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Overview

Biological Consequences of Radiation-induced DNA Damage: Relevance to Radiotherapy

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Abstract

DNA damage of exposed tumour tissue leading to cell death is one of the detrimental effects of ionising radiation that is exploited, with beneficial consequences, for radiotherapy. The pattern of the discrete energy depositions during passage of the ionising track of radiation defines the spatial distribution of lesions induced in DNA with a fraction of the DNA damage sites containing clusters of lesions, formed over a few nanometres, against a background of endogenously induced individual lesions. These clustered DNA damage sites, which may be considered as a signature of ionising radiation, underlie the deleterious biological consequences of ionising radiation. The concepts developed rely in part on the fact that ionising radiation creates significant levels of clustered DNA damage, including complex double-strand breaks (DSB), to kill tumour cells as clustered damage sites are difficult to repair. This reduced repairability of clustered DNA damage using specific repair pathways is exploitable in radiotherapy for the treatment of cancer. We discuss some potential strategies to enhance radiosensitivity by targeting the repair pathways of radiation-induced clustered damage and complex DNA DSB, through inhibition of specific proteins that are not required in the repair pathways for endogenous damage. The variety and severity of DNA damage from ionising radiation is also influenced by the tumour microenvironment, being especially sensitive to the oxygen status of the cells. For instance, nitric oxide is known to influence the types of damage induced by radiation under hypoxic conditions. A potential strategy based on bioreductive activation of pro-drugs to release nitric oxide is discussed as an approach to deliver nitric oxide to hypoxic tumours during radiotherapy. The ultimate aim of this review is to stimulate thinking on how knowledge of the complexity of radiation-induced DNA damage may contribute to the development of adjuncts to radiotherapy.
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Key words: Clustered DNA damage; DNA repair; ionising radiation; microenvironment; radiosensitisers; radiotherapy

Statement of Search Strategies Used and Sources of Information

The review is based on a search of peer-reviewed publications found using WEB of Science and PubMed search tools and the key words identified. In addition, the reference lists from other key articles were used to obtain other pertinent articles.

Introduction

Ionising radiation can be considered as a 'two-edged sword' in that it may lead to genetic modifications in exposed, surviving normal tissue, but may lead to loss of clonogenic survival of tumour cells, the latter delivering benefit after radiotherapy. One of the ways in which ionising radiation works is through damaging the DNA of exposed tumour tissue leading to cell death.

We are continually exposed to oxidative stress, with as many as 50 000 lesions of DNA modifications [1] induced daily in each cell through reactive oxygen species (ROS), formed as a by-product of aerobic metabolism. Cells have well-developed repair processes to deal with damage induced through oxidative stress as it is vital that the cellular responses are able to maintain genome integrity and stability to minimise the onset of potential tumorigenesis and the ageing process. The identification of the different types of lesion induced endogenously and the free radical mechanisms of their formation have been described in numerous reviews [2–4] and an excellent book by von Sonntag [5]. Ionising radiation also results in DNA modifications in each cell exposed at the fractionated doses conventionally used in radiotherapy. The types of lesion produced via exposure to ionising radiation are, in the main,

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chemically identical to those formed by ROS [3,4,6]. For instance, ionising radiation induces in mammalian cells around 850 pyrimidine lesions, 450 purine lesions, 1000 single-strand breaks (SSB) and 20–40 double-strand breaks (DSB)/cell/Gy with low linear energy transfer (LET) γ-radiation (Table 1 shows yields of major lesions induced by radiation [3]). The spectrum of the types of damage and their yields are similar when induced by ion particles as used in hadron therapy (Table 1).

For a typical therapeutic dose of around 2 Gy/fraction of sparsely ionising radiation, about 3000 DNA lesions are produced per cell exposed, a level of damage far lower than the level of up to 50 000 lesions produced daily through ROS. A frequently asked question is why should a 2 Gy dose of ionising radiation, which produces a lower burden of radiation-induced DNA damage relative to the plethora of damage induced daily through ROS, lead to significant loss of clonogenic survival of tumour cells.

In this review, we will address this question based on the energy distribution events/ionisations in the radiation track defining the characteristic spatial distribution of damage (clustering) and how knowledge of DNA damage may contribute to the development of adjuncts to radiotherapy.

