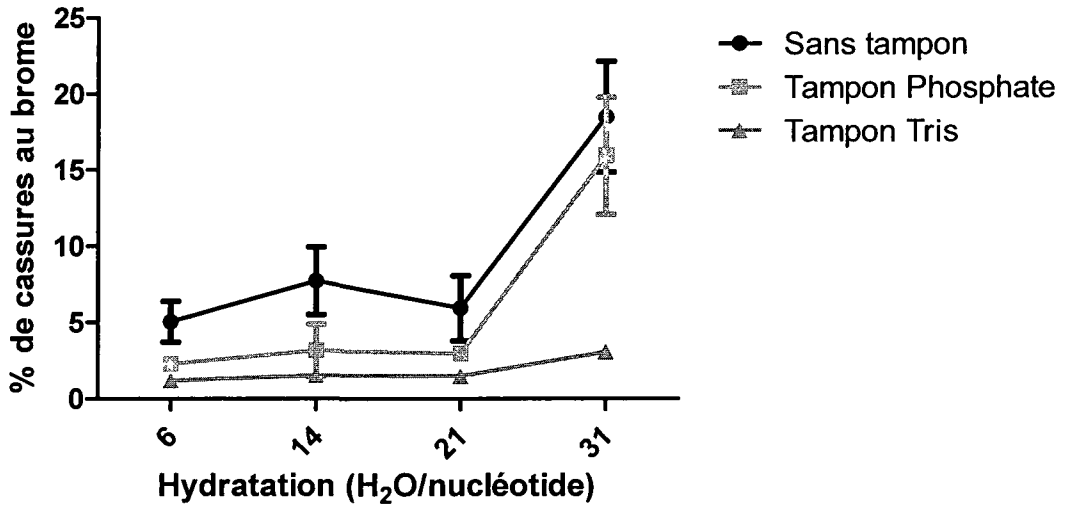

Figure VI-6 : Effet du tampon sur l'irradiation d'ADN bromé hydraté.

Pour répondre à cette question, d'autres expériences ont été menées à l'aide de ce système expérimental. En effet, à $\Gamma > 9$, il y a formation de radicaux $^{\bullet}OH$ et d'électrons solvatés issus de l'effet indirect des radiations. Cependant, il est possible de soustraire ces espèces réactives de l'équation en utilisant des capteurs de radicaux. Par exemple, le fait que les expériences d'hydratation présentées dans le premier article se soient déroulées en présence d'oxygène ($k_e = 1.9 \times 10^{10}$ L mol^{-1} s^{-1}, Buxton *et al.*, 1988) et de tampon phosphate (un capteur d'électrons solvatés à haute concentration, Jortner *et al.*, 1962) diminue radicalement la quantité d'électrons solvatés susceptibles de réagir avec le BrdU. Ainsi, en changeant le tampon présent lors de l'irradiation, il est possible de minimiser l'importance de l'effet indirect afin de pouvoir étudier la composante directe de l'effet des radiations. La figure VI-5 montre l'effet de différents tampons sur la formation de cassures à l'ADN bromé par la radiation ionisante. En absence de tampon (ligne noire), l'ADN est beaucoup plus instable, ce qui entraîne non seulement une plus grande quantité de cassures dans la région du brome, mais une augmentation générale de la formation de dommages, que ce soit pour l'ADN bromé ou non bromé. Un phénomène semblable est observé lorsque des plasmides sont irradiés par des électrons de basse énergie sur du tantale. Bien que les conditions d'irradiation soient très différentes, il est possible que cette

instabilité soit une caractéristique propre à l'ADN solubilisé, peut-être causée par une augmentation de l'oxydation. En présence de tampon phosphate, l'ADN est stabilisé, et on ne retrouve que des dommages qui sont spécifiques au brome (ligne gris foncé). Cependant, il est peu probable que ces dommages soient dus aux électrons solvatés, puisque l'irradiation se fait en présence d'oxygène et de tampon phosphate. Toutefois, les radicaux $^{\bullet}OH$ sont aussi susceptibles de réagir spécifiquement avec le BrdU pour produire des dommages. Aussi, l'irradiation a été reprise, cette fois en présence de tampon Tris, un excellent capteur de $^{\bullet}OH$ (ligne gris pâle). En présence de Tris, la formation de cassures diminue par un facteur de 5. De plus, lorsque les cassures sont observées sur gel, on constate que les cassures, qui s'étendaient sur une distance de plusieurs bases en présence de tampon phosphate, sont limitées à la base en 5′ du BrdU dans le cas du tampon Tris. Ainsi, puisque les électrons aqueux et les $^{\bullet}OH$ sont captés par l'oxygène et le Tris, respectivement, il est très probable que les cassures qui demeurent soient attribuables à l'effet direct. Cependant, comme c'était le cas pour les dommages produits par l'effet indirect, une forme d'ADN B est nécessaire à la production de cassures. Ainsi, ces résultats montrent qu'il est tout à fait possible que l'effet direct des radiations sur l'ADN bromé produise des cassures dans l'ADN cellulaire.

Chapitre VII – Discussion

VII.1 – Effet de la structure de l'ADN

Notre laboratoire a été le premier à mettre en évidence l'importance de la structure de l'ADN pour la radiosensibilisation de l'ADN par le BrdU. En effet, Cecchini et al. a démontré en 2005 que la présence d'une région ouverte de l'ADN (par exemple, une bulle formée par un ADN semi-complémentaire) augmentait la formation de cassures suite à une irradiation aux rayons gamma (Cecchini *et al.*, 2004). De plus, notre laboratoire a aussi établi que cette structure de l'ADN entraînait la formation de dimères interbrins (Cecchini *et al.*, 2005). Ces dimères interbrins sont d'une importance cruciale pour la radiosensibilisation de l'ADN par le BrdU, car si leur existence était démontrée dans les cellules, cela aurait des implications considérables quant au mécanisme de radiosensibilisation de l'ADN bromé. En effet, jusqu'à présent, ce sont les bris doubles qui sont jugés comme responsables des propriétés radiosensibilisatrices du BrdU. Les dimères interbrins, parce qu'ils créent un lien covalent entre les deux brins d'ADN, empêchent la séparation des brins d'ADN. Cette séparation est l'un des mécanismes impliqués dans au moins deux des processus cruciaux pour la cellule; la transcription et la réplication. Pour cette raison, les dimères interbrins sont considérés comme l'un des dommages les plus toxiques pour la cellule.

Notre laboratoire a donc décidé de réévaluer les bases moléculaires de la radiosensibilisation de l'ADN par le BrdU, et plus précisément de déterminer quelles étaient les conditions qui maximisaient la formation de dimères interbrins. Puisque l'importance de la structure avait déjà été établie par notre laboratoire, mon projet a porté sur un autre type de structure; la forme de la double hélice d'ADN.

VII.1.1 – ADN-A vs ADN-B

Ces dernières années, plusieurs formes d'ADN ont été observées dans les cellules. Parmi elles, la forme B et la forme A sont les plus connues et les mieux caractérisées. Les résultats présentés dans le premier article montrent encore une fois l'importance de la structure dans la radiosensibilisation de l'ADN par le BrdU. L'ADN-A ne produit que des lésions alcalines (i.e., qui sont révélées par un traitement alcalin) localisées au site du BrdU, tandis que l'ADN-B produit des cassures qui s'étalent jusqu'à 3 pb du site du radical initial, en plus d'entraîner la formation de dimères. La figure VII-1 montre le modèle que nous avons formulé en fonction des observations présentées dans cet article :

