DNA-Binding Radioprotectors

Pavel Lobachevsky¹, Alesia Ivashkevich¹, 
Olga A. Martin¹,² and Roger F. Martin¹

¹Laboratory of Molecular Radiation Biology, 
Peter MacCallum Cancer Centre
²Laboratory of Molecular Pharmacology, 
Center for Cancer Research, National Cancer Institute

¹Australia 
²USA

1. Introduction

For decades the world of radioprotectors has been dominated by the aminothiols, in particular WR1065 and its prodrug amifostine. These drugs emerged from an extensive programme of synthesis and evaluation under the auspices of the Walter Reed Army Institute of Research starting in the early 1950s (Sweeny, 1979). As discussed in detail in section 4 below, structure-activity studies on a series of aminothiols in John Ward’s lab at the University of San Diego established a relationship between net charge and radioprotective activity. Positive charge conferred a DNA binding capability, by ionic interaction, and improved radioprotective activity. This was consistent with the fact that an important aspect of the mechanism of radioprotection by WR1065 is its radical scavenging activity. Given the limited range of diffusion of hydroxyl radicals generated from ionisation of water molecules, it makes sense that the radical scavengers will be most effective when located in the close vicinity of DNA. This basic rationale prompted the synthesis and evaluation of an aminothiol tethered to a DNA intercalating agent (Laayoun et al., 1994), but there is no evidence in the literature of a systematic follow-up.

Also, the new DNA binding radioprotector methylproamine emerged not from a rational design premise, but rather, from the serendipitous discovery of radioprotective activity of a minor groove binder Hoechst 33342 synthesized by the Hoechst company as part of a program aimed at developing antihelminthics. From that starting point, a modest lead optimisation program guided by a mechanistic hypothesis showed that radioprotective activity was enhanced by the introduction of more electron-rich substituents into the phenyl ring of the molecule.

Thus, this article links two groups of radioprotectors with the common feature of DNA-binding, albeit with quite different affinities. The dissociation constant for the WR1065-DNA interaction is in the mM range (Smoluk et al., 1986), whereas that for methylproamine is a few hundred nM (Martin et al., 2004). Accordingly, the relative radioprotective potency of WR1065 and methylproamine differs by more than 2-orders of magnitude. In contrast to this focus, other publications review a much wider range of radioprotectors (Hosseinimehr, 2007; Weiss & Landauer, 2009; Citrin, 2010).
2. DNA damage and biological response to radiation

Both the hazards and potential beneficial uses of ionizing radiation (IR) were realized soon after the discovery of X-rays by Wilhelm Conrad Roentgen in 1895. Studies of Hiroshima and Nagasaki survivors, reconstructed dosimetry, and unfortunate accidents at nuclear plants documented a pattern of events following a whole-body IR exposure, confirmed by extensive animal experiments. Exposure to high doses of IR (100-150 Gy) leads to death within a few hours which results from neurological and cardiovascular breakdown. At intermediate dose levels (5-12 Gy), death occurs within a few days and is associated with gastrointestinal syndrome. At lower doses (2.5-5 Gy) death occurs within several weeks due to haematopoietic syndrome (Hall, 1973). All these effects are attributable to killing of critical cells, and the question of how IR kills cells has stimulated much research. A key milestone was the identification of DNA as the critical molecular target. This research was prompted by both the potential uses of IR, for example in cancer therapy, and by concerns about effects of IR on health. Potentially damaging exposure may come from diagnostic radiology such as computed tomography as well as from cosmic rays, the sun and radioactive nuclides in the ground (e.g. radon), during high altitude journeys, or in space. Such concerns about occupational and environmental radiation exposure have prompted much scientific and legislative activity, the latter leading to the establishment of the International Commission on Radiological Protection.

It is a commonly recognised concept that two distinct mechanisms are responsible for induction of DNA damage by IR; one involves direct ionisation of atoms in the DNA molecule and usually is referred to as the direct effect, and another that results from DNA attack by free radicals generated as a result of the radiolysis of surrounding water molecules and is referred to as the indirect effect (Hall, 1973; Hall et al., 1988). The major contributor to the indirect effect is the hydroxyl radical (HO•) as evidenced by studies using compounds that scavenge hydroxyl radicals (Roots & Okada, 1972). The direct DNA damage is considered to include the damage that is produced by hydroxyl radicals generated in water molecules intimately associated with DNA water layer since these radicals cannot be scavenged (Ward, 1994a). It is estimated that two-thirds of DNA damage is caused indirectly by scavangeable radicals (Roots & Okada, 1972).