Why Radiation Damage is More Effective than Endogenous Damage at Killing Cells

As illustrated in Figure 1, the radiation track deposits energy in the biomolecules of the cell during its passage. The pattern of these discrete energy depositions during passage of the ionising track causes chemical modifications to the biomolecules and, as a consequence, defines the spatial distribution of lesions induced. If we focus on DNA as the major biomolecule of interest, a fraction of the DNA damage sites induced by ionising radiation will have two or more lesions formed within one or two helical turns of the DNA, shown schematically in Figure 1. These sites induced by a single radiation track are termed clustered damage sites and also include DSB. Clustered damage sites may be considered as a signature of ionising radiation in contrast with isolated, endogenously induced lesions, which tend to be homogeneously distributed. It is only over the last 10 years that radiation-induced clustered damage sites have been detected in mammalian cells, with the yield of non-DSB clusters being at least four to eight times that of prompt DSB when induced by γ-radiation [7–9].

With low LET radiation, about 70% of the energy deposited induces isolated lesions, which add to the oxidative burden of the cell [10,11]. More importantly, about 30% of the energy deposited induces clustered damage sites of varying structural and chemical complexity. For densely ionising radiation [12], about 90% of the energy deposited results in clustered damage sites including DSB. The complexity of the clusters, reflecting the number of lesions present, increases with the LET of the radiation (see Figure 1 for schematic representation of clustered damage containing two lesions). It is known that clustered damaged sites including DSB, which are structurally and chemically complex, have reduced reparability when compared with that of individual lesions [13–15]. It is often overlooked that the response to, and efficiency of, repair of DNA damage may depend on the complexity of the DNA damage site and as such should be seen as a different substrate during the repair process. The importance of these substrates has recently been highlighted in the reduced repair efficiency of DNA lesions within a clustered damaged site or when associated with a complex DSB [16–20]. It is the spatial distribution of those lesions, when formed in clusters by radiation, coupled with their reduced ability to be repaired that contributes to more effective killing of tumour cells by ionising radiation.

Ionising Radiation-induced Double-strand Breaks

Due to its cytotoxicity, the most deleterious lesion induced by ionising radiation is thought to be the DSB, a break in the phosphodiester backbone of both strands of the DNA separated by about 10 base pairs or less [21,22]. As DSB are thought to be the major radio-toxic damage, it is not surprising that few, if any, DSB are induced endogenously [23,24]. Both simple and complex DSB (Figure 1) are induced by ionising radiation with 3′ blocking ends, e.g. 3′-phosphate or 3′-phosphoglycolate moieties [25,26], and possess single-stranded overhangs of variable length, whereas complex DSB have a high frequency of oxidised base modifications and abasic (AP) sites directly adjacent of the DSB ends [27–29]. The yield of DSB increases linearly with radiation dose, starting from a dose of a few mGy [30]. In addition, the more relaxed DNA is (as in transcriptionally active DNA) the DNA damage increases in yield and becomes more complex [31,32].

Table 1
The yield of major lesions induced by ionising radiations of different quality (adapted from Cadet *et al.* [3])

Radiation-induced lesions in cellular DNA	Number/Gy/cell	Number/Gy/cell
	γ-radiation	$^{12}C^{6+}$ ions (31.5 keV/μm)
5,6-thymine glycol (Tg)	582	372
5-(hydroxymethyl)-2′-deoxyuridine	174	72
5-formyl-2′-deoxyuridine	132	66
FapyG	234	132
8-oxo-7,8-dihydro-2′-deoxyguanosine	120	60
Single-strand breaks	1000	

