Radioprotection by DNA minor groove binding ligands

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Dubna, Russian Federation

International School on Nuclear Methods and Applied Research
in Environmental, Material and Life Sciences
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Introduction – what is the lecture about

Discipline – Radiation Biology

Radiobiology research area – Radioprotectors

- Specific class of radioprotectors – DNA minor groove binders
- Specific subject – mechanism of action of DNA minor groove binding radioprotectors

Introduction – brief overview of radioprotectors area

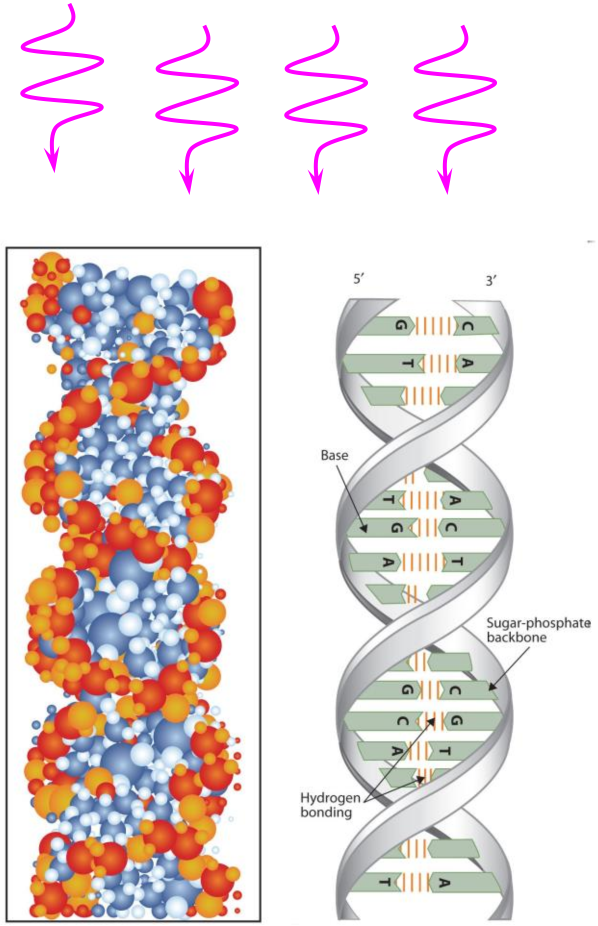
- Investigation and development of radioprotectors – a large research area in radiation biology
- It has both fundamental and applied aspects
- Fundamental aspect – assists in understanding mechanisms of radiation effects
- Applied aspect
    - Protection of normal tissues in cancer radiotherapy
    - Protection of personnel of nuclear facilities
    - Radiation safety of space missions etc.
- There are a number of compounds in practical applications, however they are not radioprotectors, but radiomitigators – they protect not from radiation damage itself but from its consequences (inflammation etc.)
- The only radioprotecting compound approved for clinical application for protection of normal tissues in cancer radiotherapy – amifostine, however it is rarely used due to its systemic toxicity

Introduction – central dogma of radiation biology

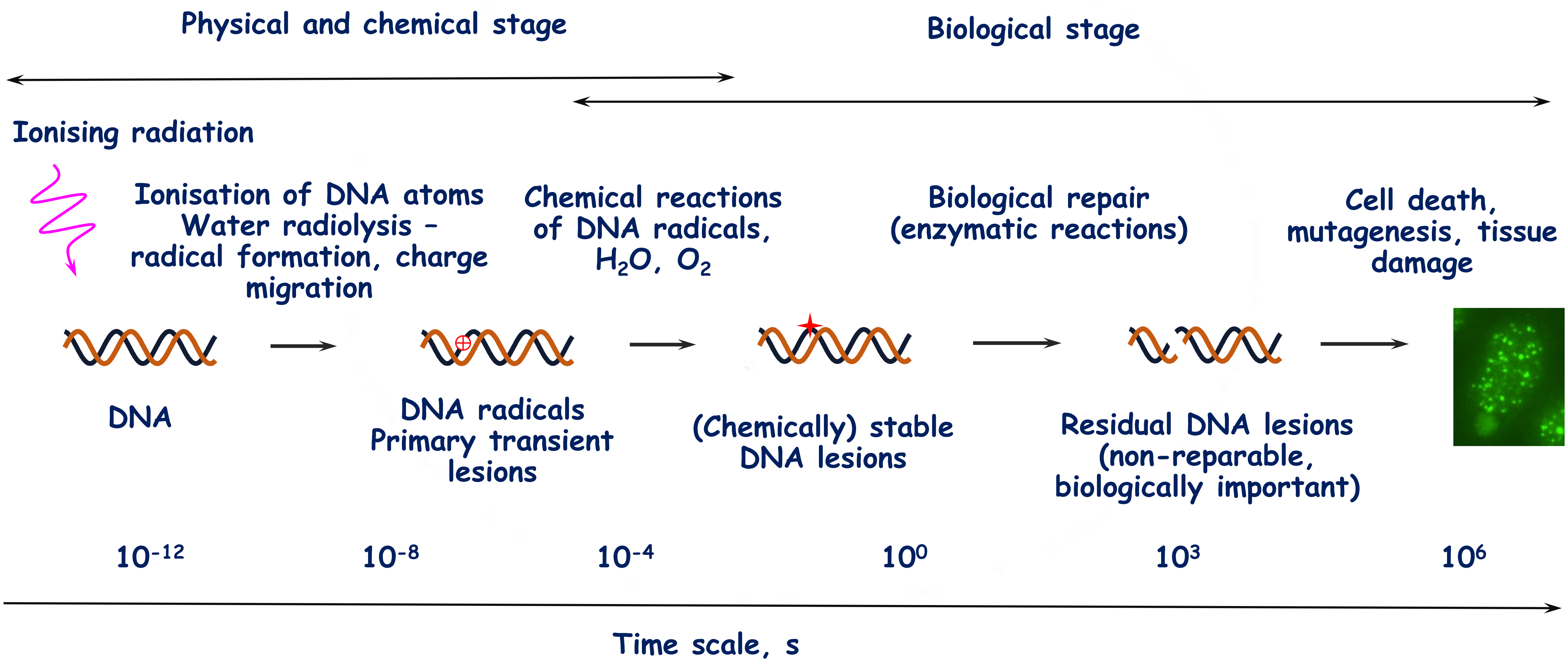
- Central dogma of radiation biology – DNA molecule represents the main target for ionising radiation in cell
- Radiation-induced DNA lesions are the primary reason for lethal, mutagenic and carcinogenic effects of ionising radiation at cellular level

Why DNA?

- DNA is a carrier of genetic information and is present in cell in one (or two) copy(ies)
- Mutagenesis, carcinogenesis – a consequence of the damage to genetic information itself; cell death – a consequence of the damage to the process of reproduction of genetic information
- The role of the damage to other cellular components (proteins, membranes) is less critical due to their multicopying



Introduction – dynamics of DNA damage and repair



Physical and chemical stage

Biological stage

Ionising radiation

Ionisation of DNA atoms
Water radiolysis –
radical formation, charge
migration

Chemical reactions
of DNA radicals,
H_2O, O_2

Biological repair
(enzymatic reactions)

Cell death,
mutagenesis, tissue
damage

DNA

DNA radicals
Primary transient
lesions

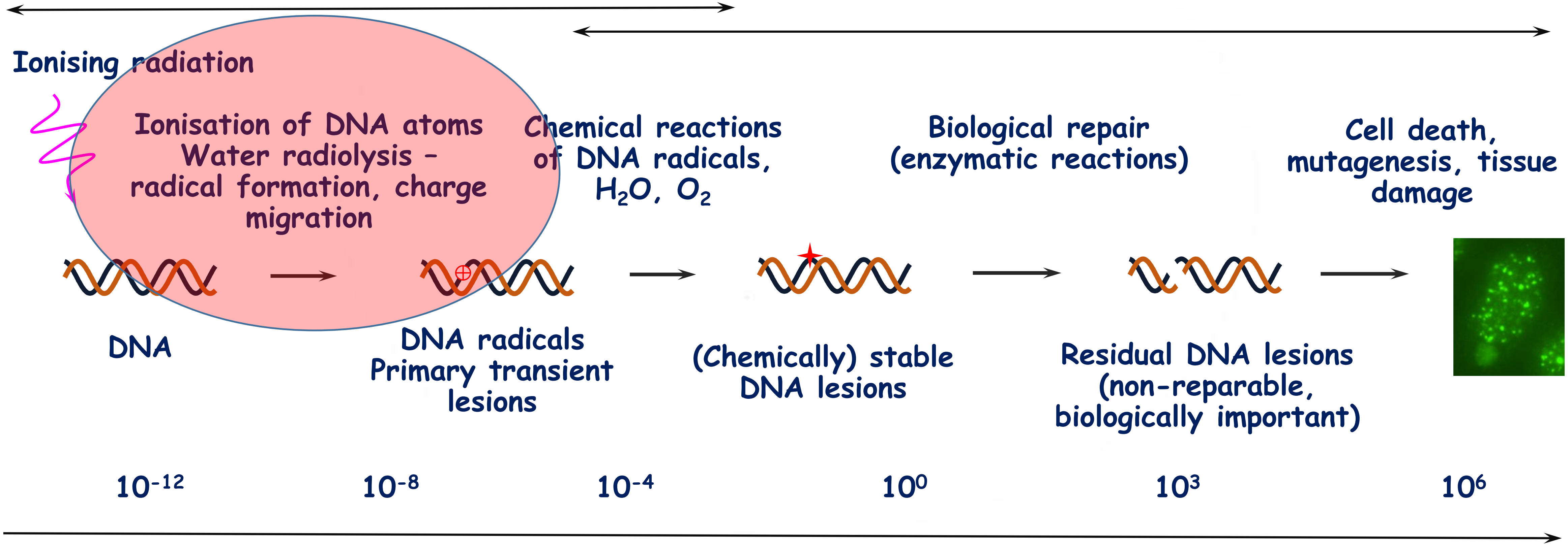
(Chemically) stable
DNA lesions

Residual DNA lesions
(non-reparable,
biologically important)

10^{-12} 10^{-8} 10^{-4} 10^{0} 10^{3} 10^{6}

Time scale, s

Introduction – dynamics of DNA damage and repair



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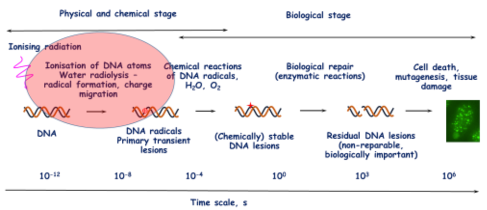
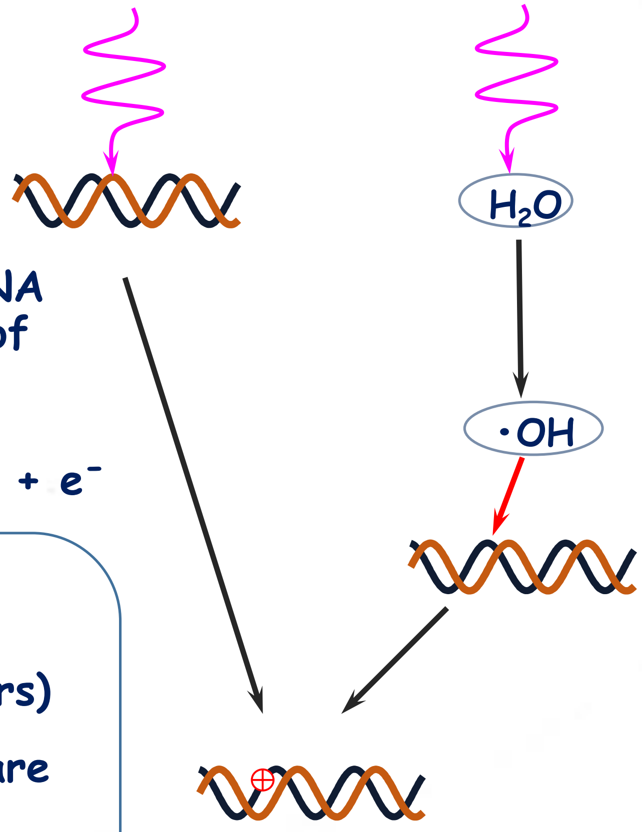
Introduction – two mechanisms of radiation action