IR induces a wide variety of mainly isolated DNA lesions, including strand breaks (single strand breaks – SSB) and damage that involves DNA bases (modified bases and abasic sites). Isolated DNA lesions are normally easily repaired by cells (Ward, 1994a; Sutherland et al., 2000), without serious biological consequences. It is believed that cytotoxic and mutagenic effects of IR originate from so called clustered DNA damage when two or more lesions occur close to each other in both DNA strands (Goodhead, 1994; Ward, 1994a; Goodhead & Nikjoo, 1997; Nikjoo et al., 1997; Nikjoo et al., 1998; Sutherland et al., 2000). This type of DNA damage was initially termed locally multiply damaged sites (LMDS) (Ward, 1994a), but more recently the term LDMS has been largely replaced by the synonym oxidative clustered DNA lesions (OCDL) (Sutherland et al., 2000; Georgakilas, 2008). Formation of strand breaks in opposite DNA strands results in a double strand break (DSB), that represents a particular case of OCDL (Purkayastha et al., 2007). Assuming that base damage (BD) occurs more frequently than SSB (Ward, 1994b; Ward, 1994a), it is expected that the majority of OCDL are not frank DSB but contain two BD or BD and SSB in opposite strands (Goodhead & Nikjoo, 1997). While isolated DNA lesions are generally repaired efficiently, OCDL are more difficult to resolve (Ward, 1981; Harrison et al., 1999; Georgakilas et al., 2008).
An attempt to process by the base excision repair (BER) a modified base that constitute an OCDL with a SSB in opposite strand may result in formation of a DSB, which also can be formed when an unprocessed OCDL interferes with either DNA replication and transcription (Bonner et al., 2008; Sedelnikova et al., 2010). Although DNA DSB can be repaired in cells by non-homologous end joining (NHEJ) and homologous recombination (HR) (Matsumoto et al., 1994; Memisoglu & Samson, 2000; Wilson et al., 2003; Cadet et al., 2010; Hinz, 2010; Lieber, 2010; Mladenov & Iliakis, 2011), DSB are among the most toxic IR-induced DNA lesions. If not properly repaired, both accumulated DSB and OCDL lead to cytotoxicity, genome instability and carcinogenesis (Jeggo & Lobrich, 2007; McKinnon & Caldecott, 2007).

It is established that a substantial level of oxidative DNA lesions may be present in normal cells and tissues, usually a few isolated oxidative DNA lesions per Mbp (Nakamura & Swenberg, 1999; De Bont & van Larebeke, 2004). These lesions are believed to be generated by free radicals that originate from endogenous reactive oxygen species (ROS) (Riley, 1994; Mikkelsen & Wardman, 2003). Two of the biologically important endogenous ROS are superoxide anion radical \( \mathrm{O}_2^- \) and hydrogen peroxide \( \mathrm{H}_2\mathrm{O}_2 \). Endogenous superoxide is produced in cells mainly as a result of mitochondrial respiration (Mikkelsen & Wardman, 2003), and then is efficiently converted to hydrogen peroxide by cellular superoxide dismutase (SOD). Although the superoxide and hydrogen peroxide are relatively long lived species and are able to diffuse in cells over considerable distance (Riley, 1994), these endogenous species are produced in cytoplasm and also they are not able to damage DNA directly. The genotoxic effect of the endogenous ROS is mainly mediated by their ability to give rise to hydroxyl radicals from hydrogen peroxide by a redox reaction with traces of reduced transitional metal ions, mainly ferrous via Fenton chemistry (Mikkelsen & Wardman, 2003). Since ROS represent potential risk for cells, an antioxidant defense system has been developed in cells to maintain a steady state level of ROS.

Exogenous cytotoxic agents can lead to the increase above the steady state in the ROS level thus creating an oxidative stress that can result in induction of additional oxidative DNA damage (Sedelnikova et al., 2010). Exposure to IR is also known to cause the oxidative stress however, interestingly, the level of ROS generated directly from radiolysis of water at biologically relevant doses is much less that the level of the endogenous ROS. This follows from the estimation that for example a 100 Gy radiation dose would be required to double the endogenous level of one of the major types of DNA base damage 7,8-dihydro-8-oxoguanine (8-oxoG) (Ward, 1994b). There is also experimental evidence that the transient increase in the cellular ROS level following irradiation is dependent on mitochondria respiration, however it is dose independent in the range of biologically relevant doses (1 – 10 Gy) with the fraction of cells exhibiting the increased ROS level being dose dependent (Leach et al., 2001). These observations underline the minimal impact of isolated DNA lesions and the critical role of clustered lesions for cyt- and genotoxic consequences of IR. The major difference between endogenous ROS and those generated by IR is that while the spatial distribution of hydroxyl radicals produced from endogenous ROS is random, IR is also able to generate clusters of hydroxyl radicals within a nanometre scale resulting in the multiple radical attack on DNA within small volume from a single track of a charged particle (Goodhead, 1994; Goodhead & Nikjoo, 1997; Nikjoo et al., 1997; Nikjoo et al., 1998). As a result, the relative frequency of OCDL is much higher for damage induced by IR as compared to endogenous oxidative DNA damage for which OCDL are very rare. The frequency of endogenous OCDL is estimated to be a few per Gbp in normal tissues (Bennett
et al., 2005), and can probably be formed as a result of two isolated oxidative lesions occurring close to each other spatially and temporally. The radiation induced OCDL can also be more complex since consisting of more than two individual lesions in a cluster (Ward, 1994a; Bennett et al., 2005).