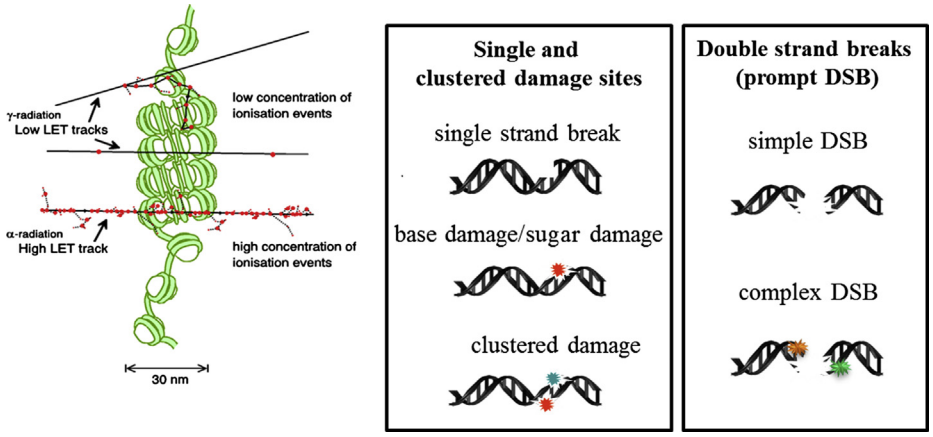

Fig 1. Schematic of the types of DNA damage, ranging from single and clustered damage sites through to simple and complex double-strand breaks (DSB), formed by passage of a single radiation track. Low linear energy transfer radiation induces lower concentrations of ionisation events and consequently less complex DNA damage sites than high linear energy transfer. The coloured stars represent base or sugar lesions.

In mammalian cells, DSB are repaired by two main pathways, namely non-homologous end joining (NHEJ) and homologous recombination. Homologous recombination provides greater repair fidelity than NHEJ, the latter being the major pathway to repair prompt DSB throughout all phases of the cell cycle (reviewed in [33,34]). Evidence indicates that the majority of DSB induced by low LET radiation are rejoined within 30–60 min, whereas a small fraction of DSB, generally <20%, are less readily repaired in mammalian cells and some may persist for >24 h [35–40]. As the LET of the irradiation increases, so does the proportion of breaks that are repaired slowly and it is thought that the slower repairing DSB reflect the increasing proportion that are complex.

NHEJ is the major pathway to repair DSB. It involves the recruitment of Ku70/80 and DNA-PKcs to the DSB termini, followed by processing of the DSB by the MRN complex (Mre11, RAD50 and Nbs1), Artemis, PNKP and APLF, gap filling by polymerases μ and λ and is completed by ligation via Ligase IV, XRCC4 and XLF (reviewed in [41]). Ku70/80 is recruited to all DSB, but DNA-PKcs is only involved in the repair of the longer lived complex DSB [36,42]. Blocked 3′ termini and, to a greater extent, base lesions and AP sites close to the termini of DSB can drastically reduce the ligation and repair of DSB [19,29]. The base excision repair (BER) pathway (the major pathway for the removal of base lesions) is also compromised when removing base lesions close to DSB termini [19], consistent with the observation that complex DSB are rejoined before base lesions are removed [29]. Any lesions present in a 5′-overhang of a DSB may be 'negated' by trans-lesion synthesis, as shown by 'negation' of an AP site before NHEJ takes place [43]. This process avoids loss of DNA sequences, but may be mutagenic.

With the introduction of ion therapy with ions such as $^{12}C^{6+}$, the consequences of complex DSB, which are more difficult to repair and require DNA-PKcs when repaired by NHEJ, needs to be considered in terms of an increased relative biological effectiveness of ion therapy and a reduction in the effects of radio-resistant hypoxic cells.

Ionising Radiation-induced Non-double-strand Break Clustered Damage

Non-DSB clustered damage sites containing two or more lesions within one to two turns of the DNA helix (see Figure 1) can be bistranded (lesions on both strands of the DNA helix) or in tandem (two or more lesions adjacent on the same strand of DNA). As with DSB, biophysical modelling confirms that as the ionisation density of the radiation increases, both the complexity and the yield of non-DSB clustered damage increases [44].

Endogenously induced isolated lesions are repaired very efficiently in cells by the BER pathway (reviewed in [45]). The BER pathway is also the major pathway responsible for the repair of non-DSB clustered DNA damage sites. It is now well established that the efficiency of repair of lesions within both bistranded and tandem clustered damage sites is reduced compared with that for the repair of isolated lesions (reviewed in [15]). The extent of the reduction in repair depends on the types of lesion within the cluster, the inter-lesion separation, the number of lesions within the cluster (complexity) and the orientation of the lesions to each other. For instance, a SSB (formed directly by ionising radiation or via the action of an AP endonuclease or glycosylase on an AP site or base lesion) or an AP site confers a strong retardation on the removal of a nearby base lesion by glycosylases [46,47] and a base lesion close to a SSB can strongly impair the repair of the SSB [48,49].