Direct mechanism

Direct interaction of ionising particle with DNA molecule

Ionisation of atoms in DNA molecule and formation of radicals

$$DNA \xrightarrow{\text{radiation}} DNA^{\cdot +} + e^-$$

Indirect mechanism

Ionisation of water molecules

Formation of $\cdot OH$ radicals (2.7/100 eV)

$$H_2O \xrightarrow{\text{radiation}} H_2O^{\cdot +} + e^-$$

$$H_2O \xrightarrow{\text{radiation}} H_2O^*$$

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

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

DNA attack by $\cdot OH$ radicals and formation of DNA radicals

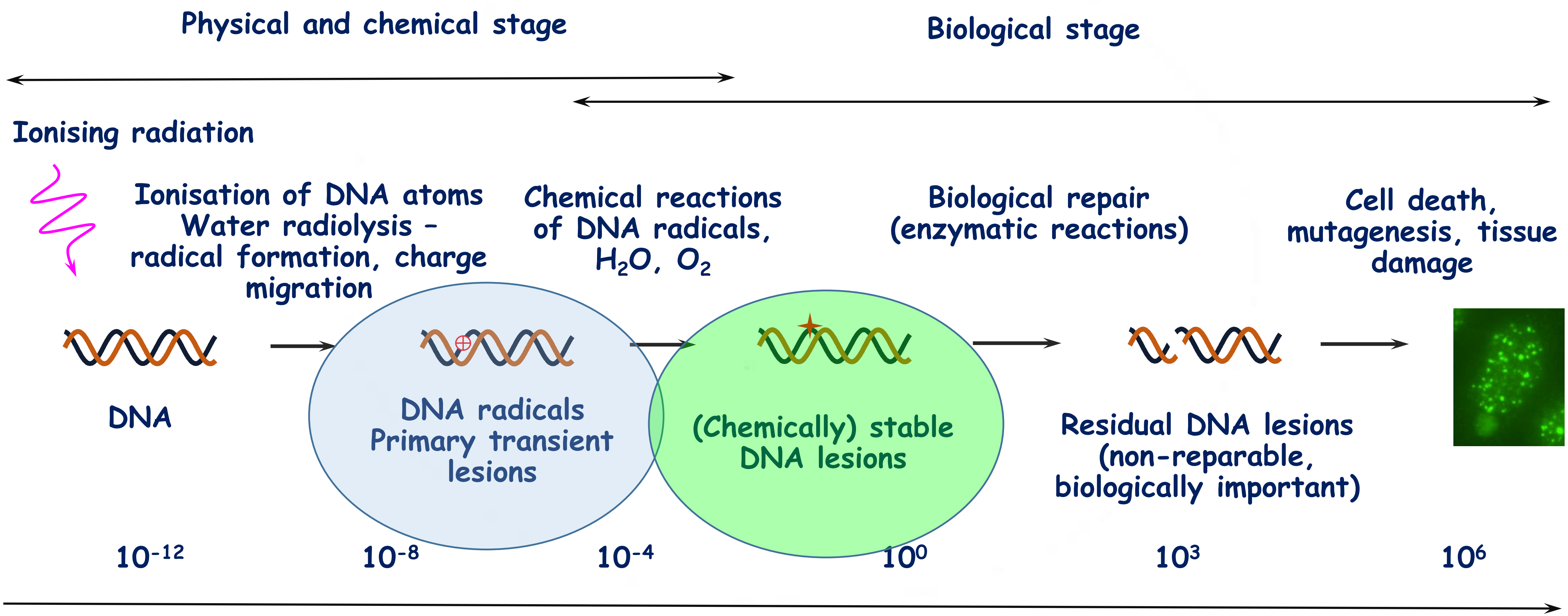
DNA radicals
Transient lesions

Contribution of indirect mechanism depends on environment (concentration of $\cdot OH$ radical scavengers)

- In aqueous solution – up to 98% of DNA lesions are produced by $\cdot OH$ radicals
- In biological medium (cells) ~ 55 – 70%

Roots & Okada, IJRB, 1972

Introduction – transient and stable DNA lesions



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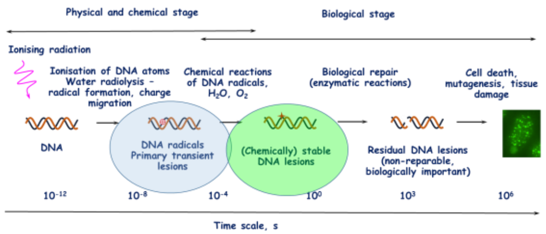
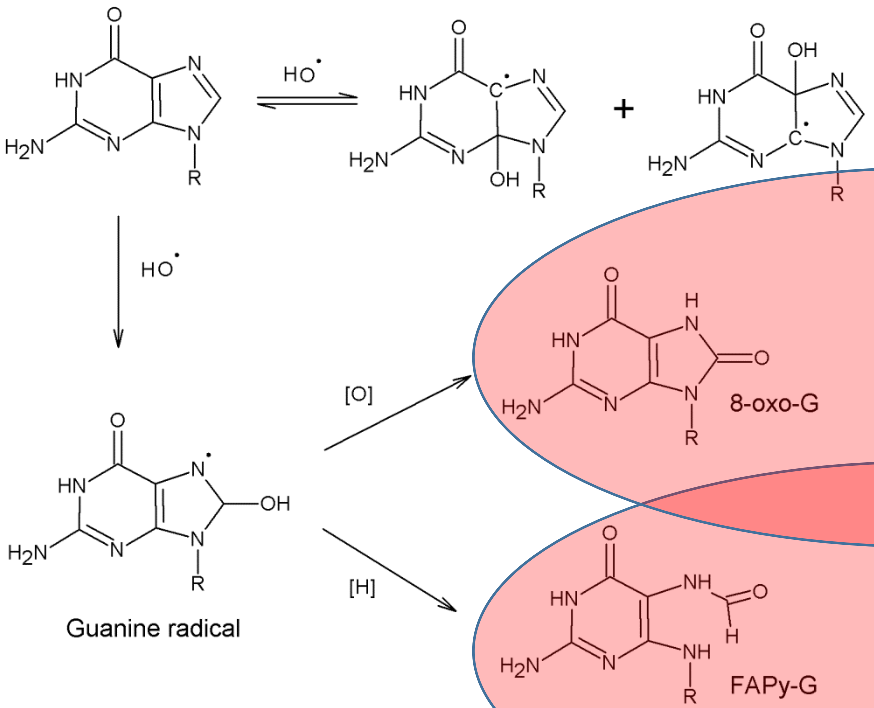
Time scale, s

Introduction – transient and stable DNA lesions (base damage)



Reactions of guanine with ·OH radical



8-oxo-G

7,8-dihydro-8-oxoguanine or

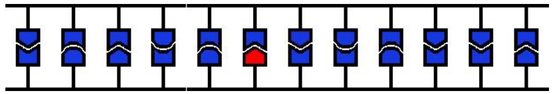
7,8-dihydro-8-hydroxyguanine

FAPy-G

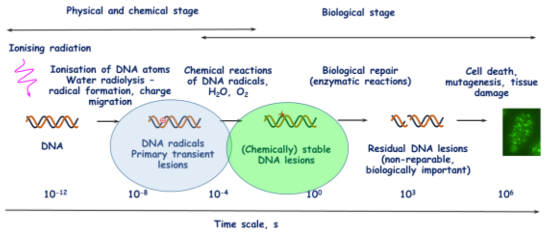
2,6-diamino-5-formamido-4-hydroxypyrimidine

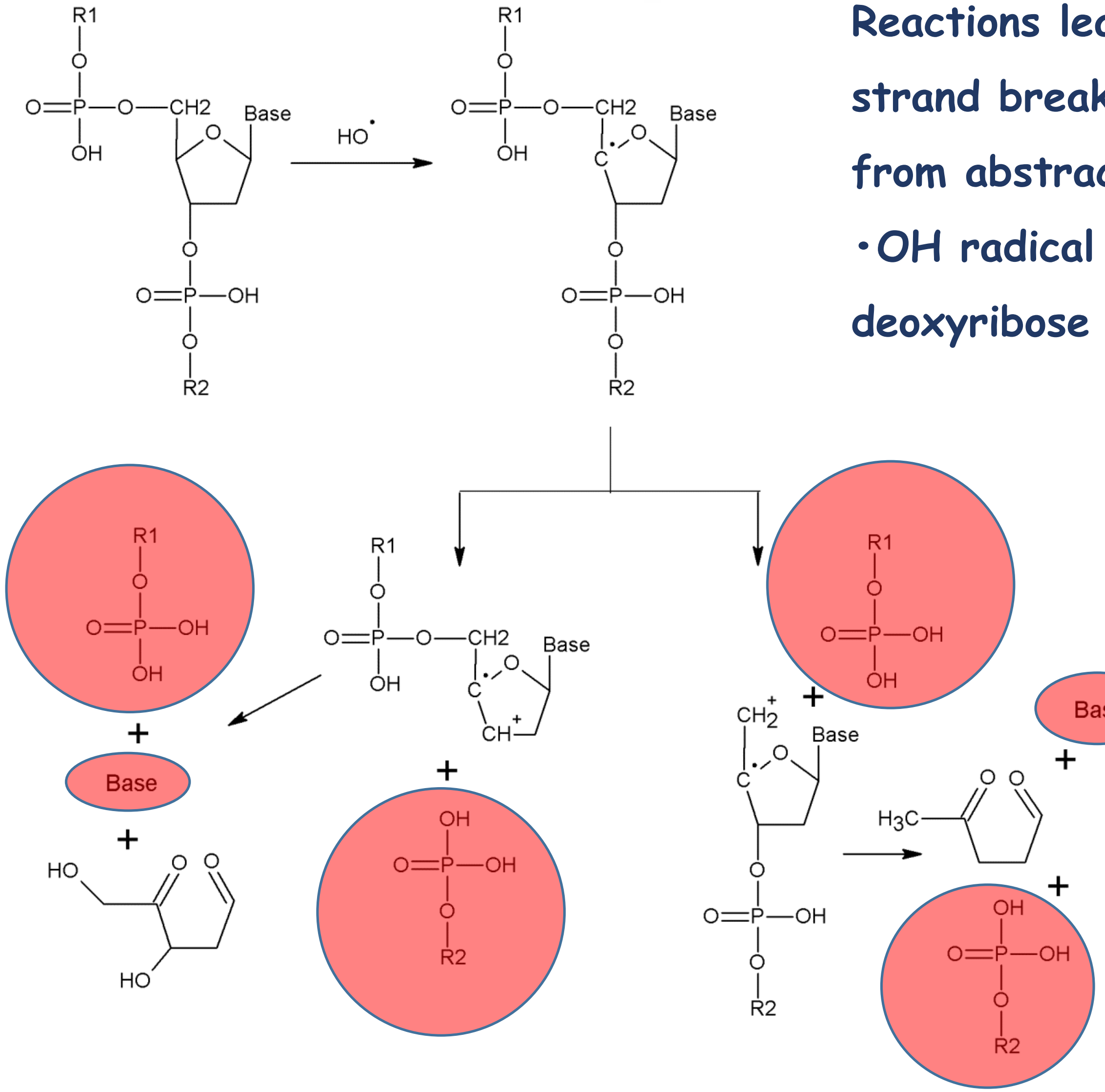
Guanine radical – transient lesion

8-oxo-G и FAPy-G – stable base damage BD (modified bases)

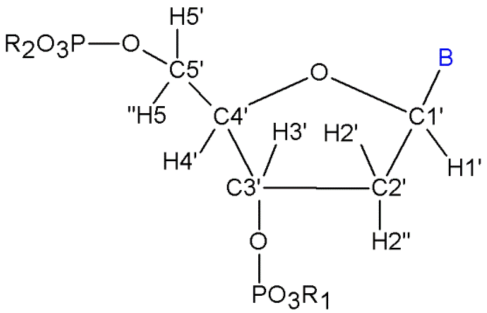


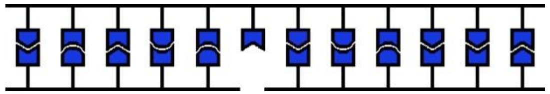
Introduction – transient and stable DNA lesions (sugar damage)