Recently, the dogma that cells subjected to IR are killed solely through direct energy deposition within a cell, with the effect being proportional to dose, has been reconsidered in view of the discovery of the radiation-induced bystander effect (RIBE). The neighbours of irradiated cells respond as if they themselves have been irradiated. The RIBE is now a well-established consequence of exposure to IR and is manifested as increased genomic abnormalities and loss of viability in unirradiated (“bystander”) cells associated with the targeted cells. Affected bystander cells exhibit increased levels of micronuclei, apoptosis, mutations, altered DNA damage and repair, and senescence arrest (Sokolov et al., 2005; Sedelnikova et al., 2007; Prise & O’Sullivan, 2009). DSB appear in the DNA of bystander cells, invoking the existence of some kind of biological “danger signal” that is sent from irradiated to bystander cell. Possible mediators of the RIBE include various inflammation-related cytokines and ROS including nitric oxide that have been found at elevated levels in medium conditioned by irradiated cells (Dickey et al., 2009; Prise & O’Sullivan, 2009; Hei et al., 2010; Ivanov et al., 2010).

3. Radiation therapy of tumours and the role of radioprotectors

The detrimental consequences of IR for cells and tissues can be harnessed in cancer radiation therapy. Radiation therapy exploits the cytotoxic effect of IR on cancer cells (Lawrence et al., 2008) and represents one of the three major treatment modalities for cancer, along with surgery and cytotoxic chemotherapy. Radiotherapy is used in approximately half of all patients diagnosed with cancer at some stage of their illness. Technological advances in the physical targeting of radiation to the tumour are extensively exploited, reflecting the simple idea that the most efficient radioprotection strategy is to exclude normal tissues from irradiated volume. These include such techniques as conformal radiotherapy, intensity modulated therapy, image guided radiotherapy etc (Brizel, 2005). Although still in the pre-clinical stage of development, microbeam radiotherapy (MRT), in which the X-ray beam is split into an array of planar parallel microbeams, shows much greater therapeutic index than conventional radiotherapy. Studies of synchrotron MRT in animal models indicated that tumours can be ablated by MRT at radiation levels that spare normal tissues (Dilmanian et al., 2002; Dilmanian et al., 2003; Crosbie et al., 2010). Nevertheless, whether used alone or in combination with other treatment modalities, the dose of radiation that can be safely delivered is limited by radiation induced injury to the normal tissues in the irradiated volume. This gives rise to the concept of “treatment to tolerance” i.e. the administration of the maximally tolerated dose (MTD) imposed by the normal tissues which is often less than required to effect a high probability of tumour eradication. Clearly, any strategy that selectively increases the MTD improves the chances of tumour cure, and one of such strategies is the selective pharmacologic modification of the normal tissue response with radiation modifiers/protectors. These agents alter the response of normal tissues to irradiation when present in tissues prior or after exposure to IR (Citrin, 2010). This approach can also be viewed as an attractive countermeasure for possible nuclear/radiological terrorism and radiation accidents, but without the important constraint to avoid protection of the tumour that is imperative in the use of radioprotectors in radiation oncology.
The US National Cancer Institute Workshop have developed recommendations for the terminology and classification of the agents used to ameliorate the biological consequences of the exposure to IR (Stone, 2003). The classification implies that there are different mechanisms of action of these agents, and therefore they may be efficient when administered appropriately with regard to the time of the exposure to IR. Accordingly, there are three groups of such agents. Prophylactic agents/protectors are administered before exposure to IR and mainly act by chemically preventing the initial radiochemical damage; mitigators are given during or soon after exposure to IR to prevent development of tissue damage; and treatment agents are administered after exposure to IR to reduce symptoms developed as a result of this exposure.

Apart from normal tissue damage, another major concern associated with cancer radiotherapy is the potential for emergence of secondary radiation-induced cancers, affecting more than 1% of patients (Hall, 2006). Such an outcome can arise in two ways, the first being the induction of mutagenic DNA damage in nearby normal tissues. The second is associated with a phenomenon similar to RIBE in \textit{in vitro} settings that has been reported by cancer radiotherapists more than 50 years ago and termed the abscopal effect (Mole, 1953; Kaminski et al., 2005). It is defined as a change in an organ or tissue distant from the irradiated region. Since these non-targeted effects include malignant transformation (Hall & Hei, 2003; Mancuso et al., 2008), the abscopal effect represents a serious risk factor in radiotherapy.

Therefore, efforts to reduce radiation toxicity in normal tissues and/or in a whole organism are of significant clinical importance and an area of active research. The development of radioprotectors can be regarded as an important strategy to achieve these objectives.

4. Aminothiols as radioprotectors

Of the thousands of compounds synthesised and tested at the Walter Reed Army Institute of Research in the 1960’s search for radioprotectors, aminothiols emerged as the most promising compounds. The persistent motif associated with radioprotective activity of aminothiols is a thiol separated from an aliphatic amino group by a two carbon chain (Brown et al., 1982). The simplest example is cysteamine (chemical formula $\text{H}_2\text{N-CH}_2\text{-CH}_2\text{-SH}$). One of the most studied aminothiols is the radioprotector WR1065 (2-[aminopropyl]amino]ethanethiol, Figure 1), which is the active thiol metabolite of amifostine (WR2721).