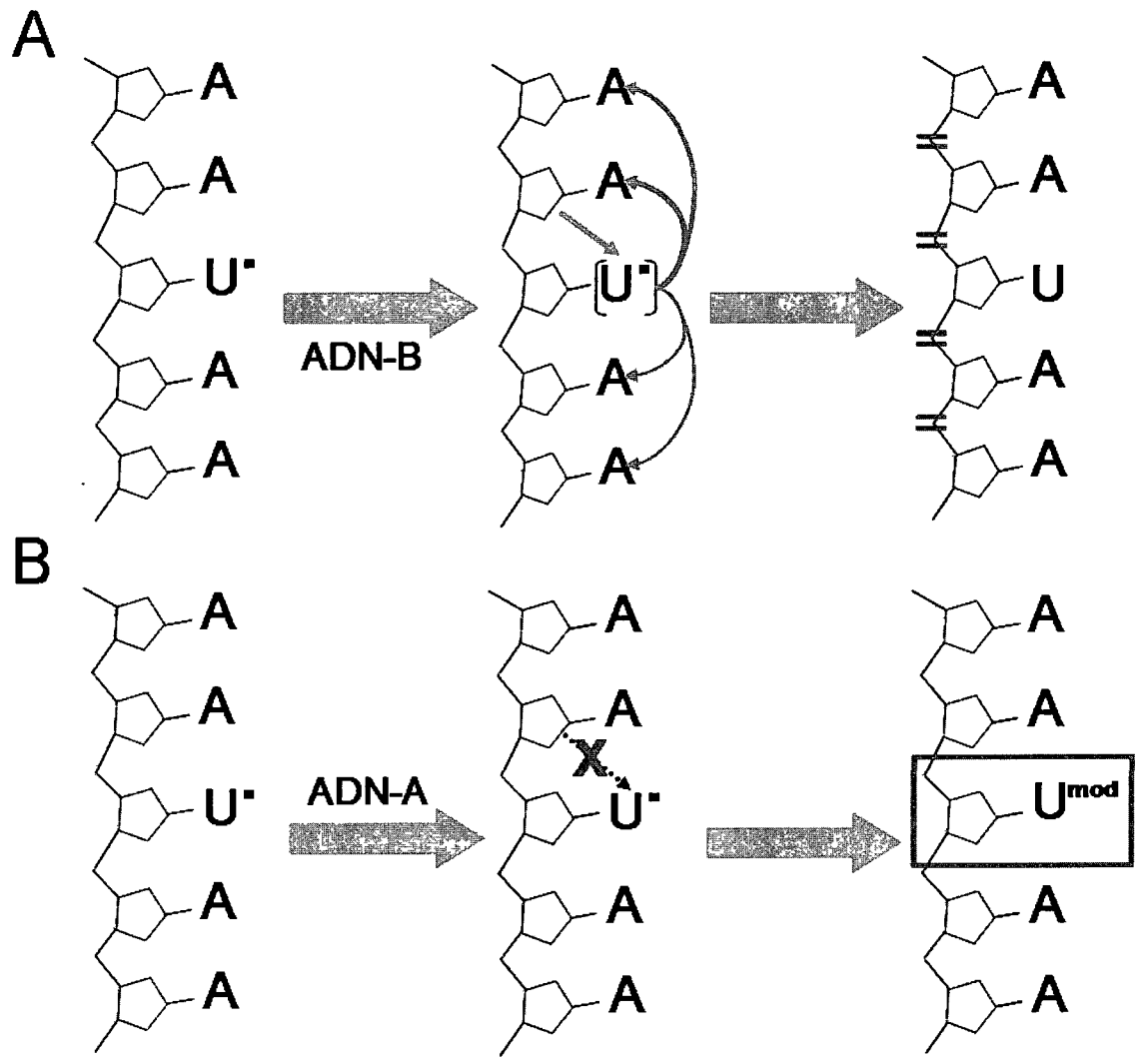

Figure VII-1 : Génération des dommages à l'ADN bromé en fonction de la conformation.

Dans les deux cas, le modèle prévoit qu'il y ait attachement de l'électron au site du BrdU et formation du radical uridine-5-yl. Cependant, lorsque l'ADN bromé adopte une forme B, la structure de l'ADN fait en sorte que le radical est en mesure de faire l'abstraction d'un hydrogène sur le sucre du nucléotide en 5′, plus précisément sur le C1′ ou le C2′. Par la suite, les réactions subséquentes mènent à la formation d'une cassure ou d'un dimère. De cette façon, il y a production de dommages qui ne sont pas localisés uniquement au site de la substitution du BrdU. Alors que pour un ADN-A, le radical, possiblement parce que la proximité des bases est différente, est

incapable de compléter l'abstraction au niveau du sucre en 5'. Il est alors possible qu'une autre réaction avec un nucléotide adjacent ait lieu, ou que le radical soit tout simplement fixé par réaction avec l'eau; le dommage demeure localisé au site de substitution, et n'apparaît qu'après un traitement alcalin. Le dommage qui résulte de l'irradiation d'un ADN bromé de forme A, bien qu'il n'ait pas été identifié précisément, correspond probablement à une lésion au niveau de l'uracile. Le groupe de Léon Sanche a en effet déjà observé le départ de fragments CN et OCN suite à l'irradiation de BrdU par des électrons de basse énergie (Abdoul-Carime *et al.*, 2000b, Dugal *et al.*, 2000). Ces fragments correspondent à la fragmentation de la base plutôt qu'à celle du sucre, ce qui indique qu'il est peu probable que le radical uracil-5-yl soit transféré au sucre.

Ces résultats ont montré une fois de plus l'importance de la structure de l'hélice pour la radiosensibilisation de l'ADN par le BrdU. En effet, la forme de l'ADN et son état d'hybridation influencent :

- la quantité de dommage;
- le type de dommage (dimère interbrin, cassure, lésion aux bases);
- la localisation du dommage (au site de substitution vs. migration du dommage).

VII.1.2 – Flexibilité de l'ADN

Un autre aspect qui semble déterminer la formation de dommages à l'ADN bromé est la flexibilité de la double hélice. En effet, notre laboratoire a déjà proposé que c'est la flexibilité résultant de l'introduction d'une région ouverte (bulle) qui est responsable de la formation du dimère interbrin, qui n'est pas observé lorsqu'un ADN parfaitement complémentaire est irradié. Cette flexibilité permettrait à d'autres bases, qui ne sont normalement pas à proximité, de réagir avec le radical uridine-5-yl pour produire d'autres types de dommages.

Un des premiers volets de mon projet de recherche a été de reproduire l'ouverture des brins qui se produit dans la cellule lors de la transcription, afin de déterminer si des processus cellulaires pouvaient augmenter l'effet du BrdU suite à une irradiation, ou encore si la radiosensibilisation de l'ADN bromé se produisait dans des zones spécifiques de la cellule (p.ex. bulles de transcription, fourches de réplication, centromères). Pour cela, j'ai utilisé un système modèle avec l'ARN polymérase d'*Escherichia coli*. Bien que ce projet n'ait pas été mené à terme, une conclusion a tout de même pu être tirée de cette série d'expériences; la flexibilité de l'ADN est importante pour la radiosensibilité de l'ADN bromé. La figure VII-2 montre que les dimères qui sont formés par les rayons gamma en présence d'une bulle disparaissent lorsque l'ARN polymérase est présente.

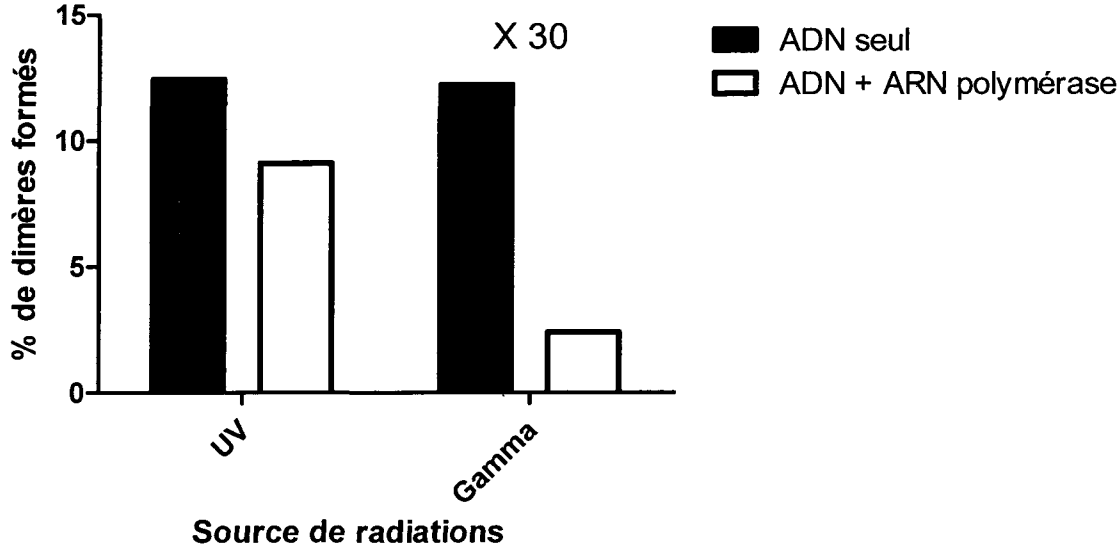

Figure VII-2 : Effet de l'ouverture des brins d'ADN par l'ARN polymérase sur les dommages causés par l'irradiation de l'ADN bromé.