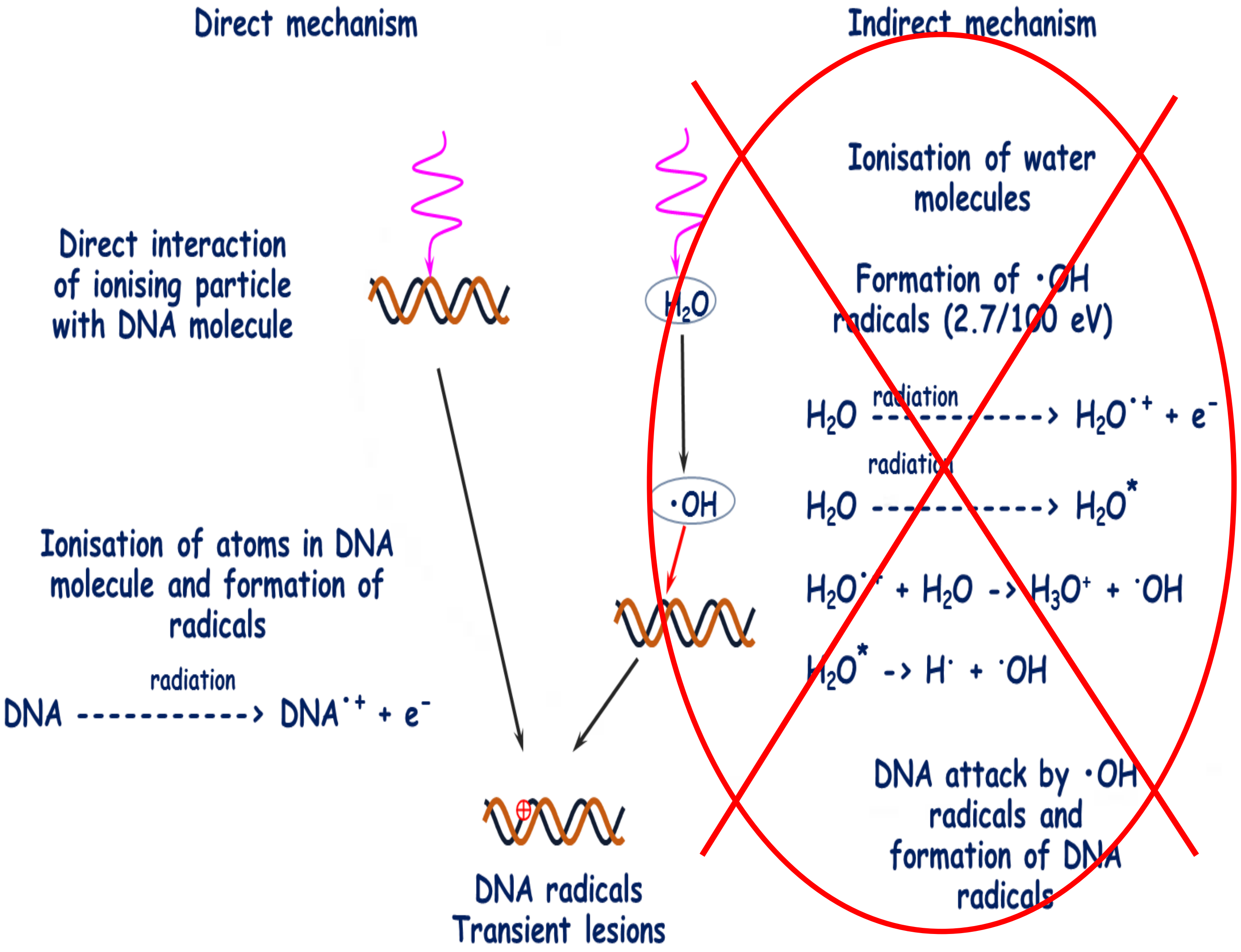
Reactions leading to single strand break (SSB) resulting from abstraction of hydrogen by •OH radical from C4-atom of deoxyribose





Single strand break (SSB) is formed and one base is lost

Introduction – radical scavengers as radioprotectors



Direct mechanism

Direct interaction of ionising particle with DNA molecule

Ionisation of atoms in DNA molecule and formation of radicals

$DNA \xrightarrow{radiation} DNA^{\cdot +} + e^-$

DNA radicals
Transient lesions

Indirect mechanism

~~Ionisation of water molecules~~

~~Formation of ·OH radicals (2.7/100 eV)~~

~~$H_2O \xrightarrow{radiation} H_2O^{\cdot +} + e^-$~~

~~$H_2O \xrightarrow{radiation} H_2O^*$~~

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

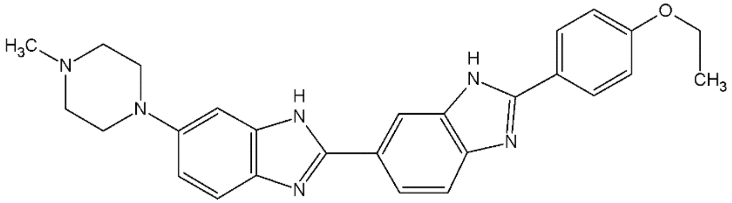
~~$H_2O^* \rightarrow H^{\cdot} + {}^{\cdot}OH$~~

~~DNA attack by ·OH radicals and formation of DNA radicals~~

- Scavengers of ·OH radicals – radioprotectors
- Reduce or eliminate indirect mechanism of action
- Examples:
  - DMSO – dimethyl sulphoxide
  - Alcohols
  - Aminothiol WR1065

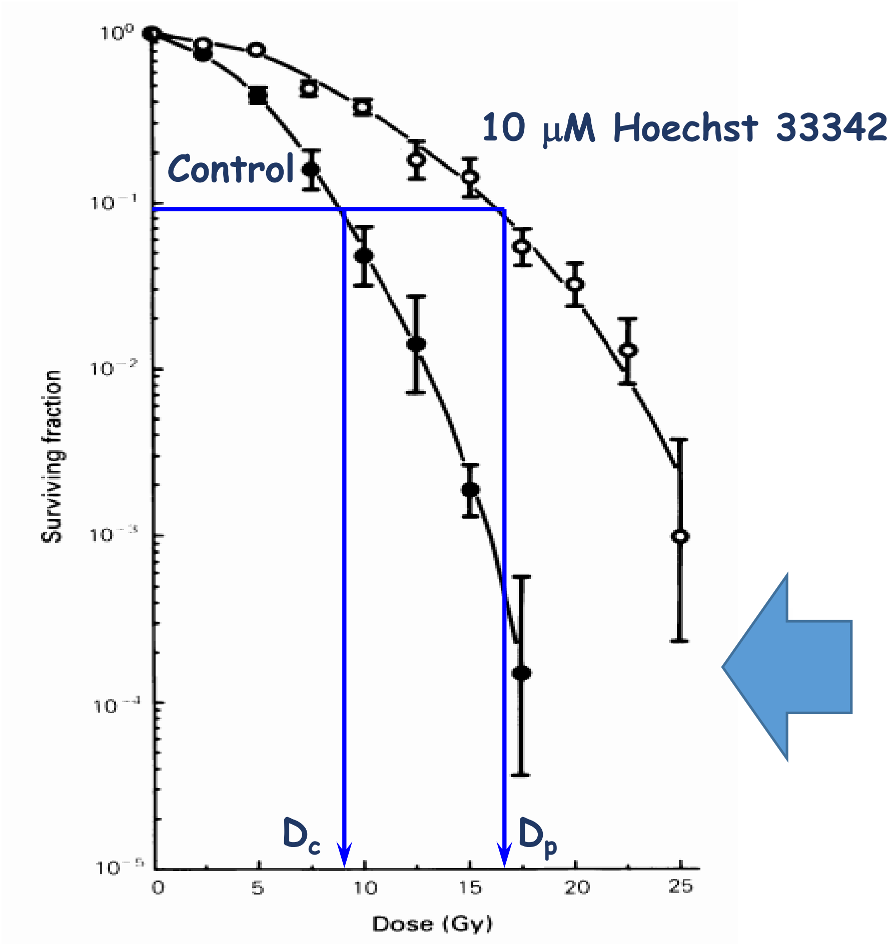
Aminothiol WR1065 – active form of approved radioprotector amifostine

DNA binding radioprotectors – historical aspect

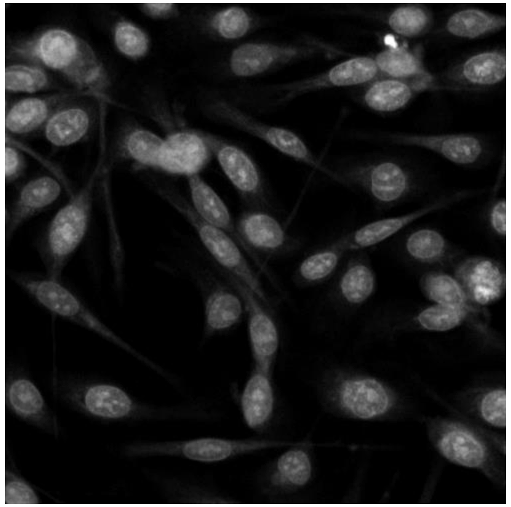


Radioprotective effect of Hoechst 33342

- Hoechst 33342 – fluorescent DNA dye widely used in molecular biological studies
- Chemical structure of Hoechst 33342 – bis-benzimidazole



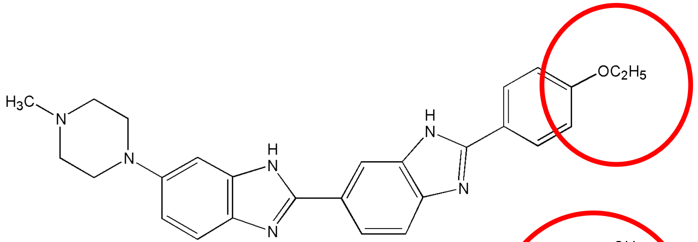
Fluorescence microscopy image of cultured cells stained with Hoechst 33342



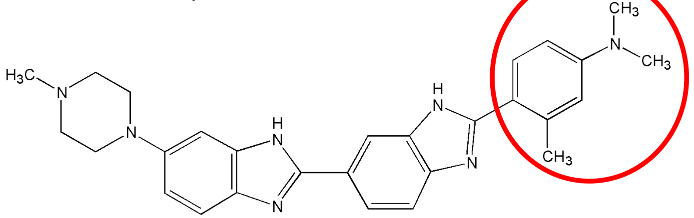
Radioprotection of KHT cell line (murine fibrosarcoma) by 30 min pre-incubation with 10 µM Hoechst 33342, DMF = D_p/D_c = 1.7

Young, S.D., Hill, R.P. Br J Cancer. 1989. V.60. P.715.

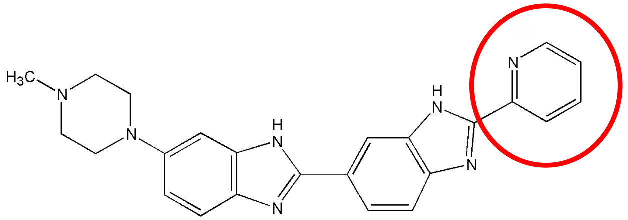
Radioprotectors – chemical structure of bis-benzimidazoles



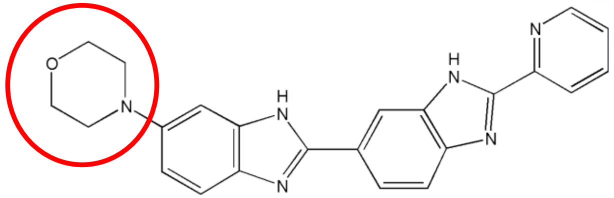
Hoechst 33342 (H42, ethoxy-phenyl)



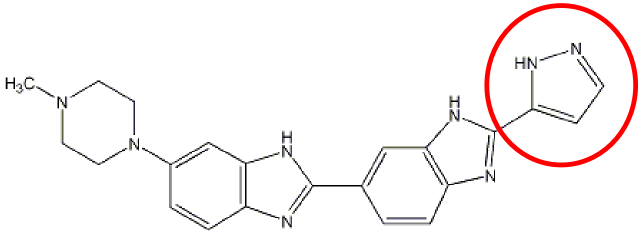
Methylproamine (MP, ortho-methyl para-di-methyl-amino)



2PH (2-Pyridine)



M2PB (Morpholino – LHS)