![Fig. 1. Structure of WR1065 (R = H) and its prodrug amifostine (R = H$_2$PO$_3$).](www.intechopen.com)

WR1065 protects cultured cells against radiation induced clonogenic death. A dose modification factor (DMF) of 1.9 is achieved for V79 cells pre-incubated 30 min with 4mM of WR1065 before irradiation (Grdina et al., 1985). DMF is defined as the ratio of radiation doses producing the same degree of radiation effect, in the presence and absence of the radiomodifier. In the context of radioprotection, and particularly for \textit{in vivo} endpoints, DRF, dose reduction factor is often used. It has been shown using neutral elution technique that...
the number of radiation induced DNA DSB in V79 cells is reduced by 4 mM WR1065 with a DMF of 1.8 (Sigdestad et al., 1987). WR1065 also protects against the mutagenic effect of radiation as demonstrated for the hypoxanthine-guanine phosphoribosyl transferase locus in V79 cells (Grdina et al., 1985). Radioprotection by WR1065 and WR2721 in vivo has been demonstrated using the Withers assay that is based on histological staining and counting of the repopulating crypt clonogens in mouse jejunum (Withers & Elkind, 1969; Withers & Elkind, 1970). A DMF of 1.8 – 2.0 has been reported for this assay (Murray et al., 1988a), however much smaller DMF values of 1.1 – 1.3 have been obtained for the DNA SSB induction end point in the same system. Reduction of the radiation induced phosphorylated histone H2AX (γH2AX) level by WR1065 has been observed in human endothelial cells in accordance with increasing clonogenic survival (Kataoka et al., 2007). The phosphorylation of the histone H2AX occurs in response to IR exposure in the regions of chromatin adjacent to the sites of radiation induced DNA DSB (Rogakou et al., 1998; Rogakou et al., 1999; Sedelnikova et al., 2003) and is considered as a marker for DNA DSB (Sedelnikova et al., 2002; Sedelnikova et al., 2003).

Amongst the different mechanisms that have been suggested for radioprotection by WR1065 and other aminothiols, the most likely are the scavenging of hydroxyl radicals, the chemical repair of DNA radicals and the depletion of oxygen (Purdie et al., 1983; Smoluk et al., 1988a). It has been demonstrated that the radioprotective ability of aminothiols is dependent on their positive charge (Aguilera et al., 1992; Zheng et al., 1992). This observation is attributed to the phenomenon of the counterion condensation that results in high local concentration of cationic aminothiols near DNA (Smoluk et al., 1988b). At neutral pH, the WR1065 molecule has a positive charge of +2 and therefore protects better than cysteamine with a charge of +1. Experiments with plasmid DNA demonstrated however, that radioprotection by aminothiols cannot be accounted solely by scavenging of hydroxyl radicals (Zheng et al., 1992). This follows from the fact that WR1065 protects DNA much better than cystamine (a disulfide form of cysteamine, chemical formula \( \text{H}_2\text{N-(CH}_2\text{)}_2\text{-S-S-(CH}_2\text{)}_2\text{-NH}_2 \)) which has the same positive charge and higher hydroxyl radical scavenging capacity (Zheng et al., 1992). Investigators comparing radioprotective effects of aminothiols on DNA damage endpoints, with clonogenic survival (Murray et al., 1988b; Aguilera et al., 1992) or repopulating crypt clonogens in the in vivo mouse jejunum model (Murray et al., 1988a), also conclude that the radioprotective mechanism is more complex than just scavenging of hydroxyl radicals. Studies aimed at investigating the role of chemical repair of DNA in radioprotection of V79 cells suggest that this becomes the dominant mechanism for aminothiols with increasing positive charge (Aguilera et al., 1992). The oxygen depletion hypothesis emerged from the studies of radioprotection in mouse skin by WR2721 under different oxygen tension that demonstrated decrease in radioprotection from a DMF of 1.95 in air, down to 1.1 and less, at 5% oxygen and less (Denekamp et al., 1982). This hypothesis has been further supported by the finding of the rapid oxygen consumption in cell culture medium following addition of WR1065 and WR2721 (Purdie et al., 1983). Cell culture studies with V79 cells have also indicated the decrease in radioprotection by WR1065 under hypoxia (DMF of 1.4) as compared to oxic conditions (DMF of 1.9) (Grdina et al., 1989).

With regard to clinical application, attention has focussed on WR2721 (amifostine, Ethyol), which is a phosphorylated form of the WR1065 (Figure 1). Amifostine has FDA approval for use as a radioprotector for a subgroup of patients undergoing radiation therapy. Following administration, amifostine is dephosphorylated by alkaline phosphatase to convert it to WR1065, which actually affords protection against IR. Amifostine has undergone extensive
testing as a potential adjuvant to radiotherapy and chemotherapy. The drug has been shown conclusively to have protective activity against both radiation and cisplatin induced toxicity without demonstrable protection of tumours (Wasserman, 1994). One randomised trial of amifostine in patients with inoperable, unresectable, or recurrent rectal cancers (Liu et al., 1992), showed a significant reduction in morbidity in the treated group. Despite these results, and those of subsequent clinical studies, including differing routes of administration, the drug has not found wide clinical acceptance in radiation oncology, because of its toxicity especially hypotension and severe malaise, and the requirement that it be administered systemically (with monitoring of blood pressure) before each radiation treatment. The topical application of WR2721 to rat colon (France et al., 1986) conferred substantial protection, namely a DMF of 1.8. Subsequent clinical trials, the most recent in 2008, employing amifostine doses up to 2 g in a 30 ml enema, did report some clinical benefit, especially for the higher of two doses (Simone et al., 2008). These results underline the low potency of this agent.