Of importance is the finding that certain clustered damage sites, especially those that are orientated 3′ to each other, have a greater dependence on the long-patch BER pathway in contrast to isolated lesions, which are repaired predominately by the short-patch repair pathway [48,50,51]. In addition, some lesions induced by ionising radiation, such as oxidised AP sites, cannot be repaired by short-patch BER and have to rely on the long-patch BER pathway. Use of the long-patch BER pathway in mammalian cells will probably result in the formation of a DSB compared with the short-patch pathway [52].

A hierarchy of repair exists for processing non-DSB clustered damage sites whereby a SSB retards the excision of a base lesion until the SSB has been repaired [48,49,53,54], thus limiting the formation of DSB. Furthermore, the excision of the first base lesion in a clustered site will prevent the excision of further base lesions [51]. This hierarchy serves to extend the lifetime of the clustered damaged site. For instance, the lifetime of lesions in a clustered damage site may be up to eight times that of the same lesions when in isolation [48]. A consequence of increasing the lifetime of the lesions within a cluster is that the chance of them encountering a replication fork before their repair is increased. As a consequence, the enhanced mutagenic potential of the lesions within the cluster increases, confirmed through the observation of elevated mutation frequencies with a number of clustered damage sites in bacteria [49,51,55,56]. In contrast, bistranded AP sites are rapidly incised, despite being in close proximity, to form DSB *in vitro* [49,50,57], in bacteria [49,58] and yeast [59]. Interestingly, bistranded AP sites do not form DSB in mammalian cells [60], but bistranded furans (obligate long-patch BER substrates) do [61], suggesting that short-patch BER of an AP site is preferred. Indeed clustered AP sites have been shown to live for days in cells [62]. It was estimated that 10% of non-DSB clustered lesions are converted to DSB during processing [63] and if there are other lesions in close proximity to these newly formed DSB, then complex DSB would be formed [9,61].

Replication-induced Double-strand Breaks

In addition to the formation of radiation-induced prompt DSB, replication DSB are formed after ionising radiation (see Figure 2 for schematic representations), detected as RAD51 foci at times >2–3 h [64,65]. It is thought that replication-induced DSB, which are chemically distinct from prompt DSB, are formed when an unrepaired non-DSB clustered damage site meets a replication fork to produce a replication-induced DSB [65,66], which requires homologous recombination for its repair [66–68]. The concept of an extended lifetime of radiation-induced lesions when in clusters is similar to that proposed to explain synthetic lethality in BRCA-deficient cells when PARP inhibition causes SSB to persist longer and as a consequence their probability of encountering a replication fork is enhanced [69,70].

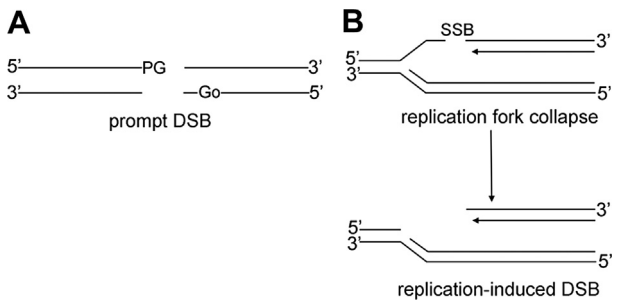

Fig 2. Schematic illustration of radiation-induced prompt and replication-induced double-strand breaks. These two types of double-strand break are chemically distinct.

Radiosensitisation of Hypoxic Tumours Revisited – A Role for Clustered DNA Damage

The variety and severity of DNA damage from ionising radiation is influenced by the tumour microenvironment. In particular, the oxygen status of a tumour has a huge role to play in local control after radiotherapy. Many tumours are comprised of poorly oxygenated hypoxic regions that are chemo- and radio-resistant [71] and patients displaying highly hypoxic tumours have a much poorer outcome than those with well-oxygenated tumours [72]. Mechanistically, the radiosensitising effect of O_2 can in part be explained through its extremely rapid reaction, within a few milliseconds, with radiation-induced DNA radicals (Figure 3), which ultimately lead to the products shown in Table 1. However, the types of base damage and the yields of base release arising through ionising radiation vary between oxygenated and hypoxic cells [5]. Additionally, the formation of different types of lesion within clustered damage sites may have consequences on the repair of the lesion within the clusters as discussed above.