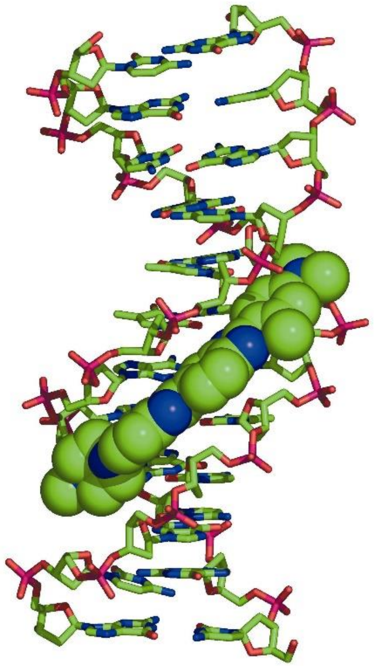


HPZ (2-Pyrazole)

- New generation of DNA binding radioprotectors
- Synthesized in the context of Radioprotector project
- Developed and investigated for normal tissue protection in cancer radiotherapy (>400 compounds)

Molecular Radiation Biology Laboratory
Research Division
Peter MacCallum Cancer Centre
Melbourne

Radioprotectors – crystal structure of DNA-ligand complex

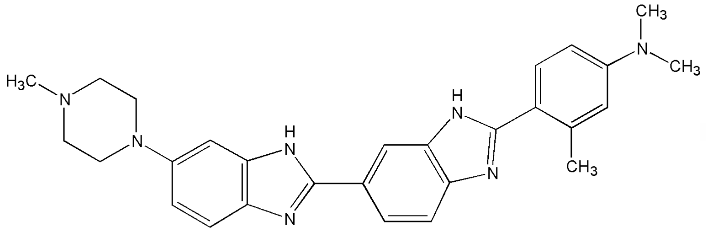


Crystal structure of MP (Methylproamine) with

GCGAATTCGCG
CGCTTAAGCGC

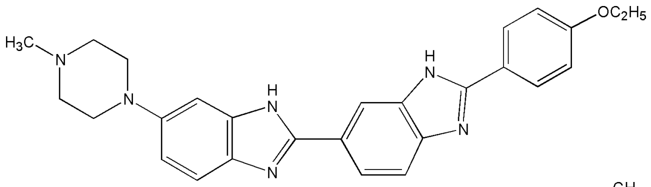
Data source:

Martin et al, 2004, *Cancer Research*, 64, 1067

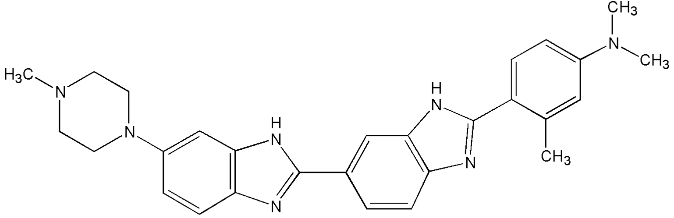


- Bis-benzimidazoles bind in DNA minor groove
- Binding site – 3-4 consecutive AT base pairs
- Hydrogen bonds are involved in high affinity site-specific binding

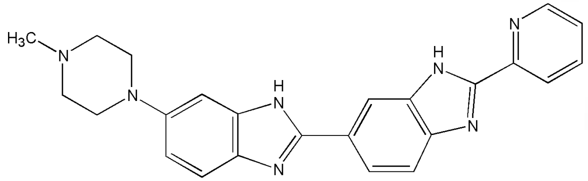
Introduction – DNA binding constants of bis-benzimidazoles



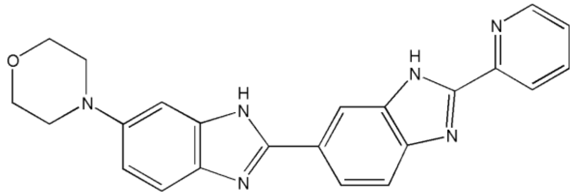
Hoechst 33342 Kd = 170 +/- 45 nM



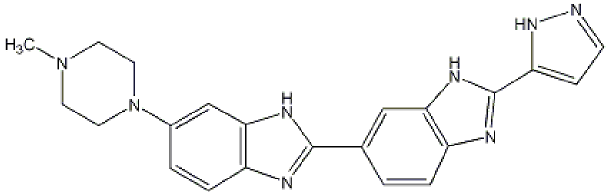
MP Kd = 100 +/- 15 nM



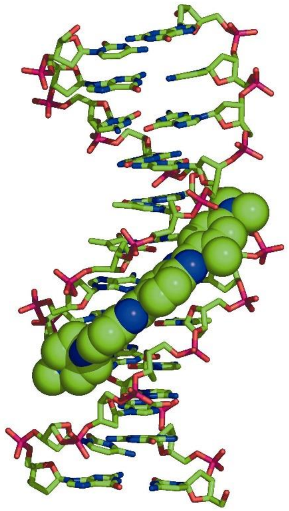
2PH Kd = 330 +/- 20 nM



M2PB Kd = 1690 +/- 150 nM



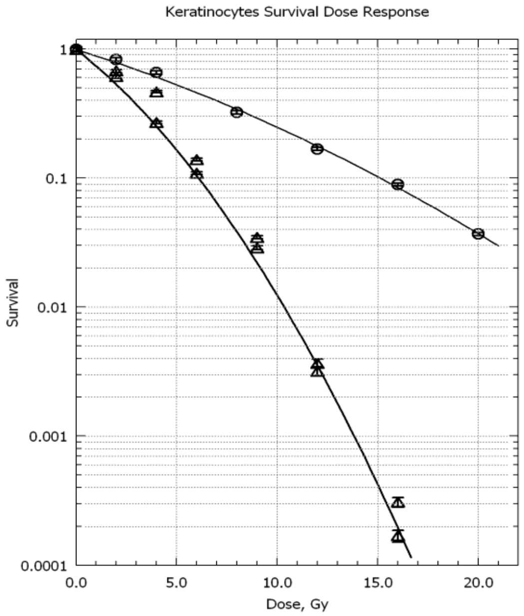
HPZ Kd = 180 +/- 10 nM



Kd = binding dissociation constant
Measured in the presence of 10% DMSO

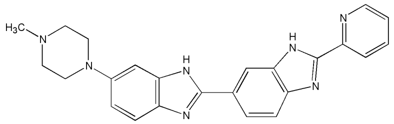
Bis-benzimidazoles feature strong high-affinity site-specific DNA binding

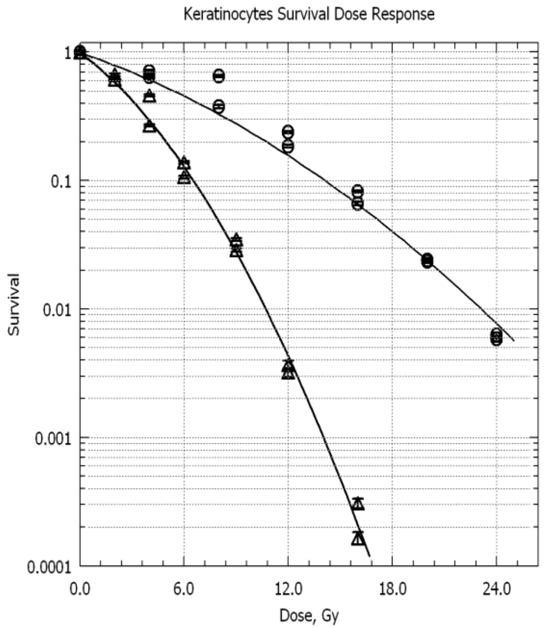
Radioprotection of cultured cells – clonogenic survival



2PH (2-Pyridyl Hoechst), 20 μM

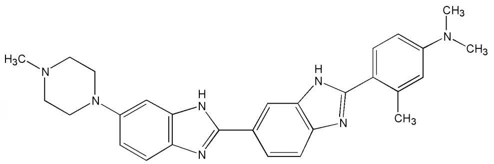
DMF = 2.48 +/- 0.07





MP (Methylproamine), 10 μM

DMF = 2.16 +/- 0.04



Human keratinocytes
Cell line FEP 18-11

γ-rays 137Cs, 0.6 Gy/min

Drug treatment:
- 30 min before irradiation
- total duration 60 min

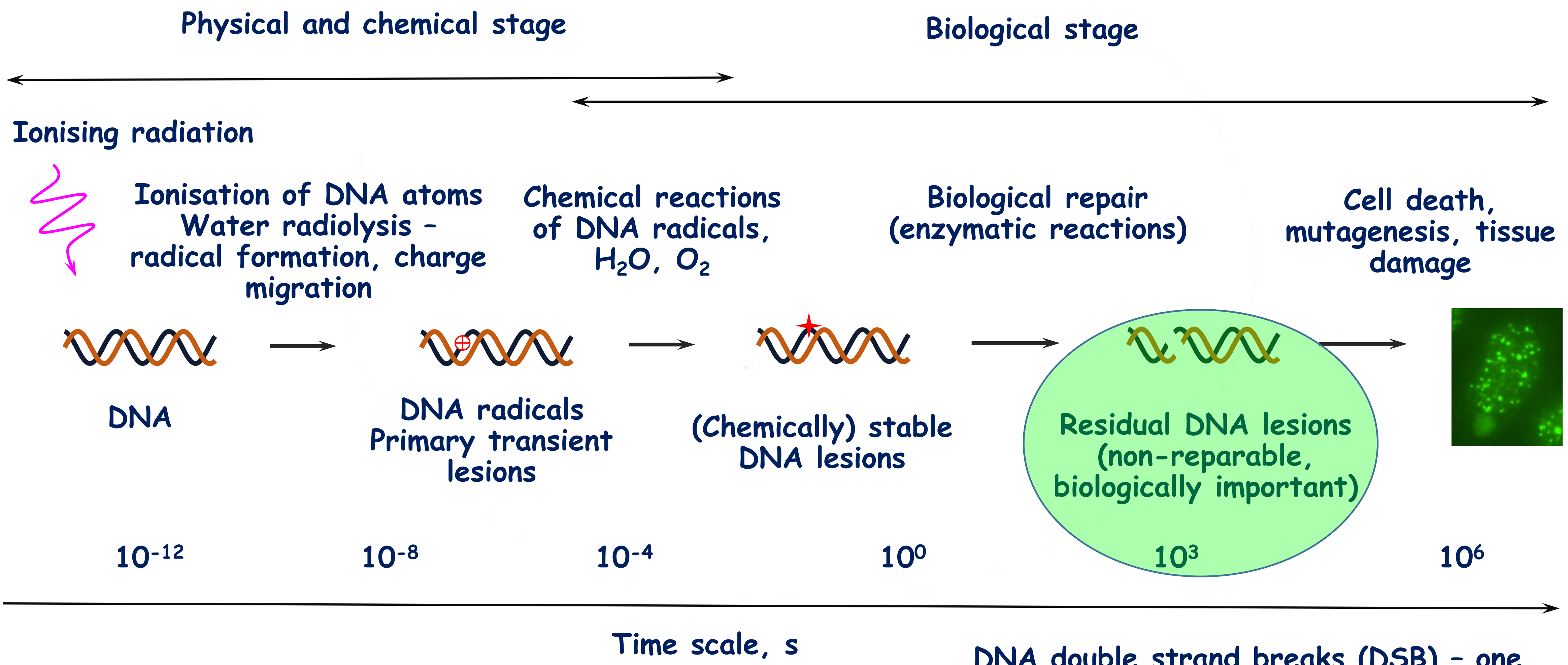
Clonogenic survival assay

Data source:
- Molecular Radiation Biology Lab
- Peter Mac

2PH and MP are more efficient than Hoechst 33342 in protecting cells from clonogenic death

Question: Does this protection correlates with protection from DNA damage?