5. Radioprotection by methylproamine

5.1 Methylproamine as a DNA binding antioxidant

Methylproamine is a radioprotector, which belongs to a family of DNA minor groove binders featuring a common bi-benzimidazole structure (Figure 2). Two commercially available bi-benzimidazoles Hoechst 33258 and Hoechst 33342 are widely used as fluorescent DNA binding dyes.

![Chemical structure of bi-benzimidazoles](image-url)

Some protective activity against IR was initially discovered for Hoechst 33342 in cultured cells (Smith & Anderson, 1984; Young & Hill, 1989) and followed by reports of radioprotection of isolated DNA (Denison et al., 1992; Martin & Denison, 1992) and in vivo radioprotection of mouse lung (Martin et al., 1996) and brain (Lyubimova et al., 2001). New analogues of Hoechst 33342 were designed to improve the radioprotective activity, resulting in synthesis of more efficient compounds proamine (Figure 2) (Martin et al., 1996) and methylproamine (Figure 2) (Martin et al., 2004). Incubation of V79 Chinese Hamster cells in 30 μM of methylproamine before and during γ-irradiation increases clonogenic survival with a DMF of 2.1 (Martin et al., 2004). In vivo radioprotection by methylproamine has been demonstrated in mouse jejunum using the Withers assay with a DMF of 1.2 – 1.3.

Methylproamine, like other DNA binding bi-benzimidazoles, has a binding preference for AT-rich sequences, the consensus binding site being 3-4 consecutive AT base pairs as
established by footprinting (Harshman & Dervan, 1985) and affinity cleavage (Martin & Holmes, 1983; Martin et al., 1990; Murray & Martin, 1994) studies, and confirmed by X-ray crystallography studies (Martin et al., 2004).

5.2 Electron transport is involved in radioprotection by methylproamine

In the 70’s and 80’s considerable effort was devoted to the development of hypoxic cell radiosensitisers and it was well established that these agents were electron-affinic. From this dogma, that withdrawing electron density from DNA confers radiosensitivity, it can be inferred that increasing electron density in DNA would have radioprotective effect. It was this simple idea that guided the modification of Hoechst 33342 by the substitution of electron rich groups, and this improved radioprotective activity. Pulse radiolysis studies of methylproamine/DNA complexes provided further information on the movement of an electron from DNA-bound radioprotector to oxidising lesions on DNA (Martin & Anderson, 1998). In these studies, the spectral changes associated with oxidation of the DNA ligand were followed by time resolved spectrophotometry. Moreover, by studying the effect of the drug loading of DNA on the rate of oxidation of the ligand, the range of electron movement from the bound ligand to oxidising lesion could be estimated. The results indicated that for methylproamine, the maximum range was several base pairs (Martin & Anderson, 1998).

The phenomenon of electron donation as the basis for radioprotection also emerged from the studies of tyrosine containing peptides which have been modelled of naturally occurring nuclear proteins involved in the endogenous radioprotection (Tsoi et al., 2010). From consideration of studies of the chemical mechanism of radioprotection by thiols, the alternative mechanisms of H-atom donation and electron donation have been discussed. The close relationship between these two mechanisms of reduction is also invoked in the combination of electron and proton transfer in a concerted mechanism. Indeed this mechanism is well established in radiation chemistry of DNA (Kumar & Sevilla, 2010). These considerations have lead to the hypothesis that radioprotection by methylproamine involves repair of transient radiation induced species on DNA by electron donation from the DNA bound ligand. An alternative mechanistic concept would invoke the DNA bound ligand as the sink for “holes” produced in irradiated DNA. It is well established that radical cations produced on DNA by powerful oxidants move to the most easily oxidisable base pair, namely GC. The presence of DNA-bound methylproamine would constitute an alternative destination for the hole, thus reducing the yield of oxidised bases. A redox potential of 0.84 - 0.9 volt has been reported for Hoechst 33342 (Adhikary et al., 2000) so it is reasonable to assume that the redox potential for methylproamine is similar and therefore consistent with the hole trapping hypothesis.

5.3 DNA bound methylproamine is responsible for radioprotection of cells

The results of pulse radiolysis studies of methylproamine-DNA complexes indicated that intramolecular electron transfer from the ligand to radiation induced oxidising species on DNA is involved in the oxidation of methylproamine, and this process can be implicated in radioprotective activity of methylproamine (Martin & Anderson, 1998). Radioprotection in vivo however occurs in a different environment than reduction of oxidising species on DNA in pulse radiolysis experiments, and other processes may contribute to radioprotection in vivo such as for example scavenging of hydroxyl radicals by free methylproamine. To clarify
the role of DNA bound methylproamine in radioprotection we have undertaken extensive
studies of radioprotection using clonogenic survival of human cultured keratinocytes as an
endpoint (Lobachevsky et al., 2011). The dose response curves of clonogenic survival have
been established for FEP-1811 keratinocytes pre-incubated with various concentrations of
methylproamine from 0.5 to 10 μM for 30 min before irradiation with $^{137}$Cs $\gamma$-rays. The DMF
calculated for each survival curve has increased from 1.01 at 0.5 μM to 1.97 at 10 μM of
methylproamine.