Il est possible que cette diminution soit due à l'interférence de la protéine avec la réaction des radicaux, ce qui diminuerait d'une part le nombre d'électrons solvatés susceptibles de réagir avec le BrdU, et qui d'autre part pourrait favoriser la formation

de ponts entre l'ADN et la protéine. En effet, la formation de ponts entre l'ARN polymérase et l'ADN a été observée dans ce système, mais elle n'était cependant pas dépendante de la présence du BrdU. Cependant, notre hypothèse est que la séparation des brins d'ADN par l'ARN polymérase, ainsi que les ponts hydrogènes qui sont formés entre l'ADN et la protéine, nuisent à la formation du dimère interbrin en éloignant physiquement les brins l'un de l'autre. Là où la région semi-complémentaire permettait une plus grande flexibilité, le mécanisme de formation d'une bulle de transcription nécessite le placement des brins d'ADN à un endroit précis dans l'espace, ce qui diminue la flexibilité de la région ouverte et nuit à l'attaque du radical uridine-5-yl sur les autres bases. Cela n'exclut pas que les régions ouvertes de l'ADN cellulaire soient plus sensibilisées par le BrdU que les autres, mais ces résultats indiquent que la flexibilité de l'ADN dans ces régions sera un facteur déterminant pour la production de dommages.

Enfin, une autre observation intéressante est que la diminution observée en présence de l'ARN polymérase est moins marquée lorsque l'ADN est soumis aux rayons UV qu'aux rayons gamma. Cette observation concorde avec d'autres résultats à l'UV, qui montrent que la photosensibilisation de l'ADN bromé est indépendante de la structure. La différence entre ces différents mécanismes de sensibilisation est très marquée, et devra être élucidée par d'autres expériences.

VII.2 – Effet de la séquence de l'ADN

Bien que la réactivité des différentes bases de l'ADN soit l'un des aspects les plus étudiés dans le domaine de la chimie des radiations, l'impact de la séquence sur la production de dommage au niveau de l'ADN cellulaire est encore largement incompris. De plus, comme la séquence de l'ADN peut influencer la structure secondaire et tertiaire de l'ADN, il est possible que la réactivité des radicaux soit

d'autant plus influencée. Pour cette raison, un des volets de mon projet de recherche a porté sur l'effet de la séquence sur la production de dommages à l'ADN bromé.

Les résultats présentés dans le second article montrent encore une fois l'importance cruciale de l'environnement où le radical est créé : la figure III-3 établit clairement à quel point la séquence influence la production des dommages à l'ADN bromé, que ce soit au niveau de la formation des dimères ou des cassures.

Toutefois, ce sont les résultats présentés à la figure III-4 qui sont les plus spectaculaires. En effet, lorsqu'il est soumis aux radiations, le BrdU produit un radical unique – le radical uridine-5-yl – qui est à l'origine de tous les produits qui sont observés par gel, que ce soit les cassures ou les dimères interbrins. La figure III-4 montre qu'à partir de ce radical unique, jusqu'à 10-12 structures de dimères distinctes peuvent être séparées par électrophorèse sur gel dénaturant, en plus de produire des cassures qui s'étendent sur l'ensemble de la région désappariée. Ces nombreux produits distincts représentent une indication supplémentaire que la réactivité est grandement augmentée au site d'une région ouverte de l'ADN. De plus, cette observation est la première indication qu'un radical formé dans une région ouverte de l'ADN est en mesure de migrer à un site distant du site de formation initial. En effet, si la migration était impossible, seule une attaque d'une des 5 bases sur le brin opposé au brin bromé serait en mesure de former des dimères interbrins. Comme l'électrophorèse sur gel est incapable de résoudre des structures symétriques, cela signifie qu'un maximum de 3 bandes distinctes pourrait être observé sur gel (figure VII-3, réaction de gauche). Entre 10 et 12 bandes distinctes peuvent être visualisées sur gel, ce qui indique qu'il est possible que le radical initial puisse transférer sur d'autres bases du brin bromé avant d'attaquer le brin opposé et ainsi générer un dimère interbrin (figure VII-3, réaction de droite). Cette constatation, si elle est appliquée à l'ADN cellulaire, implique que la structure de l'ADN est susceptible d'influencer le type et la complexité des dommages produits, et que les modèles expérimentaux utilisant uniquement de l'ADN complémentaire pourraient en réalité

ne pas représenter adéquatement ce qui se produit lors d'une irradiation de l'ADN cellulaire.

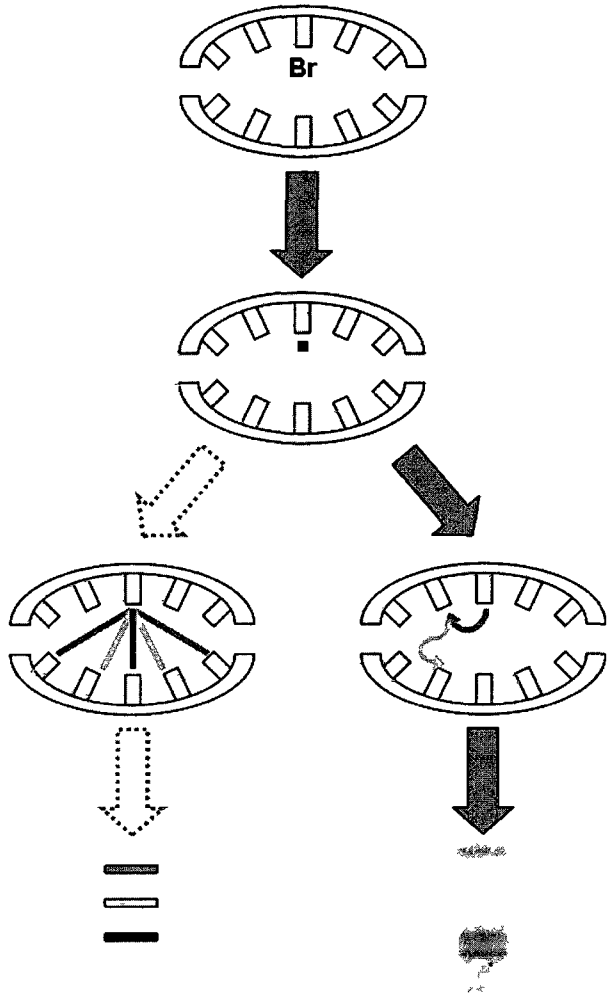

Figure VII-3 : Production de bandes multiples à partir d'un radical unique.

Cette notion, que la présence d'une structure particulière dans l'ADN affecte la réactivité des bases et le type de dommage produit, est d'autant plus intrigante qu'elle ne s'applique pas qu'à l'ADN bromé. En effet, des douze séquences bromées qui ont été étudiées dans le second article, on a observé la formation de dimères interbrins dans l'équivalent non bromé de trois séquences. Ces dimères interbrins, formés par les rayons ionisants dans des conditions hypoxiques et en absence de radicaux hydroxyles, sont spécifiques à la présence d'une zone désappariée de l'ADN. La

figure V-1 montre le patron de bandes obtenu avec les trois séquences qui ont produit des dimères dans l'ADN non bromé, ainsi que leurs équivalents obtenus avec l'ADN bromé. Bien que plusieurs bandes formées dans l'ADN bromé n'aient pas d'équivalent pour l'ADN non bromé, d'autres bandes, dont la bande centrale de GGTGG//ATTTA, est présente dans les deux ADN, et est amplifiée par la présence du BrdU. Ceci indique que, bien que la chimie de la formation des dimères interbrins est probablement très différente entre l'ADN bromé et l'ADN non bromé – entre autres, il n'y a pas de formation de cassures au niveau de l'ADN non bromé – une certaine similarité existe probablement. De façon plus importante, ces résultats révèlent que les régions ouvertes de l'ADN sont particulièrement favorables à la production de dimères interbrins, surtout si on considère que des mécanismes distincts semblent être impliqués dans la formation de dimères dans l'ADN bromé et non bromé.