Introduction – residual biologically relevant lesions



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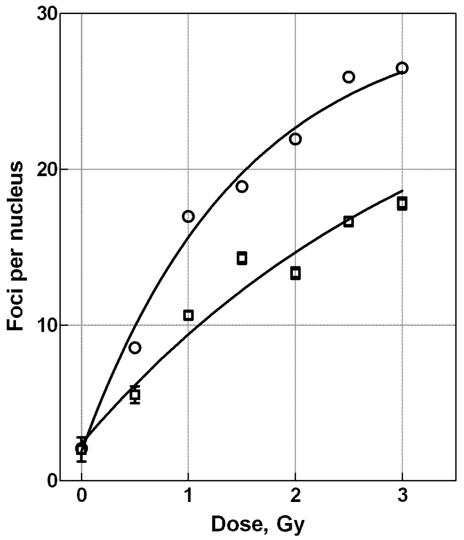
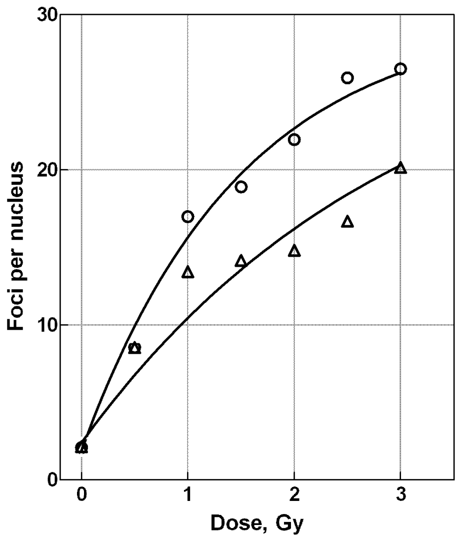
Time scale, s

DNA double strand breaks (DSB) – one
of the most critical DNA lesions

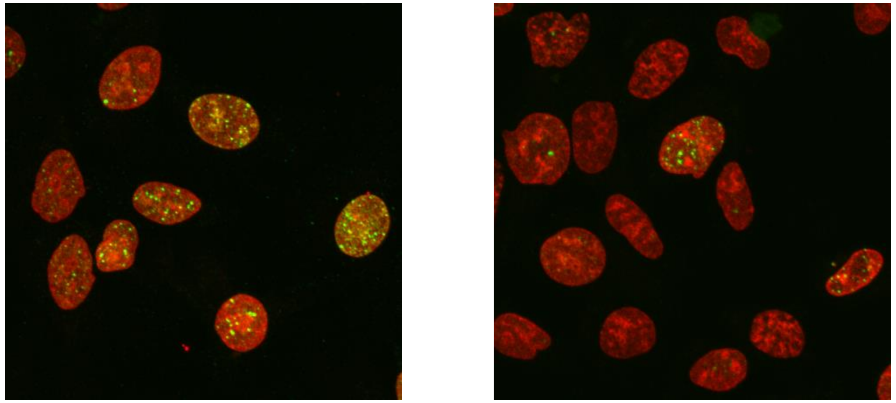
Introduction - radioprotection of cultured cells – γH2AX assay


γH2AX reduction by 2PH in keratinocyte

DMF_{2PH} = 2.30+/-0.26


γH2AX reduction by MP in keratinocytes

DMF_{MP} = 1.96+/-0.30



2PH (2-Pyridyl Hoechst), 20 μM MP (Methylproamine), 10 μM

Protection from clonogenic death correlates with protection from DNA damage (γH2AX/DSB)

Protection from DNA damage forms a basis for protection from clonogenic death

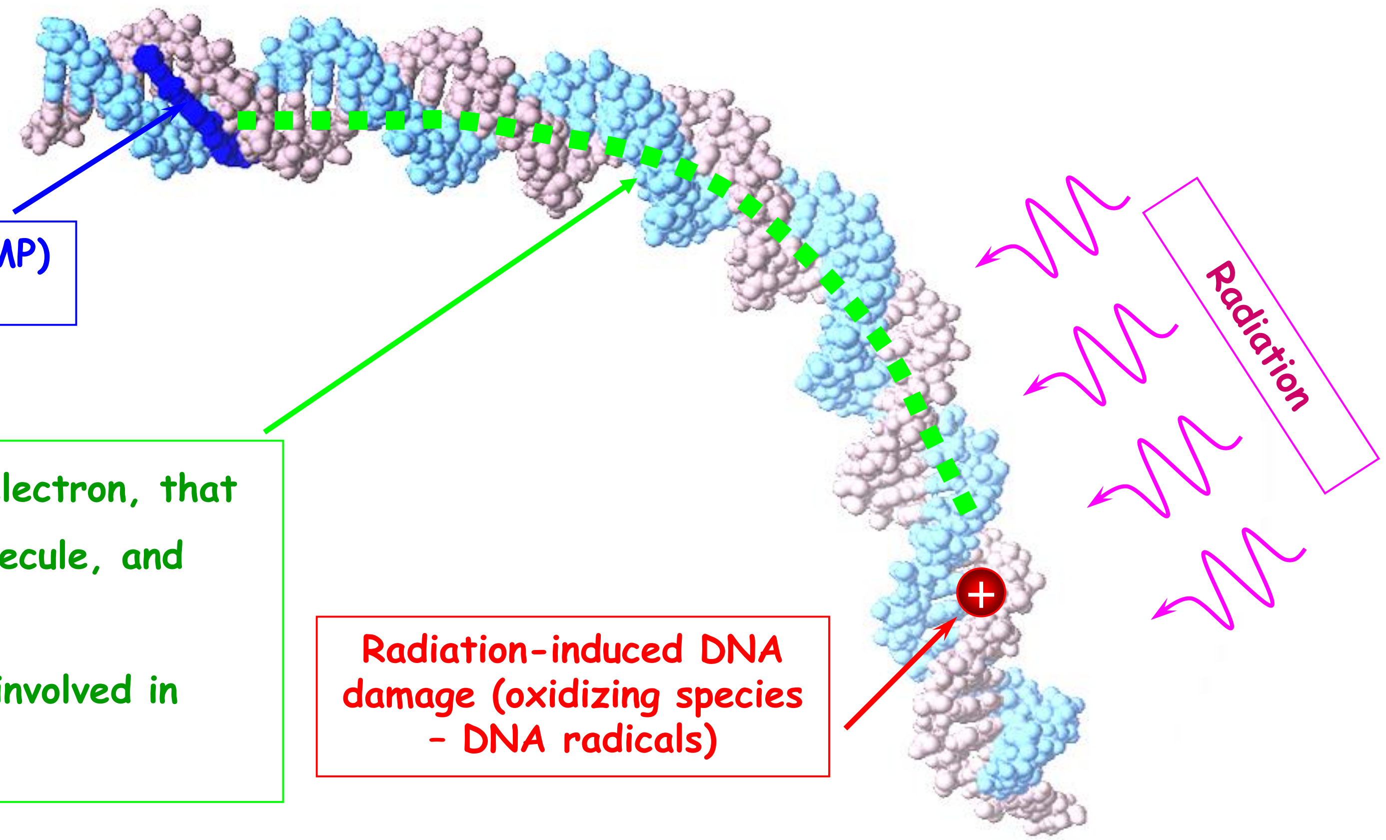
Question: What are mechanisms of protection from DNA damage?

Human keratinocytes
Cell line FEP 18-11

γ-rays 137Cs, 0.6 Gy/min
Drug treatment:
30 min before irradiation

γH2AX assay (DNA DSB)
Data source:
Molecular Radiation Biology Lab
Peter Mac

Hypothesised mechanism of radioprotection



Radioprotector (MP)
bound to DNA

- Radioprotector donates an electron, that moves through the DNA molecule, and repairs the oxidizing lesion
- Electron (hole) migration is involved in radioprotection

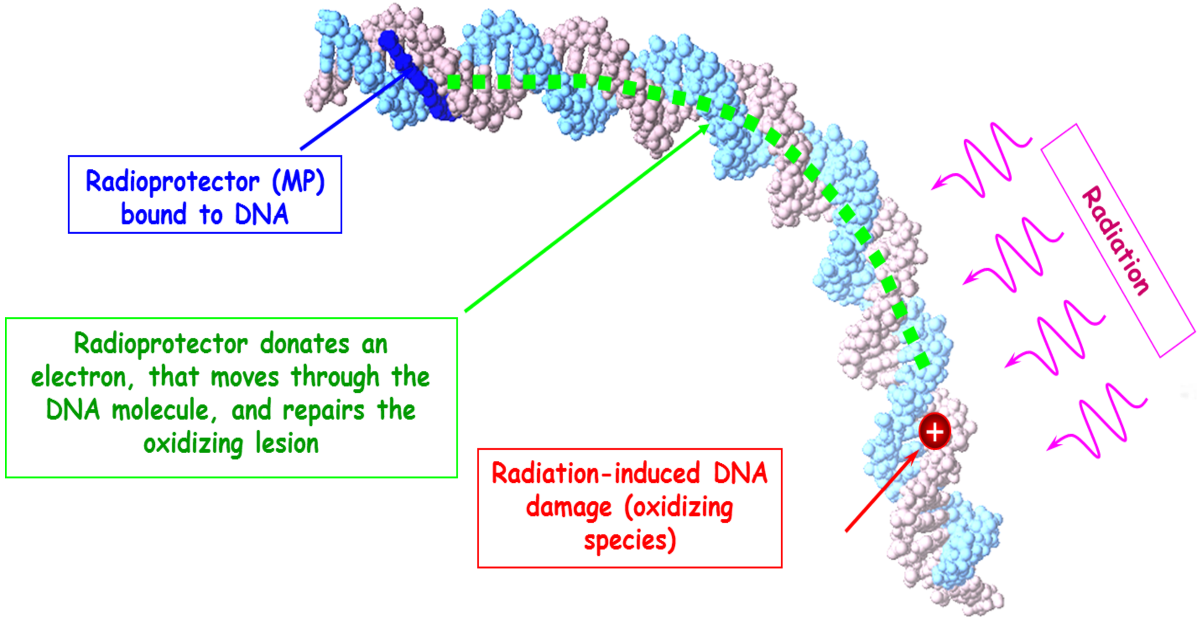
Radiation-induced DNA damage (oxidizing species – DNA radicals)

Hypothesis proposed and supported by pulse radiolysis studies of DNA-ligand complexes

Estimated electron (charge) migration range 15 – 20 base pairs

Martin & Anderson, 1998, IJROBP, 42, 827

Hypothesised mechanism of radioprotection-Questions



Questions:

1. What are those transient radiation induced DNA species that are reduced by electron donation from the ligand?

2. What is that final damage (residual lesions) that is relevant for cytotoxicity and presents a subject for radioprotection?

3. What is the range of electron/charge migration?

4. What alternative hypothesis can be suggested?

Questions – Candidate DNA lesions

Questions:

1. What are those transient radiation induced DNA species that are reduced by electron donation from the ligand?

Candidates:

Transient oxidative base damage, e.g. guanyl radical that results mainly in the stable base damage 8-oxo-G (7,8-dihydro-8-oxoguanine) and FAPY-G (2,6-diamino-4-hydroxy-5-formamidipyrimidine). Reaction of DNA bound Hoechst with purine radicals was suggested as a mechanism of radioprotection (Adhikary et al, IJRBOP, 2000, 76, 1157)

Transient sugar damage, e.g. carbon-centred (C4') deoxyribose radical that results in strand break. Radioprotection from strand breaks was observed in plasmid model (Martin & Denison, 1992, IJROBP, 23, 579)

Questions – Radiobiologically relevant DNA damage

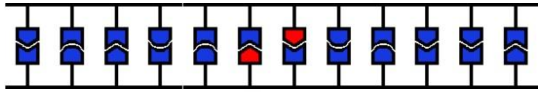
Questions:

2. What is that final damage that is relevant for cytotoxicity and presents a subject for radioprotection?

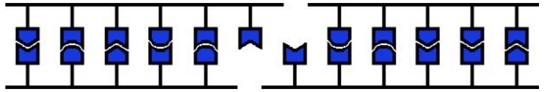
Isolated lesions (single strand breaks – SSB and base damage – BD) are unlikely candidates

Candidates:

OCDL (Oxidative Clustered DNA Lesion) can be prevented by repairing a radical that otherwise leads to BD that constitutes a part of OCDL



DSB (Double Strand Break) can be prevented by repairing a deoxyribose radical that otherwise leads to SSB as a part of DSB



Questions – Alternative hypotheses

Questions:

4. What alternative hypotheses can be suggested?

Hypothesis 0 (H0)

DNA bound ligand protects DNA by donating an electron to transient radiation induced species (local protection)

Hypothesis 1 (H1)

Free ligand in solution protects by scavenging radiation induced hydroxyl radicals (global protection)

Hypothesis 2 (H2)

DNA bound ligand protects by scavenging radiation induced hydroxyl radicals in vicinity of DNA (local protection)

Alternative hypotheses, research tools

Questions:

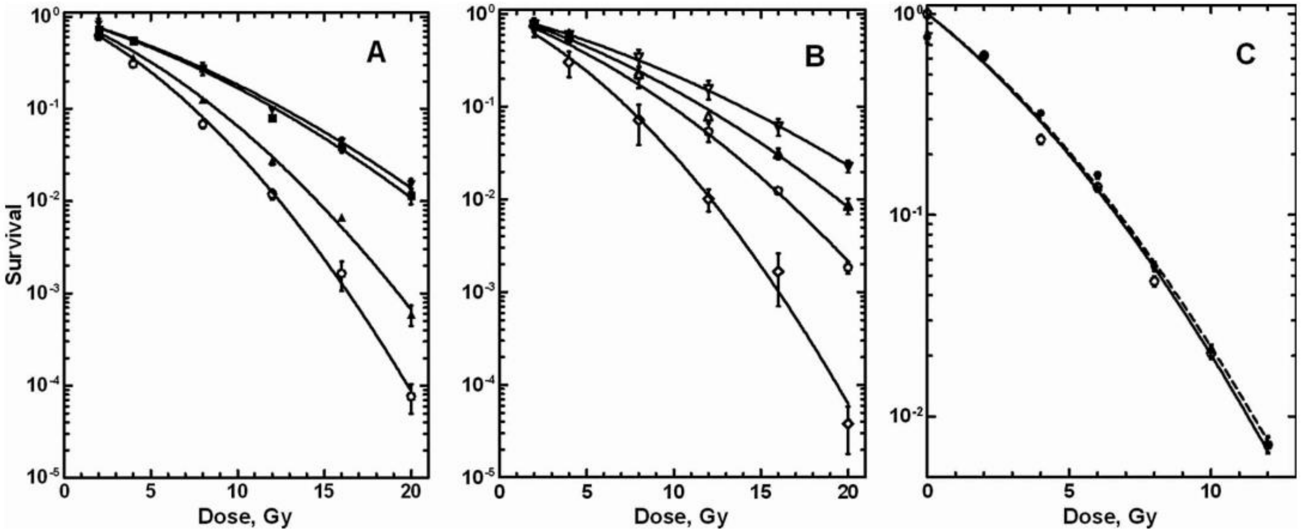
4. What alternative hypothesis can be suggested? Can be summarised by two questions:

- is radioprotection mediated by DNA bound or free in solution ligand?
- is radioprotection mediated by scavenging hydroxyl radicals or by quenching DNA radicals?

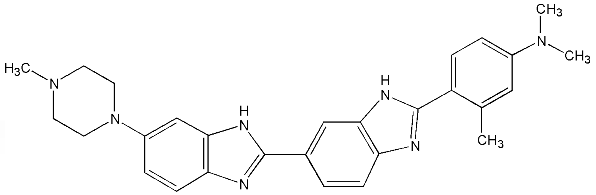
Research tools:

- To study radioprotection by bis-benzimidazoles in combination with radical scavenger
- To study extent of radioprotection at different concentration of radioprotector
- To study yield of DNA damage in the presence of radioprotector

Radioprotection at different concentration of Methylproamine



C, μM	DMF
0,5	1.01
1	1.32
2	1.37
3	1.67
4	1.64
6,7	1.82
10	1.97



Methylproamin (MP)

Radioprotection of human keratinocytes in culture

A, B: survival of keratinocytes irradiated in the presence of 0,5 – 10 μM MP

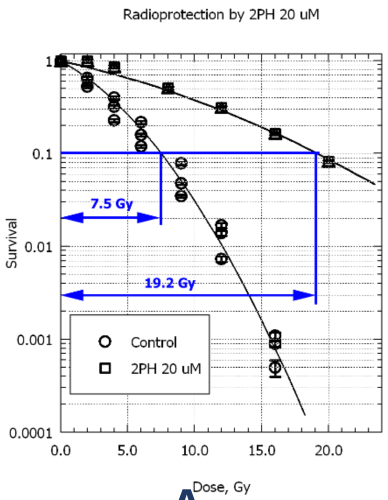
C: MP added after irradiation

Data source: Lobachevsky et al, IJRB, 2011, V.87. P.274

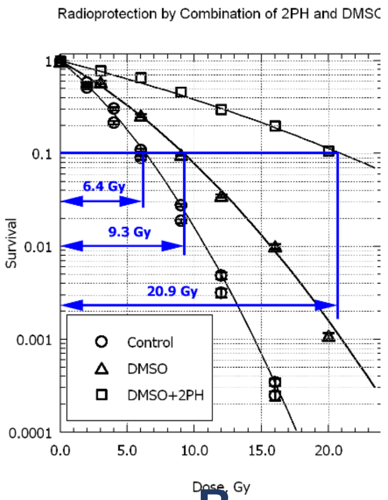
DMF increasing and approaches a plateau at MP concentrations when all binding sites are occupied by the ligand

Supports the hypothesis that DNA bound ligand is responsible for radioprotection

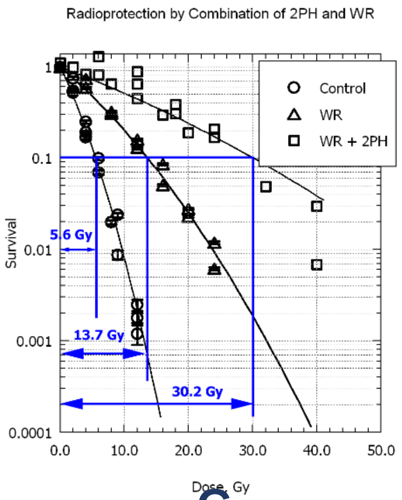
Radioprotection in combination with OH$^\cdot$ radical scavengers


Radioprotection by 2PH 20 uM

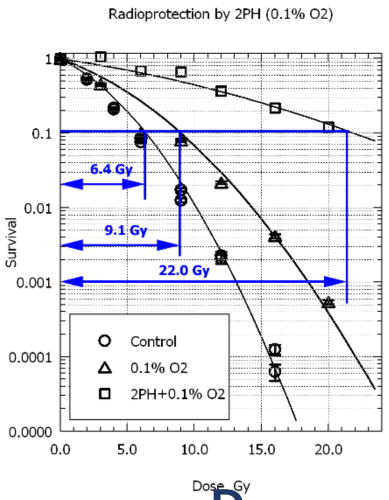
A


Radioprotection by Combination of 2PH and DMSO

B


Radioprotection by Combination of 2PH and WR

C


Radioprotection by 2PH (0.1% O2)

D

Condition	DMF
20 µM 2PH	2.55
0,25 M DMSO	1.45
2PH+DMSO	3.23
2PH/DMSO	2.23
5 mM WR1065	2.46
2PH+WR	5.42
2PH/WR	2.20
0,1% O_2	1.41
2PH+0,1% O_2	3.43
2PH/0,1% O_2	2.43

Radioprotection by 2PH of human keratinocytes in culture

A: 20 µM 2PH

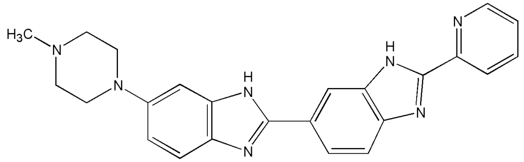
B: 20 µM 2PH + 0,25 M DMSO

C: 20 µM 2PH + 5 mM WR1065 (aminothiol)

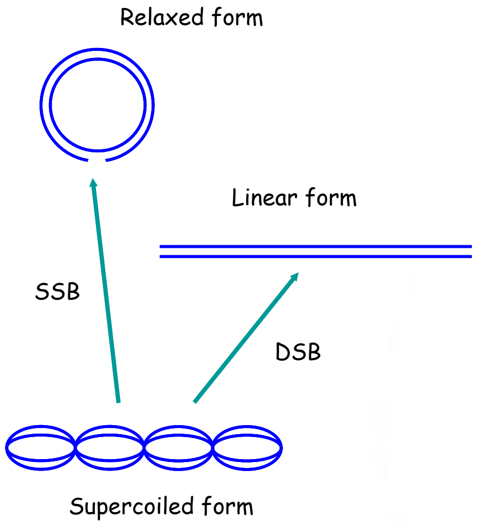
D: 20 µM 2PH, 0,1% O_2

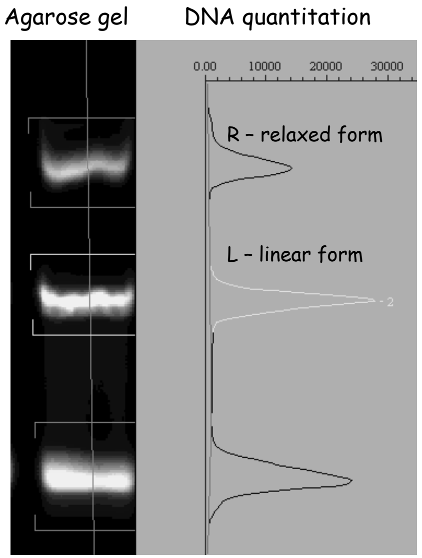
Additivity indicates different mechanism of radioprotection

Doesn't support the hypothesis (H1) that 2PH protects by scavenging hydroxyl radicals



Research tools – Plasmid DNA model





$$N_{dsb} = \frac{L}{1-L}$$

$$N_{ssb} = \ln\left(\frac{1-L}{S}\right)$$

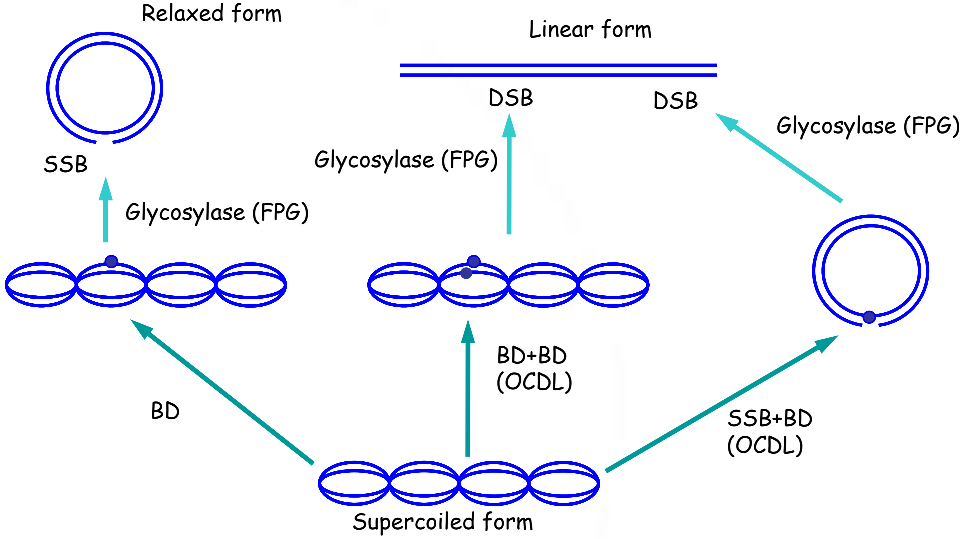
$$\longleftarrow$$

$$S = \frac{e^{-N_{ssb}}}{1+N_{dsb}}$$

$$R = \frac{1-e^{-N_{ssb}}}{1+N_{dsb}}$$

$$L = \frac{N_{dsb}}{1+N_{dsb}}$$

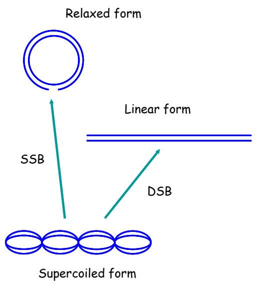
Quantitation of single strand breaks (SSB) and double strand breaks (DSB) yield from the fractions of relaxed and linear form

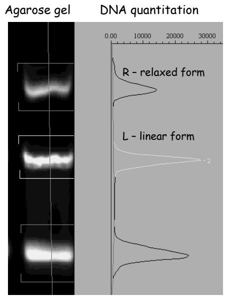


Quantitation of FPG sensitive base damage (BD)

- FPG - formamidopyrimidine-DNA N-glycosylase
- recognises oxidised purines, in particular 8-oxoG
- converts FPG sensitive BD to SSB