In parallel experiments the uptake of methylproamine in cells and nuclei has been measured
by extracting the drug from nuclear and cellular pellets and measurement by liquid
chromatography. It was found that while the cellular uptake increased as a linear function
of methylproamine concentration (up to 4 fmole/cell at 10 μM of methylproamine), the
nuclear uptake indicated the presence of the major saturated and minor linear components.

Values of DMF obtained at various concentrations of methylproamine have been analysed
in conjunction with results of cellular and nuclear uptake studies (Lobachevsky et al.,
2011). Correlation has been studied between DMF values and each of the cellular uptake,
nuclear uptake and saturated and linear components of nuclear uptake. The best
correlation have been achieved for the total nuclear uptake of methylproamine ($R^2 = 0.97$)
and the least correlation for the cellular uptake ($R^2 = 0.87$). These results, along with the
finding that the majority of the nuclear methylproamine is in the DNA bound form, support the hypothesis that it is the DNA associated drug that is responsible for
radioprotection of cells.

5.4 Methylproamine reduces radiation induced DNA damage in cells

In addition to radioprotection at the clonogenic survival endpoint, the effect of
methylproamine on the induction by radiation of $\gamma$H2AX foci has been studied (Sprung et
al., 2010; Lobachevsky et al., 2011). $\gamma$H2AX can be detected microscopically using
immunofluorescence technique as a distinct focus that is associated with a DNA DSB
(Sedelnikova et al., 2002; Sedelnikova et al., 2003). The dose response curves of $\gamma$H2AX focus
number per cell were established following irradiation of FEP-1811 keratinocytes pre-
incubated with 20 μM of methylproamine for the time intervals of 1, 5 and 15 min before
irradiation with $^{137}$Cs $\gamma$-rays (Lobachevsky et al., 2011). The results have demonstrated the
reduction by methylproamine of the number of radiation induced $\gamma$H2AX foci. The extent of
this reduction is consistent with pre-incubation interval as indicated by DMF values.
of 1.4, 1.9 and 3.5 for 1, 5 and 15 min respectively. The efficient reduction of the number of radiation induced γH2AX foci by pre-incubation with methylproamine has been also demonstrated with three lymphoblast cell lines derived from the blood of the radiotherapy patients with different DNA repair capacity (Sprung et al., 2005; Sprung et al., 2008). In these experiments (Sprung et al., 2010), cells have been irradiated with $^{137}$Cs γ-rays following 15 min pre-incubation with 20 μM methylproamine. Radioprotection has been observed in all three cell lines including those obtained from a radiosensitive patient and with a defective DNA ligase IV that is critical for DNA DSB repair pathway. This finding demonstrates the ability of methylproamine to reduce the amount of radiation induced DNA damage.

To further investigate the effect of methylproamine on the radiation induced DNA damage in cells, the pulsed field gel electrophoresis (PFGE) assay has been exploited (Sprung et al., 2010). For this assay, lymphoblast cells have been irradiated with γ-ray doses of 20, 40 and 80 Gy in the presence or without 20 μM methylproamine. DNA was extracted from the cells, analysed on PFGE and the fraction of lower molecular weight DNA released from the wells has been quantified (Sprung et al., 2010). The results demonstrate substantial decrease of the low molecular weight fraction in all irradiated samples pre-treated with methylproamine as compared to the irradiated only samples thus indicating prevention by methylproamine of DNA fragmentation due to radiation induced DSB.

5.5 Methylproamine protects against breaks and base damage in plasmid DNA

A series of observations such as the role of DNA bound methylproamine in the radioprotection of cells, the requirement for methylproamine to be present in cells at the time of irradiation and demonstration that electron transport is involved in radioprotection support the hypothesis that the chemical reduction of transient radiation induced oxidative species on DNA by donation of an electron from methylproamine constitutes the main mechanism of radioprotection. This hypothesis however, in conjunction with the observation that methylproamine prevents formation of radiation induced DSB in cells, as demonstrated by pulsed field gel electrophoresis studies and the reduction of the yield of γH2AX foci, prompts the question of what are those oxidative DNA species that are reduced by methylproamine and how the chemical reduction can repair or prevent the formation of a DNA DSB. A further insight into the mechanisms of radioprotection can be obtained from investigation of DNA damage of isolated DNA using a plasmid model.