VII.3 – Structure des dimères interbrins : effet de l'environnement

Si les deux premiers articles ont montré l'importance de la structure et de la séquence de l'ADN – l'importance de l'environnement où le radical initial est généré, donc – pour la formation de dimères, le troisième article montre plus précisément l'effet qu'a cet environnement sur la structure du dimère qui est formé. Dans le troisième article, nous avons étudié les sites impliqués dans la formation des dimères interbrins dans trois des douze séquences qui avaient été étudiées dans le second article. Nous avons modifié une méthode développée en 1991 par Millard et al. (Millard *et al.*, 1991), où l'emplacement des dimères était déterminé par clivage aléatoire de l'ADN ponté. Cette méthode produisait des cassures à toutes les bases de l'ADN, sauf aux sites de pontage. Une diminution des cassures sur gel d'électrophorèse signalait donc la présence d'un dimère interbrin. Cependant, cette méthode s'appliquait difficilement si deux dimères étaient localisés sur des bases adjacentes, et représentait au mieux une démonstration indirecte de la présence de dimères interbrins. Pour cette raison, nous

avons modifié la méthode pour nous permettre de visualiser seulement les bases qui avaient été endommagées par la présence du dimère.

La figure IV-3 montre que pour les trois séquences qui ont été étudiées, et dont les dimères interbrins ont un patron de bandes très différent sur gel d'électrophorèse, la localisation des dimères interbrins est aussi très différente. Ces résultats confirment aussi notre hypothèse initiale, présentée dans le second article, que le transfert de radical est possible dans un ADN désapparié, autant sur le brin bromé que sur le brin opposé. En effet, dans le cas de AA^{Br}UAA//AATAA et GG^{Br}UGG//AATAA, d'autres bases que le BrdU sont impliquées dans la formation du dimère, ce qui implique que le radical initial formé au site du BrdU a transféré aux autres nucléotides avant d'attaquer le brin opposé pour former le dimère.

Cette hypothèse est aussi confirmée par les résultats obtenus pour les mêmes séquences irradiées à l'UV. Dans le cas d'ADN bromé irradié par les rayons UV, un radical cation est aussi créé, préférentiellement sur la base en 5′ du BrdU (cette préférence dépend du potentiel d'oxydation des bases adjacentes). Dans l'ADN double brin, le radical cation a tendance à migrer le long de l'hélice pour se localiser sur les guanines, à cause de leur faible potentiel d'oxydation. Étonnamment, dans le cas de AA^{Br}UAA//AATAA et AA^{Br}UAA//CCCCC, la guanine en 5′ de l'extrémité de la bulle sur le brin opposé au BrdU est extrêmement réactive. Cela suggère que non seulement le radical cation peut être transféré sur le brin opposé dans un ADN mésapparié, mais aussi que ce transfert est impliqué dans la formation de dimères. Cette hypothèse a été confirmée lorsque les résultats obtenus avec GG^{Br}UGG//AATAA ont été analysés; lorsque des guanines entourent le BrdU, la guanine du brin opposé n'est plus impliquée dans la formation du dimère.

Ainsi, le troisième article montre clairement qu'à partir d'un radical initial unique, une variété impressionnante de structures peut être formée. De plus, nos résultats (figure V-1) montrent aussi que cette réactivité ne s'applique pas qu'à l'ADN

substitué avec le BrdU, mais aussi à l'ADN non substitué, et qu'elle ne dépend pas du type de radiation (ionisante ou UV) qui est employé pour créer le radical. Cette facilité à former des dimères semble être spécifique à l'ADN mésapparié, puisqu'elle n'est pas observée dans l'ADN parfaitement complémentaire. Or, bien que des mésappariements multiples existent dans la cellule, par exemple durant la transcription ou la réplication, ou encore dans les centromères, il y a très peu d'études qui se sont intéressées à connaître comment ces régions sont affectées par les radiations. Étant donnée la spécificité de structure que nous avons observée pour la formation de dimères, il est possible que ces régions de l'ADN cellulaire soient particulièrement favorables à la formation de dimères interbrins.

Puisque nous connaissons maintenant les sites privilégiés de formation des dimères interbrins dans l'ADN bromé, il sera plus facile de trouver les structures des dimères interbrins en utilisant la spectrométrie de masse. Des essais préliminaires avaient donné un nombre trop élevé de candidats, mais puisqu'on connaît maintenant les bases les plus susceptibles de réagir ensemble, il sera plus facile de sélectionner les candidats les plus intéressants.

Bien sûr, le but ultime de ce projet est d'identifier la structure de quelques dimères interbrins, et de confirmer s'ils sont produits par les radiations dans l'ADN cellulaire bromé. Si c'est le cas, il faudra examiner l'impact de ces dimères sur la cellule, ainsi que les mécanismes qui sont impliqués dans la réparation de ce type de lésion. Enfin, l'utilisation du BrdU en tant que radiosensibilisateur en clinique devra éventuellement être réévaluée.

Il est important de mentionner ici quelques mots sur la réparation des dimères interbrins. En effet, comme il en a été question dans la section 1.2 du chapitre V, même si beaucoup d'efforts ont été consacrés à l'identification des mécanismes qui régissent la réparation des dimères interbrins, on en sait toujours très peu à ce sujet. D'autant plus qu'une quantité croissante d'études montrent que la voie de réparation

dépend des caractéristiques du dimère (Cai *et al.*, 2007, Smeaton *et al.*, 2008, Smeaton *et al.*, 2009). Par exemple, Noll et al. (Noll *et al.*, 2006) a proposé que le décrochement (unkooking) du dimère par le système NER n'était peut-être pas une caractéristique générale de la réparation des dimères, comme on le croyait auparavant, mais que cette étape était plutôt spécifique aux psoralènes, qui représente l'un des systèmes les plus utilisés pour étudier les dimères. À ce stade, il est donc difficile de prévoir comment le dimère interbrin causé par le BrdU risque d'être réparé, d'autant plus qu'il semble se former préférentiellement dans des régions où la structure de l'ADN n'est pas sous forme double brin (par exemple, les zones de transcription ou les épingles à cheveux), et dont l'existence nécessite possiblement une voie de régulation pour inhiber le travail de la NER dans ces régions. Afin d'éclaircir le mécanisme de réparation, l'utilisation de lignées cellulaires déficientes en réparation serait possible, notamment l'utilisation de lignées Fanconi, qui sont particulièrement sensibles aux agents produisant des dimères. Cependant, une des difficultés importante qui est associée à ce système est que le BrdU produit à la fois des dimères et des cassures, qui sont susceptibles de solliciter les mêmes voies de réparation. Cette prochaine étape représente donc un grand défi, qui devrait nous permettre de mieux comprendre le mécanisme de radiosensibilisation de l'ADN par le BrdU. Les connaissances recueillies jusqu'à présent devraient faciliter la transition vers l'étude *in vivo*.

En effet, mes travaux de thèse ont montré que les caractéristiques de l'environnement où se trouve le BrdU sont critiques pour la production de dommages, et que :

- la structure de l'ADN (état d'hybridation, forme A/B);
- la séquence de l'ADN entourant le BrdU;
- le type de radiation utilisé (radiations ionisantes, UVB).

Influençaient :

- la quantité de dommage produit;
- le type de dommage produit (lésions aux bases, cassures, dimères interbrins);
- l'emplacement du dommage.

Tous ces facteurs ont probablement une incidence importante sur la létalité des dommages qui sont produits lorsque des cellules dont l'ADN a été substitué avec le BrdU sont soumises aux radiations. Bien que d'autres travaux soient nécessaires afin de comprendre comment le BrdU endommage l'ADN cellulaire, les résultats présentés ici montre la facilité avec laquelle l'ADN bromé est en mesure de produire des dimères interbrins lorsque soumis à la radiation. Ce résultat est particulièrement intéressant, puisqu'il permet de fournir une piste de réflexion quant à l'échec des essais cliniques utilisant le BrdU. En effet, la dernière étude de phase III utilisait aussi un agent formant des dimères interbrins. Donc, il est possible qu'aucun avantage supplémentaire n'ait été observé à l'ajout du BrdU, puisque les systèmes de réparation de la cellule étaient déjà sollicités par le même type de dommage. Ainsi, dans l'éventualité où de nouvelles études cliniques seraient ouvertes, il serait préférable d'utiliser le BrdU en conjonction avec un agent qui cible un autre mécanisme.

Toutefois, ce qui ressort principalement des résultats présentés ici, c'est l'importance de la structure pour la formation des dimères interbrins. Cette observation soulève deux autres questions :

Pourquoi les mésappariements favorisent-ils l'apparition de dimères interbrins?
Quel est l'impact de la structure secondaire et tertiaire sur la formation de dommages à l'ADN cellulaire?