The difficulties of treating hypoxic tumours with radiotherapy can be partially overcome by altering the cellular microenvironment. Enhancing radiation-induced DNA damage relies upon the presence or indeed absence (of a radioprotector) of a free radical reactant exactly at the time of radiotherapy. Chemical radiosensitisers, in particular electron affinic molecules like nitroaromatic compounds, react and may conjugate with free radicals generated at DNA bases [73] in a similar manner to O_2, inducing strand breaks (described in [74]) and modified base lesions (Figure 3). However, early success with 2-nitroimidazoles (e.g. misonidazole) had limited clinical use because of severe neurotoxicity and the 5-nitroimidazole, nimorazole [75], and the nitrotriazole, sanazole [76], are now the main electron-affinic compounds being assessed for clinical radiosensitiser use.

Another hypoxic cell radiosensitiser that is gaining renewed interest since first being recognised as long ago as 60 years [77] is nitric oxide (NO). NO is a natural compound produced in tissue by nitric oxide synthases, but it is also amenable to drug delivery. Delivery of NO (or indeed any radiosensitiser) to a solid tumour, specifically to the hypoxic regions, is however a challenge.

Although the effect NO offers may in part be through increasing tumour oxygenation (reviewed in [78]), it can also influence the types of damage induced by radiation under hypoxic conditions [79–81]. NO as a stable free radical reacts rapidly with DNA radicals formed by ionising radiation ($k \sim 10^9\ M^{-1}\ s^{-1}$) [79] (Figure 3). The presence of NO in mammalian cells irradiated under hypoxia leads to increased numbers of SSB and DSB [79,80] and this is particularly evident hours after irradiation, when most simple strand breaks would have been repaired [81,82].

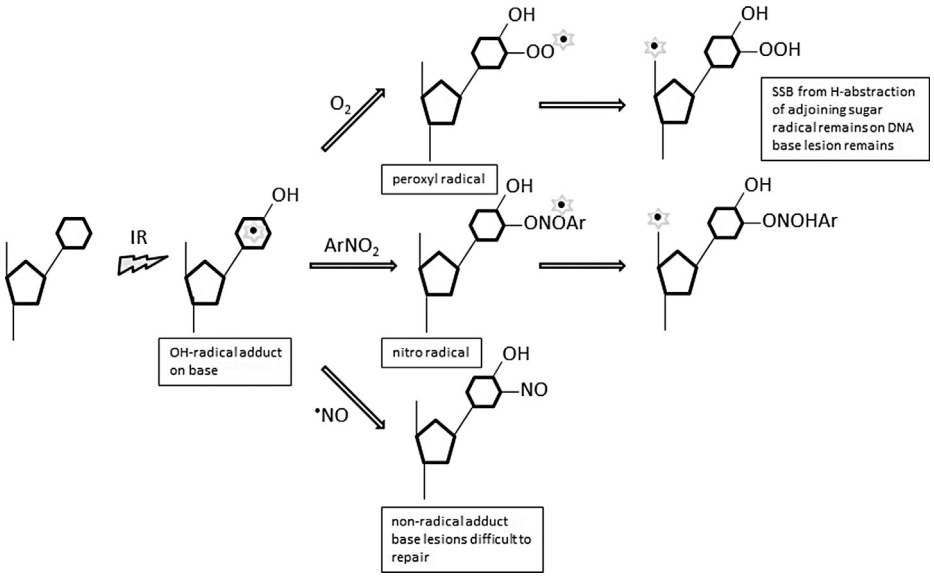

Fig 3. Schematic showing the comparison of the pathways for reaction of hydroxyl radical adducts of DNA nucleobases with oxygen and the hypoxic cell radiosensitisers, nitro-arenes and nitric oxide (•NO). Whereas the products of reactions with oxygen and nitro-arenes are still free radicals, only •NO forms a non-radical product.

These effects of NO are in contrast to similar equally rapid reactions of DNA radicals with O_2 when long-lived peroxyl radicals are formed, which may induce the formation of SSB through secondary free radical reactions (Figure 3). [83]. In addition, NO reacts with DNA base radicals to form non-radical modified bases (Figure 3). For example, 8-azaguanine nucleotide, a cytotoxic triazole form of guanine, has been identified in plasmid DNA irradiated in the presence of NO [81]. These modified base lesions, especially if present in a clustered damage site, may be difficult to repair by conventional mechanisms and as a consequence may lead to replication fork collapse to give replication DSB (Figure 2) and ultimately cell death, as shown by Folkes and O'Neill [82].