Plasmid DNA is a convenient tool to assay DNA strand breakage. It exploits conformational changes of the supercoiled plasmid to the relaxed open circle form following induction of a SSB and to the linear form following induction of a DSB (Freifelder & Trumbo, 1969; Cowan et al., 1987; Lobachevsky et al., 2004). Three plasmid forms can be separated using agarose gel electrophoresis and numbers of SSB and DSB calculated from fractions of the linear and relaxed forms (Cowan et al., 1987). Combination of the plasmid DNA breakage assay and treatment of DNA with base excision repair enzymes (endonucleases) that recognise various DNA base lesions and convert them to strand breaks allows quantitation of radiation induced base lesions (Milligan et al., 2000a). One of such enzymes is the endonuclease formamidopyrimidine-DNA N-glycosylase (FPG) from Escherichia coli (O’Connor & Laval, 1989). FPG recognises oxidised purines, in particular 8-oxoG (Chetsanga & Lindahl, 1979; Milligan et al., 2002) and possesses both glycosylase and endonuclease activity to excise the modified base and then produce a nick at this abasic site (O’Connor & Laval, 1989), thus converting the base damage to a SSB.
Early experiments with plasmid DNA model have demonstrated ability of methylproamine analogues Hoechst 33342 and Hoechst 33258 to protect DNA from radiation induced SSB (Denison et al., 1992; Martin & Denison, 1992) with a linear increase in the DMF from approximately 2 to more than 10 with the ligand concentration changing from 5 to 50 µM (Martin & Denison, 1992). Two modes of protection have been suggested: the site-specific and global protection. The site-specific protection occurs at the site of the ligand binding on DNA and has been suggested to involve direct block of the radical attack by the ligand occupying DNA minor groove and/or electron or H-atom transfer from the ligand to DNA. However, since only limited DNA regions (less than 20%) can by occupied by bound ligand, the site-specific protection can not account completely for the observed extent in the SSB reduction (up to DMF of 10). Therefore the idea of global protection has been suggested that implies radioprotection between ligand binding sites. Given that the linear relationship between the extent of radioprotection and the ligand concentration has been observed, the most likely mechanism of global protection detected in these experiments is the scavenging of hydroxyl radicals by free ligand in solution. This suggestion is supported by the much smaller extent of radioprotection by 25 µM of Hoechst 33258 in the presence of 100 mM mannitol that efficiently scavenges hydroxyl radicals: a DMF of 1.4 as compared to 9.6 for 25 µM of Hoechst 33258 in phosphate buffer (Martin & Denison, 1992), and the fact that majority of the ligand binding sites (94-100%) are occupied within the range of the studied concentrations of Hoechst 33258 (5 – 50 µM) indicating that the radioprotection by DNA bound ligand wouldn’t change significantly at this condition.

Although the site-specific protection by methylproamine analogues partially prevents formation of SSB, and the global protection by DNA bound ligand in cells can also potentially reduce the yield of SSB, it is unlikely that these mechanisms of radioprotection by methylproamine will affect the yield of DSB as to account for the observed radioprotection at the clonogenic survival and \( \gamma H2AX \) induction endpoints with DMF of 2 and more. A more likely mechanism is the chemical reduction by DNA bound methylproamine of the initial oxidative DNA lesion that results in base damage constituting a part of OCDL. Such OCDL represent a difficult challenge for the cellular DNA repair machinery and potentially can result in enzymatic DNA DSB. However, following reduction by methylproamine of the radical precursor of the lesion that otherwise would constitute a part of an OCDL, the formation of this OCDL can be prevented, thus preventing potential formation of an enzymatic DSB. The ability of methylproamine to reduce oxidative base lesions has also been demonstrated using plasmid DNA model.

In our experiments pBR322 plasmid DNA has been irradiated with \(^{137}\)Cs \( \gamma \)-rays in the PTP buffer (Figure 3) containing thiocyanate ions, as described by (Milligan et al., 2000b; Milligan et al., 2001). In this buffer, the thiocyanate ion (SCN\(^{-}\)) is the main scavenger of radiation induced hydroxyl radicals (HO\(^{\bullet}\)) that otherwise would be responsible for the induction of the majority of DNA breakage.

Interaction of SCN\(^{-}\) with HO\(^{\bullet}\) results in formation of highly reactive radical species SCN\(^{\bullet}\) and/or (SCN\(^{-}\))\(^{2\bullet}\) that are the major mediators of the radiation induced DNA damage in this buffer. In contrast to HO\(^{\bullet}\) radicals that are efficient in induction of DNA breaks, SCN\(^{\bullet}/(SCN)_{2}^{\bullet}\) radicals produce oxidative lesions of DNA bases. Guanine is considered as the most frequently damaged site oxidation of which results in formation of guanyl radical that eventually is converted mainly to 8-oxoG. Compared to HO\(^{\bullet}\) radicals, with a reduction potential (E) of 2.3 V, SCN\(^{\bullet}/(SCN)_{2}^{\bullet}\) are more moderate oxidants (E = 1.62/1.32 V), but
nevertheless powerful enough to oxidise guanines in DNA (E = 1.29 V). Thus irradiation in PTP buffer results in selective damage; primarily oxidation of guanine.

Fig. 3. Loss of supercoiled plasmid with increasing radiation dose. A solution of 7.5 μg/mL of pBR322 (11.4 μM bp) in a buffer containing 5 mM sodium phosphate, pH 7.0, 1 mM sodium thiocyanate and 110 mM sodium perchlorate (PTP buffer) has been irradiated with $^{137}$Cs $\gamma$-rays without (circles) or with 5 μM of methylproamine (squares). Irradiated samples were analysed by agarose gel electrophoresis to assay frank SSB (open symbols) or after treatment with FPG to assay total frank SSB and enzymatic SSB (base damage) (closed symbols).