Plasmid DNA model – protection from SSB and BD





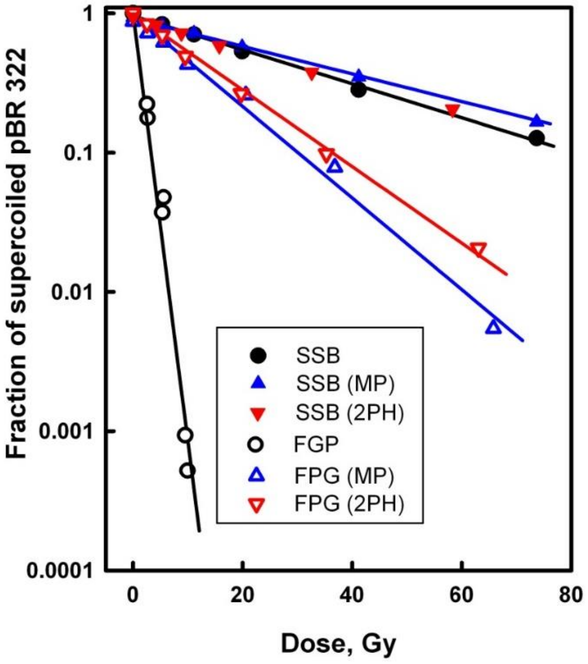
$$S=\frac{e^{-N_{ssb}}}{1+N_{dsb}}$$

$$R=\frac{1-e^{-N_{ssb}}}{1+N_{dsb}}$$

$$L=\frac{N_{dsb}}{1+N_{dsb}}$$

$$N_{dsb}=\frac{L}{1-L}$$

$$N_{ssb}=\ln\left(\frac{1-L}{S}\right)$$



Y_0 – control damage yield

Y_p – yield of damage in the presence of radioprotector

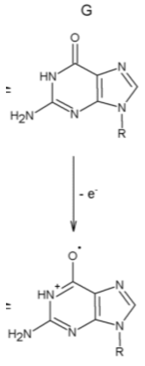
$1-\frac{Y_p}{Y_0}$ – fraction of protected damage

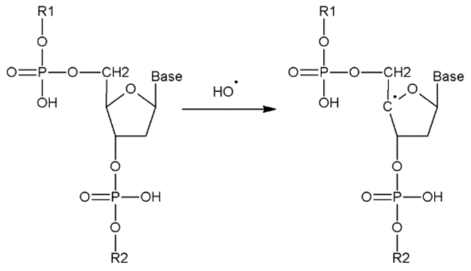
$\mathrm{DMF}=\frac{Y_0}{Y_P}$ – Dose Modifying Factor

	DMF		Fraction protected	
Ligand/ damage	SSB	BD	SSB	BD
MP, 2 μM	1.2	9.4	0.18	0.9
2PH, 1 μM	1.1	18.1	0.13	0.94

Protection from FPG-sensitive BD is more efficient than from SSB

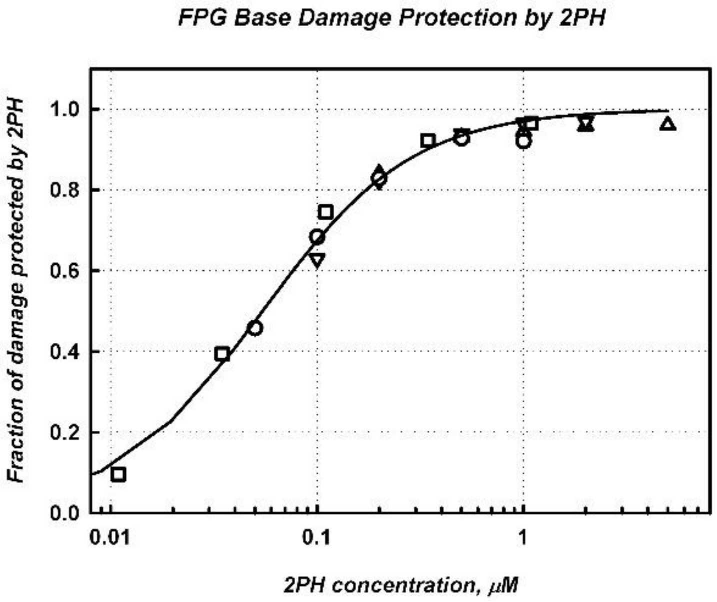
- DNA lesions – precursors of BD are the subject for radioprotection (e.g. guanyl radical)
- DNA lesions – precursors of SSB OP are not the subject of radioprotection (sugar radicals)

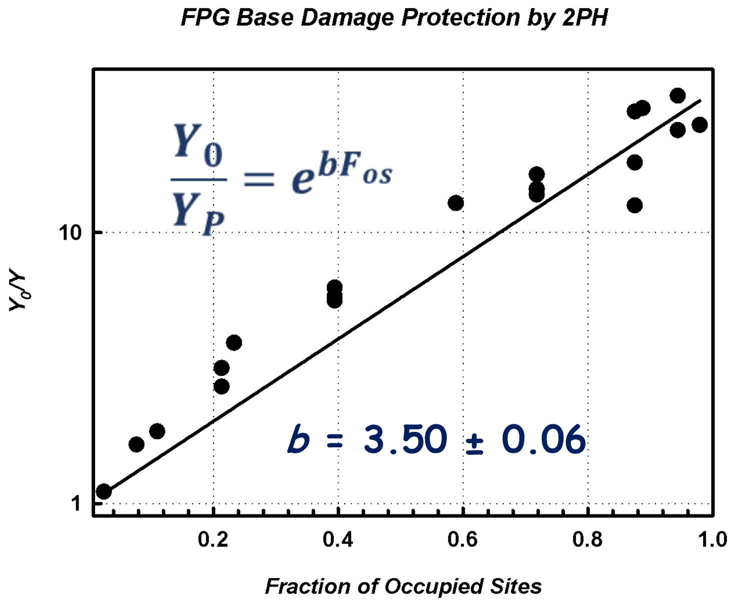




Protection from BD – effect of ligand concentration

$1 - \frac{Y_P}{Y_0}$





$$\frac{Y_0}{Y_P} = e^{bF_{os}}$$

b = 3.50 ± 0.06

$$\frac{Y_0}{Y_P} = e^{bF_{os}}$$

$$b = \frac{2R}{N_s}$$

b = 3.50 +/- 0.06
R = 1.75 N_S

Y_0 – control damage yield

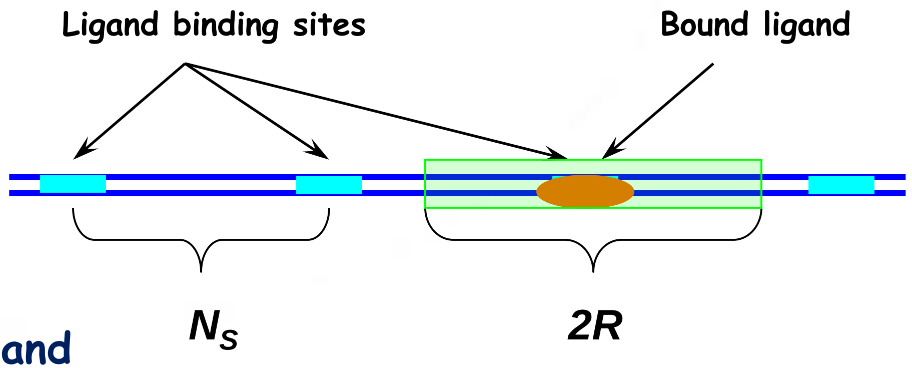
Y_p – yield of damage in the presence of radioprotector

$1 - \frac{Y_P}{Y_0}$ – fraction of protected damage

F_{OS} – fraction of DNA/ligand binding sites occupied by ligand

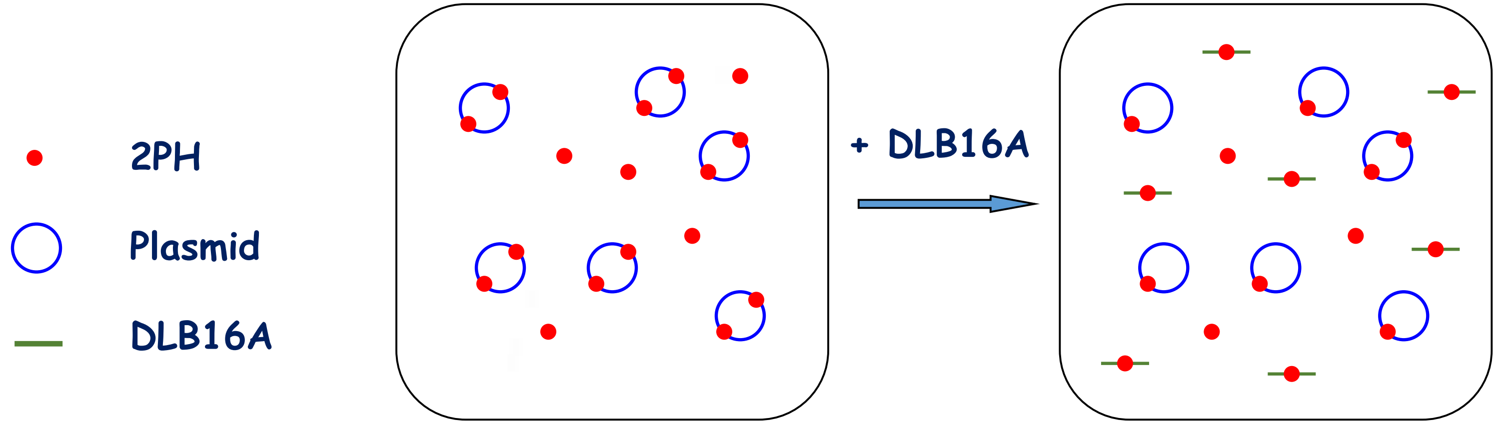
N_S – average distance between neighboring binding sites, bp

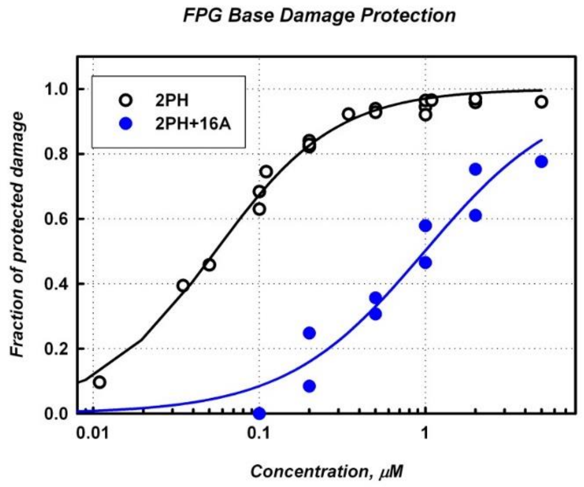
R – radioprotection (electron transfer) range, bp



Assuming N_S = 25 - 30, R = 40 - 52 bp

Protection from BD – effect of ballast DNA



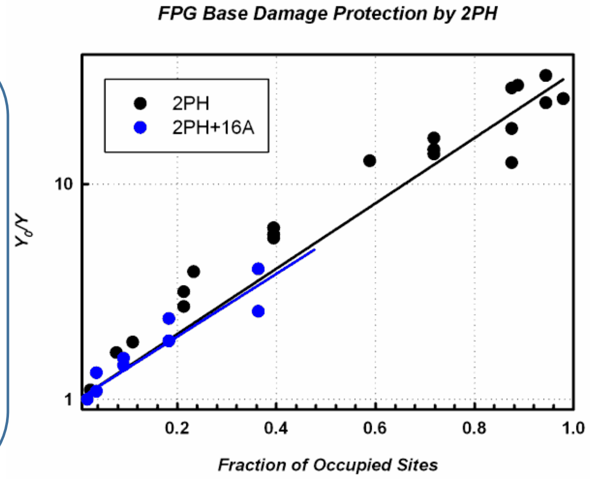


Addition of ballast DNA reduces the fraction of sites occupied by ligand F_{OS}

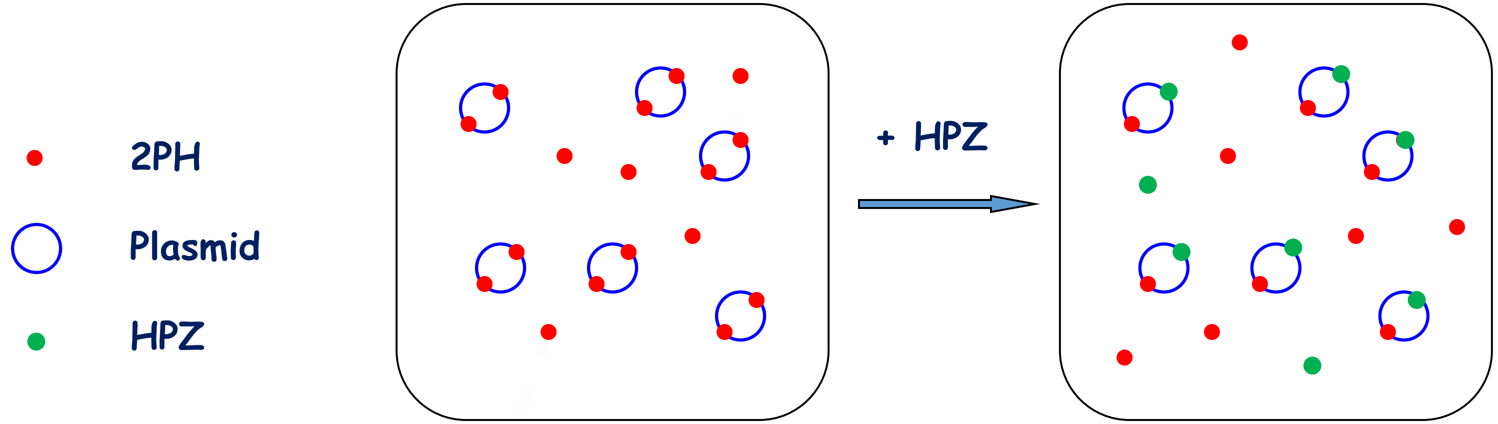
DLB16A – 16-mer self complementary oligodeoxynucleotide containing AATT binding site