Bien que beaucoup de questions demeurent, tant au sujet de l'origine que des conséquences de la formation de dimères interbrins dans l'ADN, il existe tout de même quelques pistes de réflexion intéressantes, qui pourraient mener à une meilleure compréhension de l'impact des dimères interbrins dans le dommage causé à l'ADN par les radiations ionisantes. Ce sont ces pistes de réflexion, en lien avec les deux questions précédentes, qui seront abordées en guise de conclusion.

Chapitre VIII – Conclusion et perspectives

VIII.1 – Formation de dimères interbrins : est-ce que la flexibilité de l'ADN est la clé?

L'impact des radiations sur les mésappariements des bases de l'ADN a surtout été étudié pour les mésappariements simples, en utilisant soit 2 bases non canoniques (par exemple, A/C), soit une base supplémentaire (bulge). Ces mésappariements se produisent naturellement dans la cellule (Bhattacharyya et Lilley, 1989, Wang et Griffith, 1991), par exemple par déamination d'une base, ou encore lorsque l'ADN polymérase « glisse » lors de la réplication et incorpore des bases supplémentaires, et sont reconnus par les systèmes de réparation par excision des nucléotides, de recombinaison homologue et de réplication de translésions. Ces mésappariements entraînent des interruptions dans la structure de la double hélice de l'ADN, ce qui nuit au transfert des radicaux dans l'ADN (Giese, 2002, Wagenknecht, 2006). Cependant, l'impact des radiations sur les mésappariements multiples est encore largement inconnu, bien qu'il existe plusieurs exemples dans la cellule (Huertas et Azorin, 1996, Li *et al.*, 1991). Par exemple, plusieurs structures en épingle sont impliquées dans la régulation de la transcription. Les protéines HMG lient les structures cruciformes afin d'enlever certains blocages retrouvés durant la transcription (Wadkins, 2000, Waga *et al.*, 1990). D'autres protéines importantes, comme la β-caséine, EGF et c-myc, sont régulées par la liaison de structures secondaires à l'ADN (Altiok et Groner, 1994, Chen *et al.*, 1993, Duncan *et al.*, 1994). Enfin, les centromères, qui contiennent eux aussi des mésappariements multiples, adoptent parfois des structures en fermeture-éclair, où les bases sont empilées les unes sur les autres (Chou et Chin, 2001, Ferrer *et al.*, 1995).

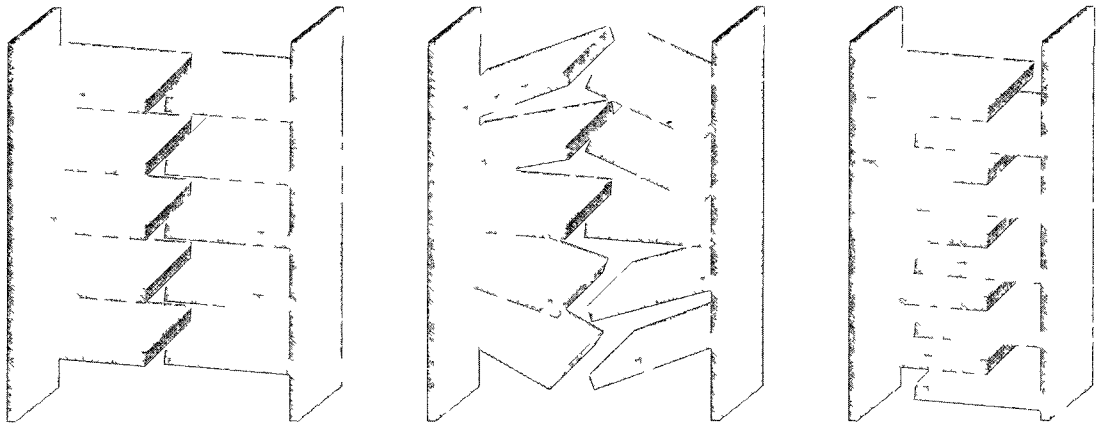

Figure VIII-1 : Structure de l'ADN en présence de mésappariements multiples. En présence de mésappariements multiples, la structure normale de la double hélice (gauche) est modifiée. Deux modèles existent : le modèle de la bulle (centre), où les bases s'écartent les unes des autres, et le modèle en fermeture-éclair (droite), où les bases s'imbriquent les unes dans les autres.

Lorsque la formation de dimère interbrin a été observée pour la première fois par notre groupe, nous avons proposé que c'est cette structure en fermeture-éclair qui favorisait l'attaque du radical uridin-yl sur le brin opposé. À la figure VIII-1, on peut voir l'agencement des bases de l'ADN. Alors que l'image de gauche représente un ADN sans mésappariement, l'image centrale représente le modèle de la bulle, où les bases tendent à s'écarter les unes des autres, tandis que l'image de droite représente le modèle en fermeture-éclair. Nous avons proposé que la proximité des bases dans la structure en fermeture-éclair favorise la réaction du radical initial avec les bases sur le brin opposé, ce qui entraînerait la formation de dimères interbrins, ainsi que la formation de cassures sur le brin opposé au brin bromé.

Lorsque cette structure a été modélisée par simulation informatique, notre groupe a trouvé que la présence de mésappariements perturbait la structure canonique de l'ADN, ce qui se traduisait par une augmentation de la mobilité des bases impliquées dans le mésappariement, ainsi que des bases adjacentes (Gantchev *et al.*, 2005). Ainsi, de nouveaux sites devenaient accessibles pour réagir avec le radical initial. De plus, dans le cas de l'ADN bromé, l'électron tendait à se localiser plus longtemps au site de substitution du BrdU dans un ADN comportant un désappariement que dans un ADN

parfaitement complémentaire, ce qui se traduisait par une augmentation de la formation de dommages (Gantchev et Hunting, 2008, Gantchev et Hunting, 2009).

Ainsi, ce modèle veut que la présence de mésappariements rapproche les bases l'une de l'autre, ce qui favorise l'attaque du radical sur le brin opposé plutôt que sur le ribose du nucléotide en 5′, qui mène normalement à la formation de cassures. La flexibilité accrue révèle de nouveaux sites, ce qui fait que de nouveaux produits de réaction peuvent être observés, incluant les dimères interbrins. Mon projet a aussi mis en évidence que c'est probablement la flexibilité, plus que la simple présence d'une région ouverte de l'ADN, qui facilite la formation des dimères interbrins. En effet, l'ouverture mécanique des brins d'ADN par l'ARN polymérase ne semble pas faciliter la production de dimères ou de cassures. Dans ce cas, bien que l'ARN polymérase ouvre l'hélice d'ADN pour permettre la transcription, il est probable que les deux brins soient tenus à l'écart l'un de l'autre, ce qui nuit à l'interaction du radical initial avec le brin opposé.

La présence de mésappariements consécutifs, ainsi que l'ouverture des brins d'ADN par l'ARN polymérase durant la transcription, ne sont que deux exemples parmi tant d'autres des modifications de structure que l'ADN subit constamment dans la cellule. Comme notre groupe l'a démontré, ces modifications peuvent avoir des conséquences importantes sur la formation de dommages par les radiations ionisantes. Bien que de grands progrès aient été accomplis ces dernières années dans le domaine de la chimie radicalaire de l'ADN, nous croyons que pour être en mesure de mieux comprendre l'impact des radiations sur l'ADN cellulaire, la nature dynamique de l'ADN devra être pris en compte.

VIII.2 – Impact de la structure tertiaire sur la formation de dommages à l'ADN cellulaire

Lorsqu'on pense à l'ADN, la première image qui nous vient en tête est généralement celle de la double hélice. On oublie souvent que l'ADN est avant tout une structure dynamique, en constant changement. Lorsqu'on a commencé à s'intéresser aux conséquences de l'irradiation sur l'ADN cellulaire, c'est surtout l'ADN double brin qui a été étudié, d'abord à cause des difficultés associées à la reproduction des processus qui impliquent l'ADN cellulaire, mais aussi parce que, même aujourd'hui, on en sait encore très peu sur ce qui se passe en temps réel dans la cellule.

Malgré tout, nous avons obtenu, au fil des années, une image un peu plus précise de ce qui se produit lorsque la cellule est exposée aux radiations. Par exemple, le groupe de Charlier a déjà établi que les formes A, B, et Z de l'ADN répondaient différemment aux radiations (Michalik *et al.*, 1995, Tartier *et al.*, 1994), et que la liaison du répresseur *lac* augmentait ou diminuait la formation de cassures simple brin, en fonction du changement de conformation occasionné par la liaison de la protéine (Franchet-Beuzit *et al.*, 1993). L'ADN, qui dans la cellule est organisé en nucléosome, est moins sensible aux radiations que l'ADN seul (Warters et Lyons, 1992), tandis que les régions qui sont activement transcrites sont plus sensibles que les régions inactives. La variation de la radiosensibilité pendant les différentes phases du cycle cellulaire représente un autre exemple que différents processus cellulaires affectent la réponse de la cellule aux radiations, bien que dans ce cas précis il reste encore beaucoup de phénomènes incompris.