The yield of FPG sensitive lesions (FPG enzymatic SSB) in pBR322 following irradiation in the thiocyanate buffer is more than 10-fold higher than the yield of frank SSB (Figure 3, Table 1).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Yield of SSB and BD $10^{-2}$ per plasmid per Gy</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frank SSB</td>
<td>BD (enzymatic SSB)</td>
</tr>
<tr>
<td>PTP</td>
<td>2.45 ± 0.05</td>
<td>31.8 ± 1.2</td>
</tr>
<tr>
<td>PTP+1.25 μM methylproamine</td>
<td>1.76 ± 0.03</td>
<td>3.16 ± 0.29</td>
</tr>
<tr>
<td>PTP+2.5 μM methylproamine</td>
<td>1.67 ± 0.01</td>
<td>2.45 ± 0.13</td>
</tr>
<tr>
<td>PTP+5 μM methylproamine</td>
<td>1.52 ± 0.01</td>
<td>2.08 ± 0.09</td>
</tr>
</tbody>
</table>

Table 1. Effect of methylproamine on the yield of frank and enzymatic SSB in irradiated pBR322 plasmid DNA.
Methylproamine at concentration as low as 1.25 µM protects plasmid DNA against enzymatic SSB with a DMF of 10 while against frank SSB with a moderate DMF of 1.4 (Table 1), thus demonstrating much higher extent of protection against base damage than against frank SSB. A possible candidate lesion for repair by methylproamine is a guanyl radical cation that results, if not repaired, in formation of 8-oxoG, a modified base that is recognised and converted to SSB by FPG. The most critical question with regard to mechanisms of such efficient protection against base damage is whether radioprotection is mediated by DNA bound or free methylproamine and is achieved via the reduction by methylproamine of oxidative lesions on DNA or scavenging SCN•/(SCN)2•- radicals in solutions that cause DNA lesions.

The results presented in Table 1 for radioprotection at 1.25 and 5 µM of methylproamine indicate a moderate decrease in the yield of base damage from 3.16x10⁻² to 2.08x10⁻² (34%) that resulted from 4-fold increase in methylproamine concentration. While addition of 1.25 µM methylproamine to PTP buffer prevents formation of 90% of base damage, second addition of 1.25 µM (from 1.25 to 2.5 µM) prevents formation of only 22% of the remaining base damage. It is important to note that the change in the fraction of pBR322 DNA binding sites occupied by methylproamine is minimal (estimated from 85 to 97% as methylproamine concentration changes from 1.25 to 2.5 µM). The results therefore are consistent with the hypothesis that the radioprotection against base damage is mainly mediated by DNA bound methylproamine. In general, these results demonstrate the ability of methylproamine to protect against radiation induced base damage and therefore support the hypothesis that reduction of the oxidative DNA lesions by methylproamine accounts for radioprotection of cells.

6. Cytotoxicity of DNA binding ligands

The strong high affinity binding of bibenzimidazoles in the minor groove of DNA is a factor that determines the high radioprotective potency of methylproamine: substantial radioprotection of clonogenic survival is achieved at concentrations as low as a few µM in cell culture medium (a DMF 1.6 at 2 µM) (Lobachevsky et al., 2011), and at even lower concentrations for base damage in the plasmid model (a DMF of 10 at 1.25 µM) (Table 1). On the other hand, it is logical to expect that the tightly DNA associated bisbenzimidazole molecule will interfere with normal DNA metabolic processes such as replication, transcription, repair etc and such an interaction may result in adverse cytotoxic and mutagenic effects. While no effect of methylproamine on the clonogenic survival of human keratinocytes has been detected following 60 min incubation with 10 µM of the drug, at 20 µM of methylproamine the clonogenic survival has been reduced to 80% (Lobachevsky et al., 2011). Mechanisms of this cytotoxicity have not been fully investigated. The cytotoxicity of Hoechst 33342 has prompted consideration and further development of bibenzimidazoles as potential antitumour agents (Baraldi et al., 2004). Inhibition of DNA synthesis has been demonstrated in V79 cells exposed to 5 and 10 µM of Hoechst 33342. This inhibition results in substantial changes in the progression of cells through cell cycle as manifested by appearance of increased S-phase population and S/G₂ block (Durand & Olive, 1982). One of the potential mechanisms of the Hoechst 33342 cytotoxicity is its interaction with topoisomerase I. Inhibition of the topoisomerase I activity by Hoechst 33342 and 33258 has been demonstrated using both the relaxation and cleavage assays (Chen et al., 1993). The
inhibition activity of the Hoechst ligands in the relaxation assay might indicate their interaction with the binding of topoisomerase I with DNA. In the cleavage assay, a DNA SSB is induced that is attributed to the trapping by Hoechst ligands of topoisomerase I cleavable complexes (Chen et al., 1993; Bailly, 2000).

7. Conclusion

Given the central role of DNA as radiobiological target, the design of radioprotectors which bind to DNA is an obvious strategy, however neither of the DNA binding radioprotectors discussed in this paper arose from such a deliberate design plan. WR1065 and amifostine emerged from an empirical drug development program, and the association between radioprotective efficacy and DNA binding only became evident in retrospect. In the case of methylproamine, derived from a minor groove DNA binding ligand developed for an entirely different purpose, and found to have a serendipitous radioprotective activity, incorporation electron-rich substituents has improved radioprotective activity compared to Hoechst 33342. No doubt this rational thread might be followed more explicitly in the design of future radioprotectors, but it remains to be seen whether it is possible to design DNA-binding radioprotectors that are devoid of any toxicity derived from the DNA-binding property.

8. Acknowledgement

This work was supported by licensing agreement between Sirtex Medical Inc and Peter MacCallum Cancer Centre and by the Intramural Research Program of the National Cancer Institute, National Institutes of Health.

9. References


www.intechopen.com


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

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

© 2011 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.