Ionising Radiation: A Double-edged Sword

The complexity of ionising radiation-induced DNA damage underlies its deleterious biological consequences and this property of ionising radiation is exploited in radiotherapy for the treatment of cancer, including the potential use of some adjuncts to enhance radiosensitivity of hypoxic cells. These concepts rely in part on the fact that fractionated doses of ionising radiation create significant levels of clustered DNA damage, including complex DSB, to kill tumour cells, particularly as clustered damage sites are difficult for the cell to repair. Additionally, tumour cells are often deficient in the effective pathways required to repair these types of damage, e.g. BRCA1 deficiency affecting the homologous recombination pathway. However, the lower doses of irradiation that are received by the normal tissues have to be considered, as it is these cells that may not receive a cytotoxic dose, but may have non-DSB clustered damage induced. X-ray doses as low as 10–100 cGy have been seen to result in clusters of AP sites in primary human fibroblasts [84]. These poorly repaired clustered damage sites may lead to mutations, chromosomal aberrations and, ultimately, secondary cancers. The balance between sterilising tumour cells and sparing normal tissue is also a crucial consideration in radiotherapy, especially as targeted radiation delivery, e.g. intensity-modulated radiotherapy, image-guided radiotherapy and ion therapy, gains in prominence to enhance the curative effects in eradicating cancer. Normal tissue close to tumour tissue will receive higher doses from the above targeted radiation deliveries than from present conventional radiation delivery and this area is worthy of further research.

Chemically Targeting Clustered DNA Damage Sites as Adjuncts to Radiotherapy

The protein kinase ataxia-telangiectasia mutated (ATM) plays a facilitating step in the repair of a subset (about 10%) of radiation-induced complex DSB [14]. We have recently shown that DNA-PKcs is recruited preferentially during the repair of complex DSB and that retardation of phosphorylation of DNA-PKcs by an ATM inhibitor results in 'slowing' the release of not only DNA-PKcs but also Ku80 from complex DSBs [36]. As a result, the lifetime of complex but not simple DSB is extended by an ATM inhibitor, known to enhance radiosensitivity.

Similarly, several radiation-induced non-DSB clustered damage sites rely upon the long-patch BER pathway, whereas most endogenously induced isolated lesions rely upon short-patch BER. Thus, reducing the efficiency of the long-patch BER pathway through an inhibitor may lead to an increase in the lifetime of lesions within a cluster. As a consequence, the persistence of the clustered damage, if in

S phase cells, increases the probability of encountering a replication fork, leading to its collapse to result in a replication-induced DSB. For instance, inhibition of FEN1, a protein involved in long-patch BER, blocked LP-BER causing enhancement of the cytotoxicity of the DNA-alkylating agent Temozolomide in colon cancer cells [85].

From a better understanding of the spectrum of DNA damage induced by radiation, it may be possible to target the repair of radiation-induced clustered damage and complex DNA DSB through inhibiting specific proteins that are preferentially required for repair of these types of damage but importantly do not interfere significantly with the repair of endogenous damage or simple DSB.

To enhance the radiosensitivity of hypoxic cells, agents have previously been developed as donors or modulators of NO, such as diazeniumdiolates (NONOates) [86,87], nitrite [88], NO-donating non-steroidal anti-inflammatory drugs [80], dinitroazetidines [89], insulin and electrical stimulus [90] and cytokine activation of macrophages [91]. Pro-drugs incorporating a 'trigger-effector' system are activated through free radical reactions by cellular reductases in hypoxic tissue, and were described by Denny *et al.* [92]. This concept has recently been applied to the non-radiotherapy based phase I study of TH-302 [93]. Bioreductive activation of pro-drugs to release NO is an alternative approach to specifically targeting hypoxia to fix radiation-induced DNA damage and a potential route [94] to deliver NO to hypoxic tumours for radiotherapy.

Conclusion

The aim of the review was to stimulate some concepts whereby the knowledge on the spectrum of DNA damage induced by radiation against the background plethora of endogenously induced DNA damage may be used to develop strategies to target specific types of damage that utilise repair proteins not required in the repair of endogenous damage. The cellular microenvironment, which can be altered through drug intervention, and the energy of the ionising radiation, influence the type and abundance of DNA damage arising. The ability of the cell to repair specific and different lesions determines the ultimate fate of the cell and it is the multitude of possible variations in DNA damage complexity that, in part, influence radiation lethality.

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