Dans bien des cas, il n'existe pas d'évidence directe de l'effet de structures secondaires et tertiaires, comme les structures en épingles et les cruciformes, sur l'augmentation ou la diminution des dommages causés à l'ADN par les radiations ionisantes. Cependant, les répétitions inversées, ainsi que les micro- et les minisatellites, sont capables d'adopter ces conformations, et on a observé une augmentation de la susceptibilité à la formation de cassures et aux réarrangements chromosomaux (Freudenreich, 2007, Jonstrup *et al.*, 2008, Lukusa et Fryns, 2008). On a proposé que cette fragilité pouvait provenir des fourches de réplication bloquées

par la présence de ces structures, ou de l'attaque de la région simple brin de la boucle par les nucléases (Lobachev *et al.*, 2007). Dans le cas de l'exposition de l'ADN cellulaire aux radiations, cette dernière hypothèse est particulièrement intéressante, puisque l'ADN simple brin est aussi plus sensible que l'ADN double brin à l'attaque radicalaire. Ainsi, la fragilité intrinsèque de ces sites pourrait se refléter dans la réponse aux radiations.

Cette observation, lorsqu'elle est combinée aux résultats présentés ici, souligne la possibilité que des types de dommages, qui n'ont jamais été observés jusqu'à présent avec les expériences utilisant l'ADN double brin nu, soient produits dans l'ADN cellulaire. Cela suggère aussi que certaines structures d'ADN réagissent différemment lorsqu'exposées aux radiations, et que des structures dynamiques, comme les structures en épingle, les jonctions de Holiday et les régions activement transcrites de l'ADN, pourraient générer des lésions inattendues, comme des dimères interbrins. Ces lésions pourraient s'avérer d'autant plus toxiques qu'elles seraient produites dans des régions impliquées dans des processus de régulation cruciaux pour la cellule. En effet, dans le cas où une structure particulière est requise pour une fonction donnée, une atteinte à la structure signifierait aussi une atteinte à la fonction.

Ainsi, bien que la plupart des travaux sur l'exposition de l'ADN cellulaire aux radiations se soient concentrés sur la caractérisation et la quantification des lésions, il est possible que l'emplacement des lésions soit un facteur aussi important. Pour ce faire, l'étude de structures alternatives d'ADN, comme les structures en épingle et les mésappariements multiples, pourrait s'avérer très utile.

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"While I'm still confused and uncertain, it's on a much higher plane, d'you see, and at least I know I'm bewildered about the really fundamental and important facts of the universe." Treatle nodded. "I hadn't looked at it like that," he said, "But you're absolutely right. He's really pushed back the boundaries of ignorance." – Terry Pratchett, Equal Rites

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Références

Abdoul-Carime, H, Huels, M A, Brüning, F, Illenberger, E et Sanche, L. (2000a) Dissociative electron attachment to gas-phase 5-bromouracil, *J. Chem. Phys.*, **113,** 2517-2521.

Abdoul-Carime, H, Dugal, P C et Sanche, L. (2000b) Damage induced by 1-30 eV electrons on thymine- and bromouracil-substituted oligonucleotides, *Radiat. Res.*, **153,** 23-28.

Altiok, S et Groner, B. (1994) Beta-casein mRNA sequesters a single-stranded nucleic acid-binding protein which negatively regulates the beta-casein gene promoter, *Mol. Cell. Biol.*, **14,** 6004-6012.

Bagshaw, M A, Doggett, R L, Smith, K C, Kaplan, H S et Nelsen, T S. (1967) Intra-arterial 5-bromodeoxyuridine and x-ray therapy, *Am. J. Roentgenol. Radium Ther. Nucl. Med.*, **99,** 886-894.

Bansal, K M, Patterson, L K et Schuler, R H. (1972) The production of halide ion in the radiolysis of aqueous solutions of the 5-halouracils, *J. Phys. Chem.*, **76,** 2386-2392.

Behe, M et Felsenfeld, G. (1981) Effects of methylation on a synthetic polynucleotide: The B--Z transition in poly(dG-m5dC).poly(dG-m5dC), *Proc. Natl. Acad. Sci. U. S. A.*, **78,** 1619-1623.

Bhattacharyya, A et Lilley, D M. (1989) The contrasting structures of mismatched DNA sequences containing looped-out bases (bulges) and multiple mismatches (bubbles), *Nucleic Acids Res.*, **17,** 6821-6840.

Boudaiffa, B, Cloutier, P, Hunting, D, Huels, M A et Sanche, L. (2000) Resonant formation of DNA strand breaks by low-energy (3 to 20 eV) electrons, *Science,* **287,** 1658-1660.

Buxton, G V, Greenstock, C L, Helman, W P et Ross Alberta, B. (1988) Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution, *J. Phys. Chem. Ref. Data,* **17,** 513-886.

Cai, Y, Patel, D J, Geacintov, N E et Broyde, S. (2007) Dynamics of a benzo[a]pyrene-derived guanine DNA lesion in TGT and CGC sequence contexts: Enhanced mobility in TGT explains conformational heterogeneity, flexible bending, and greater susceptibility to nucleotide excision repair, *J. Mol. Biol.*, **374,** 292-305.

Cecchini, S, Girouard, S, Huels, M A, Sanche, L et Hunting, D J. (2005) Interstrand cross-links: A new type of gamma-ray damage in bromodeoxyuridine-substituted DNA, *Biochemistry,* **44,** 1932-1940.

Cecchini, S, Girouard, S, Huels, M A, Sanche, L et Hunting, D J. (2004) Single-strand-specific radiosensitization of DNA by bromodeoxyuridine, *Radiat. Res.*, **162,** 604-615.

Chapman, J D, Reuvers, A P, Borsa, J et Greenstock, C L. (1973) Chemical radioprotection and radiosensitization of mammalian cells growing in vitro, *Radiat. Res.*, **56,** 291-306.

Chen, L L, Clawson, M L, Bilgrami, S et Carmichael, G. (1993) A sequence-specific single-stranded DNA-binding protein that is responsive to epidermal growth factor recognizes an S1 nuclease-sensitive region in the epidermal growth factor receptor promoter, *Cell Growth Differ.*, **4,** 975-983.

Chou, S H et Chin, K H. (2001) Zipper-like watson-crick base-pairs, *J. Mol. Biol.*, **312,** 753-768.

Coleman, C N et Mitchell, J B. (1999) Clinical radiosensitization: Why it does and does not work, *J. Clin. Oncol.*, **17,** 1-3.

Debije, M G, Strickler, M D et Bernhard, W A. (2000) On the efficiency of hole and electron transfer from the hydration layer to DNA: An EPR study of crystalline DNA X-irradiated at 4 K, *Radiat. Res.*, **154,** 163-170.

Dewey, W C, Sedita, B A et Humphrey, R M. (1966) Radiosensitization of X chromosome of chinese hamster cells related to incorporation of 5-bromodeoxyuridine, *Science,* **152,** 519-521.

Dewey, W C et Humphrey, R M. (1965) Increase in radiosensitivity to ionizing radiation related to replacement of thymidine in mammalian cells with 5-bromodeoxyuridine, *Radiat. Res.*, **26,** 538-553.

Dugal, P -, Abdoul-Carime, H et Sanche, L. (2000) Mechanisms for low-energy (0.5-30 eV) electron-induced pyrimidine ring fragmentation within thymine- and halogen-substituted single strands of DNA, *J. Phys. Chem. B,* **104,** 5610-5617.

Duncan, R, Bazar, L, Michelotti, G, Tomonaga, T, Krutzsch, H, Avigan, M et Levens, D. (1994) A sequence-specific, single-strand binding protein activates the far upstream element of c-myc and defines a new DNA-binding motif, *Genes Dev.*, **8,** 465-480.

Eisbruch, A, Robertson, J M, Johnston, C M, Tworek, J, Reynolds, K R, Roberts, J A et Lawrence, T S. (1999) Bromodeoxyuridine alternating with radiation for advanced uterine cervix cancer: A phase I and drug incorporation study, *J. Clin. Oncol.*, **17,** 31-40.