5′-CGCGCGAATTCGCGCG-3′
3′-GCGCGCTTAAGCGCGC-5′

- ➢ Extent of protection is reduced upon addition of ballast DNA
- ➢ Extent of protection is the same as a function of occupied sites F_{OS}
- ➢ Bound ligand is responsible for radioprotection

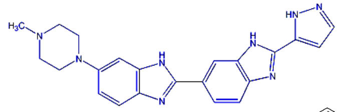


Protection from BD – effect of parasite ligand

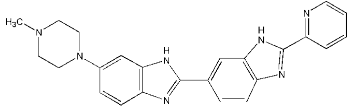


Addition of parasite ligand HPZ reduces the fraction of sites occupied by 2PH F_{OS}

- HPZ – DNA binding bis-benzimidazole
- Strong DNA binder
- Lacks radioprotective properties in cell culture

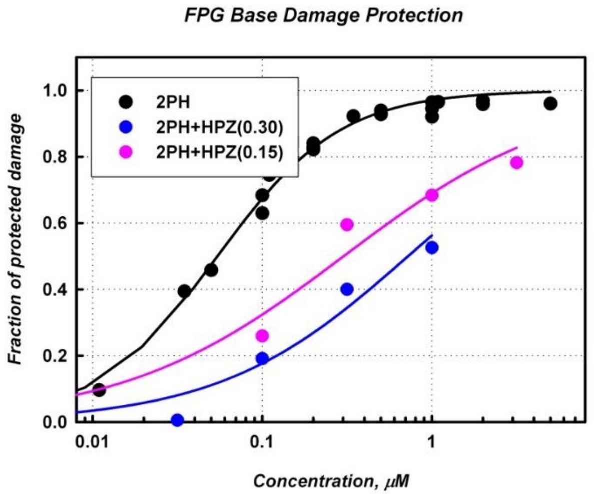


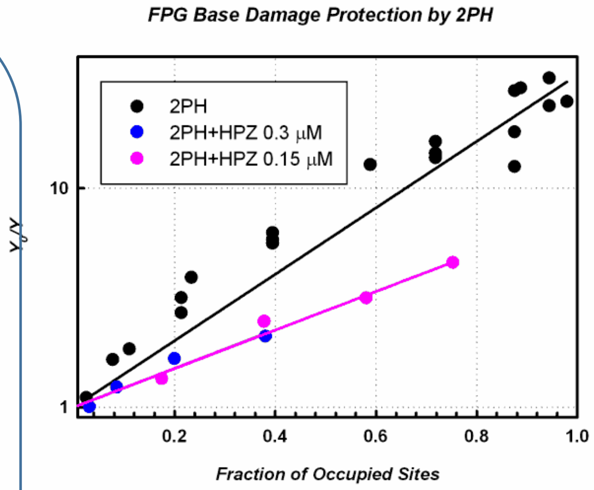
HPZ, K_d = 180 nM



TTA, K_d = 330 nM

- Extent of protection is reduced upon addition of parasite ligand HPZ
- Extent of protection is somewhat reduced as a function of occupied sites F_{OS} (parasite ligand affects electron migration?)
- Bound ligand is responsible for radioprotection
- Free ligand is not involved in radioprotection



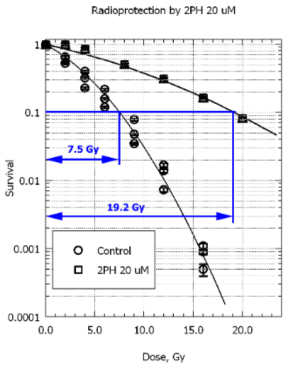


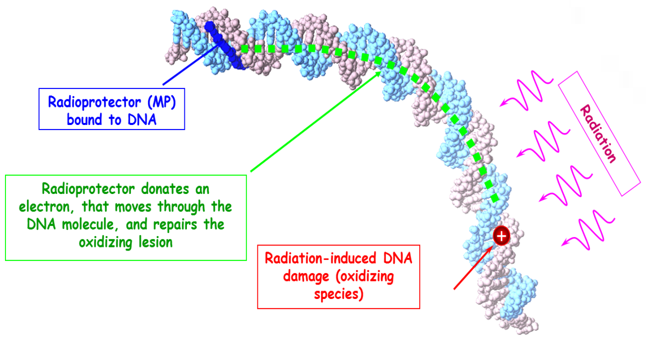
R (2PH) = 40 - 50 bp
R (2PH+HPZ) = 25 - 30 bp

Reduction of DNA radicals and protection from cell death

How reduction of DNA radicals (transient oxidising species) accounts for radioprotection of cells from clonogenic death

or are DSB DNA subject for radioprotection?

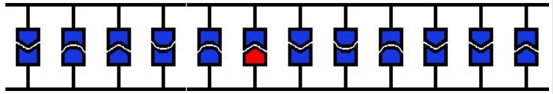
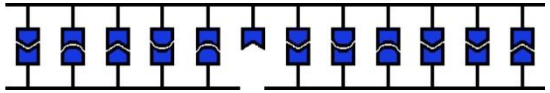
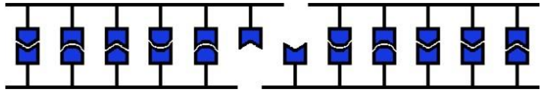
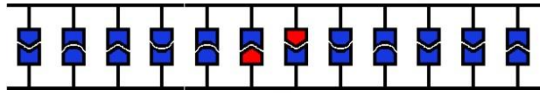
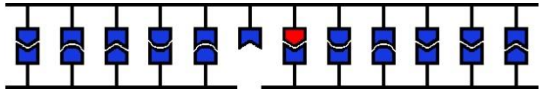
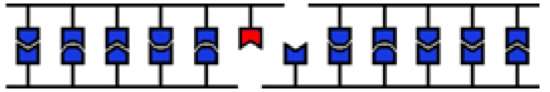




Radioprotector (MP) bound to DNA

Radioprotector donates an electron, that moves through the DNA molecule, and repairs the oxidizing lesion

Radiation-induced DNA damage (oxidizing species)

Damage type	Description		Cyto toxicity	Protection
Base damage (BD)	Isolated BD		No	Yes
Single strand break (SSB)	Isolated SSB		No	No
Complex damage	Two SSB opposite		Yes	No
	Two BD opposite		Yes	Yes
	SSB and BD opposite		Yes	Yes
	BD and DSB		Yes	No

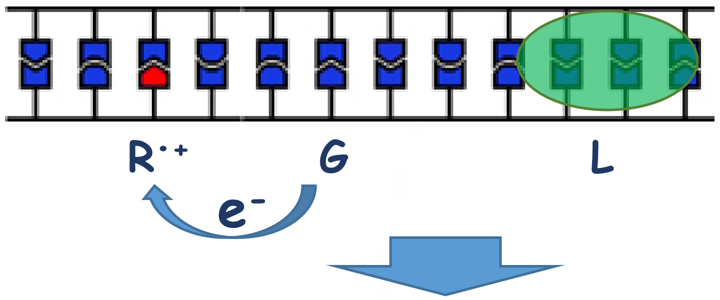
Radioprotection by DNA ligands - Summary

Questions - Answers

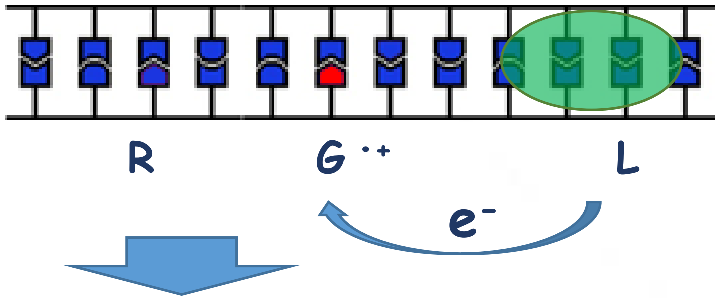
- Is DNA bound ligand responsible for radioprotection? – Yes, DNA bound ligand plays a key role in radioprotection
- What is the charge migration distance? – The charge migration distance or radioprotection range is 40 -50 base pairs
- What DNA oxidising species are the subject for radioprotection? – DNA radicals that are precursors of base damage (modified bases) are the subject of radioprotection
- Are alternative mechanisms possible (scavenging of hydroxyl radicals)? – Alternative mechanisms don't contribute significantly to radioprotection
- How reduction of DNA radicals provides radioprotection from cell death or are DSB DNA subject for radioprotection? – Reduction of DNA radicals that are precursors od base damage constituting complex DNA lesions can prevent formation of lethal damage

Mechanism of radioprotection – new view

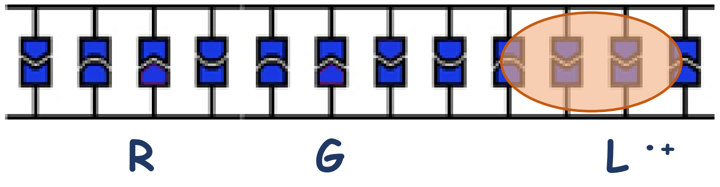
Formation of a base radical



Formation of guanyl radical



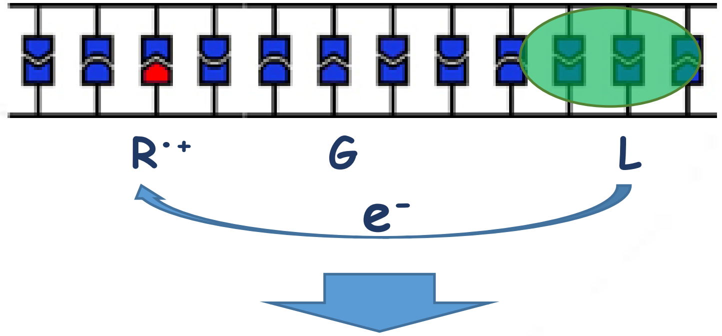
Reduction of guanyl radical



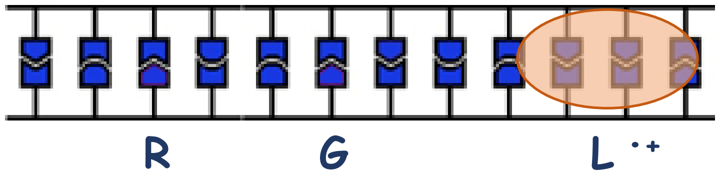
RedOx potential

C	1.6 V
T	1.7 V
A	1.42 V
G	1.29 V
Ligand (MP)	0.9 V

Formation of a base radical



Reduction of a base radical
Guanyl radical is not formed



Mechanism of radioprotection – is it unique?

Our data support the hypothesis that reduction of DNA base radicals is involved in radioprotection by minor groove binders

Question: is this mechanism unique for minor groove binders?

Similar mechanism was suggested to be involved in reduction of guanyl radicals by Tyrosine associated with DNA via cationic oligopeptide Lys2-Tyr-Lys2 (Milligan et al, 2010, Org Biomol Chem, 8, 2553) and might be one of the mechanisms of natural protection of genomic DNA by histones

Radioprotection by DNA minor groove binding ligands

Thanks for your attention

International School on Nuclear Methods and Applied Research
in Environmental, Material and Life Sciences
(NUMAR-2024)
25 – 28 February 2024