Eisch, A J et Mandyam, C D. (2007) Adult neurogenesis: Can analysis of cell cycle proteins move us "Beyond BrdU"? *Curr. Pharm. Biotechnol.*, **8,** 147-165.

Feig, M et Pettitt, B M. (1998) A molecular simulation picture of DNA hydration around A- and B-DNA, *Biopolymers,* **48,** 199-209.

Ferrer, N, Azorin, F, Villasante, A, Gutierrez, C et Abad, J P. (1995) Centromeric dodeca-satellite DNA sequences form fold-back structures, *J. Mol. Biol.*, **245,** 8-21.

Franchet-Beuzit, J, Spotheim-Maurizot, M, Sabattier, R, Blazy-Baudras, B et Charlier, M. (1993) Radiolytic footprinting. beta rays, gamma photons, and fast neutrons probe DNA-protein interactions, *Biochemistry,* **32,** 2104-2110.

Freudenreich, C H. (2007) Chromosome fragility: Molecular mechanisms and cellular consequences, *Front. Biosci.*, **12,** 4911-4924.

Gantchev, T G et Hunting, D J. (2009) Probing the interactions of the solvated electron with DNA by molecular dynamics simulations: II. bromodeoxyuridine-thymidine mismatched DNA, *J. Mol. Model.*, **15,** 9-23.

Gantchev, T G et Hunting, D J. (2008) Probing the interactions of the solvated electron with DNA by molecular dynamics simulations: Bromodeoxyuridine substituted DNA, *J. Mol. Model.*, **14,** 451-464.

Gantchev, T G, Cecchini, S et Hunting, D J. (2005) Dynamic conformational states of DNA containing T.T or BrdU.T mispaired bases: Wobble H-bond pairing versus cross-strand inter-atomic contacts, *J. Mol. Model.*, **11,** 141-159.

Giese, B. (2002) Electron transfer in DNA, *Curr. Opin. Chem. Biol.*, **6,** 612-618.

Herbert, A et Rich, A. (1999) Left-handed Z-DNA: Structure and function, *Genetica,* **106,** 37-47.

Herbert, A, Lowenhaupt, K, Spitzner, J et Rich, A. (1995) Chicken double-stranded RNA adenosine deaminase has apparent specificity for Z-DNA, *Proc. Natl. Acad. Sci. U. S. A.*, **92,** 7550-7554.

Horton, N C et Finzel, B C. (1996) The structure of an RNA/DNA hybrid: A substrate of the ribonuclease activity of HIV-1 reverse transcriptase, *J. Mol. Biol.*, **264,** 521-533.

Huertas, D et Azorin, F. (1996) Structural polymorphism of homopurine DNA sequences. d(GGA)n and d(GGGA)n repeats form intramolecular hairpins stabilized by different base-pairing interactions, *Biochemistry,* **35,** 13125-13135.

Iliakis, G, Kurtzman, S, Pantelias, G et Okayasu, R. (1989) Mechanism of radiosensitization by halogenated pyrimidines: Effect of BrdU on radiation induction of DNA and chromosome damage and its correlation with cell killing, *Radiat. Res.*, **119,** 286-304.

Jiang, H, Zacharias, W et Amirhaeri, S. (1991) Potassium permanganate as an in situ probe for B-Z and Z-Z junctions, *Nucleic Acids Res.*, **19,** 6943-6948.

Jonstrup, A T, Thomsen, T, Wang, Y, Knudsen, B R, Koch, J et Andersen, A H. (2008) Hairpin structures formed by alpha satellite DNA of human centromeres are cleaved by human topoisomerase IIalpha, *Nucleic Acids Res.*, **36,** 6165-6174.

Jortner, J, Ottolenghi, M, Rabani, J et Stein, G. (1962) Conversion of solvated electrons into hydrogen atoms in the photo- and radiation chemistry of aqueous solutions, *J. Chem. Phys.*, **37,** 2488-2495.

Kinsella, T J, Russo, A, Mitchell, J B, Rowland, J, Jenkins, J, Schwade, J, Myers, C E, Collins, J M, Speyer, J et Kornblith, P. (1984) A phase I study of intermittent intravenous bromodeoxyuridine (BUdR) with conventional fractionated irradiation, *Int. J. Radiat. Oncol. Biol. Phys.*, **10,** 69-76.

La Vere, T, Becker, D et Sevilla, M D. (1996) Yields of OH in gamma-irradiated DNA as a function of DNA hydration: Hole transfer in competition with OH formation, *Radiat. Res.*, **145,** 673-680.

Lafer, E M, Valle, R P, Moller, A, Nordheim, A, Schur, P H, Rich, A et Stollar, B D. (1983) Z-DNA-specific antibodies in human systemic lupus erythematosus, *J. Clin. Invest.*, **71,** 314-321.

Lawrence, T S, Davis, M A et Normolle, D P. (1995) Effect of bromodeoxyuridine on radiation-induced DNA damage and repair based on DNA fragment size using pulsed-field gel electrophoresis, *Radiat. Res.*, **144,** 282-287.

Lehnert, S. (2007) Biomolecular action of ionizing radiation, Éditions Taylor & Francis, New York.

Leif, R C, Stein, J H et Zucker, R M. (2004) A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase, *Cytometry A.*, **58,** 45-52.

Levin, V A, Prados, M R, Wara, W M, Davis, R L, Gutin, P H, Phillips, T L, Lamborn, K et Wilson, C B. (1995) Radiation therapy and bromodeoxyuridine chemotherapy followed by procarbazine, lomustine, and vincristine for the treatment of anaplastic gliomas, *Int. J. Radiat. Oncol. Biol. Phys.*, **32,** 75-83.

Li, H, Xiao, J, Li, J, Lu, L, Feng, S et Droge, P. (2009) Human genomic Z-DNA segments probed by the Z alpha domain of ADAR1, *Nucleic Acids Res.*, **37,** 2737-2746.

Li, X, Sevilla, M D et Sanche, L. (2003) DFT investigation of dehalogenation of adenine-halouracil base pairs upon low-energy electron attachment, *J. Am. Chem. Soc.*, **125,** 8916-8920.

Li, Y, Zon, G et Wilson, W D. (1991) Thermodynamics of DNA duplexes with adjacent G.A mismatches, *Biochemistry,* **30,** 7566-7572.

Limoli, C L et Ward, J F. (1993) A new method for introducing double-strand breaks into cellular DNA, *Radiat. Res.*, **134,** 160-169.

Ling, L L et Ward, J F. (1990) Radiosensitization of chinese hamster V79 cells by bromodeoxyuridine substitution of thymidine: Enhancement of radiation-induced toxicity and DNA strand break production by monofilar and bifilar substitution, *Radiat. Res.*, **121,** 76-83.

Liu, L F et Wang, J C. (1987) Supercoiling of the DNA template during transcription, *Proc. Natl. Acad. Sci. U. S. A.*, **84,** 7024-7027.

Lobachev, K S, Rattray, A et Narayanan, V. (2007) Hairpin- and cruciform-mediated chromosome breakage: Causes and consequences in eukaryotic cells, *Front. Biosci.*, **12,** 4208-4220.

Lukusa, T et Fryns, J P. (2008) Human chromosome fragility, *Biochim. Biophys. Acta,* **1779,** 3-16.

Malinge, J M, Perez, C et Leng, M. (1994) Base sequence-independent distorsions induced by interstrand cross-links in cis-diamminedichloroplatinum (II)-modified DNA, *Nucleic Acids Res.*, **22,** 3834-3839.

McGinn, C J et Kinsella, T J. (1992) The experimental and clinical rationale for the use of S-phase-specific radiosensitizers to overcome tumor cell repopulation, *Semin. Oncol.*, **19,** 21-28.

Mellor, J. (2006) Dynamic nucleosomes and gene transcription, *Trends Genet.*, **22,** 320-329.

Mertens, R et Sonntag, C v. (1994) Determination of the kinetics of vinyl radical reactions by the characteristic visible absorption spectra of vinylperoxyl radicals, *Angewandte Chemie International Edition in English,* **33,** 1262-1264.

Michalik, V, Spotheim Maurizot, M et Charlier, M. (1995) Calculation of hydroxyl radical attack on different forms of DNA, *J. Biomol. Struct. Dyn.*, **13,** 565-575.

Millard, J T, Weidner, M F, Kirchner, J J, Ribeiro, S et Hopkins, P B. (1991) Sequence preferences of DNA interstrand crosslinking agents: Quantitation of interstrand crosslink locations in DNA duplex fragments containing multiple crosslinkable sites, *Nucleic Acids Res.*, **19,** 1885-1891.

Milligan, J R, Ng, J Y, Wu, C C, Aguilera, J A, Fahey, R C et Ward, J F. (1995) DNA repair by thiols in air shows two radicals make a double-strand break, *Radiat. Res.*, **143,** 273-280.

Motohashi, N et Saito, Y. (1993) Competitive measurement of rate constants for hydroxyl radical reactions using radiolytic hydroxylation of benzoate, *Chem. Pharm. Bull.*, **41,** 1842-1845.

Mroczka, N et Bernhard, W A. (1993) Hydration effects on free radical yields in DNA X-irradiated at 4 K, *Radiat. Res.*, **135,** 155-159.

Noll, D M, Mason, T M et Miller, P S. (2006) Formation and repair of interstrand cross-links in DNA, *Chem. Rev.*, **106,** 277-301.

Patterson, L K et Bansal, K M. (1972) Pulse radiolysis studies of 5-halouracils in aqueous solutions, *J. Phys. Chem.*, **76,** 2392-2399.

Phillips, T L, Levin, V A, Ahn, D K, Gutin, P H, Davis, R L, Wilson, C B, Prados, M D, Wara, W M et Flam, M S. (1991) Evaluation of bromodeoxyuridine in glioblastoma multiforme: A northern california cancer center phase II study, *Int. J. Radiat. Oncol. Biol. Phys.*, **21,** 709-714.

Pollard, T D et Earshaw, W C. (2002). *The S phase and DNA replication,* Cell Biology, 1st, Ed Elservier Science, Philadelphia. 697-711.

Prados, M D, Seiferheld, W, Sandler, H M, Buckner, J C, Phillips, T, Schultz, C, Urtasun, R, Davis, R, Gutin, P, Cascino, T L, Greenberg, H S et Curran, W J,Jr. (2004) Phase III randomized study of radiotherapy plus procarbazine, lomustine, and vincristine with or without BUdR for treatment of anaplastic astrocytoma: Final report of RTOG 9404, *Int. J. Radiat. Oncol. Biol. Phys.*, **58,** 1147-1152.

Quinones-Hinojosa, A, Sanai, N, Smith, J S et McDermott, M W. (2005) Techniques to assess the proliferative potential of brain tumors, *J. Neurooncol.*, **74,** 19-30.

Rajski, S R et Williams, R M. (1998) DNA cross-linking agents as antitumor drugs, *Chem. Rev.*, **98,** 2723-2796.

Regulus, P, Duroux, B, Bayle, P A, Favier, A, Cadet, J et Ravanat, J L. (2007) Oxidation of the sugar moiety of DNA by ionizing radiation or bleomycin could induce the formation of a cluster DNA lesion, *Proc. Natl. Acad. Sci. U. S. A.*, **104,** 14032-14037.

Roots, R et Okada, S. (1972) Protection of DNA molecules of cultured mammalian cells from radiation-induced single-strand scissions by various alcohols and SH compounds, *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, **21,** 329-342.

Saenger, W. (1984) Principles of nucleic acid structure, Éditions Springer-Verlag, New York.

Saffhill, R et Ockey, C H. (1985) Strand breaks arising from the repair of the 5-bromodeoxyuridine-substituted template and methyl methanesulphonate-induced lesions can explain the formation of sister chromatid exchanges, *Chromosoma,* **92,** 218-224.

Sanche, L. (2002) Nanoscopic aspects of radiobiological damage: Fragmentation induced by secondary low-energy electrons, *Mass Spectrom. Rev.*, **21,** 349-369.

Sano, K, Hoshino, T et Nagai, M. (1968) Radiosensitization of brain tumor cells with a thymidine analogue (bromouridine), *J. Neurosurg.*, **28,** 530-538.

Sano, K, Sato, F, Hoshino, T et Nagai, M. (1965) Experimental and clinical studies of radiosensitizers in brain tumors, with special reference to BUdR-antimetabolite continuous regional infusion-radiation therapy (BAR therapy), *Neurol. Med. Chir. (Tokyo),* **7,** 51-72.

Schyman, P, Zhang, R B, Eriksson, L A et Laaksonen, A. (2007) Hydrogen abstraction from deoxyribose by a neighbouring uracil-5-yl radical, *Phys. Chem. Chem. Phys.*, **9,** 5975-5979.

Sczepanski, J T, Jacobs, A C, Majumdar, A et Greenberg, M M. (2009a) Scope and mechanism of interstrand cross-link formation by the C4'-oxidized abasic site, *J. Am. Chem. Soc.*, **131,** 11132-11139.

Sczepanski, J T, Jacobs, A C, Van Houten, B et Greenberg, M M. (2009b) Double-strand break formation during nucleotide excision repair of a DNA interstrand cross-link, *Biochemistry,* **48,** 7565-7567.

Smeaton, M B, Hlavin, E M, Noronha, A M, Murphy, S P, Wilds, C J et Miller, P S. (2009) Effect of cross-link structure on DNA interstrand cross-link repair synthesis, *Chem. Res. Toxicol.*, **22,** 1285-1297.

Smeaton, M B, Hlavin, E M, McGregor Mason, T, Noronha, A M, Wilds, C J et Miller, P S. (2008) Distortion-dependent unhooking of interstrand cross-links in mammalian cell extracts, *Biochemistry,* **47,** 9920-9930.

Szybalski, W. (1974) X-ray sensitization by halopyrimidines, *Cancer Chemother. Rep.*, **58,** 539-557.

Tartier, L, Michalik, V, Spotheim-Maurizot, M, Rahmouni, A R, Sabattier, R et Charlier, M. (1994) Radiolytic signature of Z-DNA, *Nucleic Acids Res.*, **22,** 5565-5570.

Thamann, T J, Lord, R C, Wang, A H et Rich, A. (1981) The high salt form of poly(dG-dC).poly(dG-dC) is left-handed Z-DNA: Raman spectra of crystals and solutions, *Nucleic Acids Res.*, **9,** 5443-5457.

Urtasun, R C, Kinsella, T J, Farnan, N, DelRowe, J D, Lester, S G et Fulton, D S. (1996) Survival improvement in anaplastic astrocytoma, combining external radiation with halogenated pyrimidines: Final report of RTOG 86-12, phase I-II study, *Int. J. Radiat. Oncol. Biol. Phys.*, **36,** 1163-1167.

Voet, D et Voet, J G. (1995). *Structure des acides nucléiques et leur manipulation,* Biochimie, 2nd, Ed DeBoeck Université, Paris, Bruxelles. 848-860.

Wadkins, R M. (2000) Targeting DNA secondary structures, *Curr. Med. Chem.*, **7,** 1-15.

Waga, S, Shirakawa, H, Mizuno, S et Yoshida, M. (1990) The selective binding of HMG1 to the cruciform DNA structure and the subsequent resumption of transcription, *Nucleic Acids Symp. Ser.*, **(22),** 81-82.

Wagenknecht, H A. (2006) Electron transfer processes in DNA: Mechanisms, biological relevance and applications in DNA analytics, *Nat. Prod. Rep.*, **23,** 973-1006.

Wang, C R, Hu, A et Lu, Q B. (2006) Direct observation of the transition state of ultrafast electron transfer reaction of a radiosensitizing drug bromodeoxyuridine, *J. Chem. Phys.*, **124,** 241102.

Wang, W, Becker, D et Sevilla, M D. (1993) The influence of hydration on the absolute yields of primary ionic free radicals in gamma-irradiated DNA at 77 K. I. total radical yields, *Radiat. Res.*, **135,** 146-154.

Wang, Y, Pantelias, G E et Iliakis, G. (1994) Mechanism of radiosensitization by halogenated pyrimidines: The contribution of excess DNA and chromosome

damage in BrdU radiosensitization may be minimal in plateau-phase cells, *Int. J. Radiat. Biol.*, **66,** 133-142.

Wang, Y H et Griffith, J. (1991) Effects of bulge composition and flanking sequence on the kinking of DNA by bulged bases, *Biochemistry,* **30,** 1358-1363.

Warters, R L et Lyons, B W. (1992) Variation in radiation-induced formation of DNA double-strand breaks as a function of chromatin structure, *Radiat. Res.*, **130,** 309-318.

Webb, C F, Jones, G D, Ward, J F, Moyer, D J, Aguilera, J A et Ling, L L. (1993) Mechanisms of radiosensitization in bromodeoxyuridine-substituted cells, *Int. J. Radiat. Biol.*, **64,** 695-705.