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New molecular targets in radiotherapy: DNA damage signalling and repair in targeted and non-targeted cells

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Abstract

Ionising radiation plays a key role in therapy due to its ability to directly induce DNA damage, in particular DNA double-strand breaks leading to cell death. Cells have multiple repair pathways which attempt to maintain genomic stability. DNA repair proteins have become key targets for therapy, using small molecule inhibitors, in combination with radiation and or chemotherapeutic agents as a means of enhancing cell killing. Significant advances in our understanding of the response of cells to radiation exposures has come from the observation of non-targeted effects where cells respond via mechanisms other than those which are a direct consequence of energy-dependent DNA damage. Typical of these is bystander signalling where cells respond to the fact that their neighbours have been irradiated. Bystander cells show a DNA damage response which is distinct from directly irradiated cells. In bystander cells, ATM- and Rad3-related (ATR) protein kinase-dependent signalling in response to stalled replication forks is an early event in the DNA damage response. The ATM protein kinase is activated downstream of ATR in bystander cells. This offers the potential for differential approaches for the modulation of bystander and direct effects with repair inhibitors which may impact on the response of tumours and on the protection of normal tissues during radiotherapy.

Keywords

DNA damage; radiotherapy; bystander effects; DNA repair

1. Introduction – Ionising radiation induced DNA damage and repair

The use of ionising radiation in radiotherapy is based around the principle of direct radiation exposure leading to critical DNA damage which ultimately results in tumour cell death. Over 50 years of research has mapped out the pathways which sense DNA damage within cells leading to downstream activation of repair processes. Ionising radiation is unlike many other genotoxic agents in that the yields and distributions of DNA damage are highly complex. For example, multiple types of lesions can be induced which include various types of base damage (up to 20 have been characterised), breaks in the helix either as single-(ssb) or double-strand breaks (dsb) and covalent bond formation leading to DNA-protein crosslinks and DNA-DNA crosslinks. The yields of these individual lesions also vary for a typical radiation dose of 1Gy from around 3000 base damages to around 30-40 dsb. Another significant factor is that this damage is not uniformly distributed throughout the genome after exposures. Radiation deposits its energy via “radiation tracks” where energy deposition

events are localised to each other as these tracks cross a cell nucleus, leading to clustering of lesions on the DNA within 0-20 bp. The degree of clustering is determined by the ionisation density or the radiation quality with more densely ionising radiation such as neutrons and alpha-particles producing more clustered damage than sparsely ionising radiation such as X-rays and γ -rays. Of these lesions it is thought that DNA dsb are highly toxic to cells alongside some classes of clustered lesions, as they are more difficult to repair (Goodhead, 1994).

Cells have evolved a battery of repair pathways for maintaining genomic stability. For ionising radiation two main pathways exist to repair dsb, non-homologous end-joining and homologous recombination (see figure 1). Non-homologous end-joining is the main pathway by which cells repair ionising radiation damage as it does not require a template for repair and involves some limited processing of the damaged ends prior to re-ligation of the dsb. Key components involve the Ku heterodimer which binds to the dsb ends and enables recruitment of a range of proteins starting with DNA PKcs to form DNA-PK and others including Artemis with finally DNA ligase IV binding to seal the break. The non-homologous end-joining pathway is sometimes referred to as being error prone due to the lack of template. The alternative homologous recombination pathway is active during S and G2-phase as it allows an error free repair using a sister chromatid as a template. This involves an excision repair type process with strand invasion into the sister chromatid, involving multiple proteins including Rad51, BRCA2 and Rad54.

DNA dsb can be repaired by either HR or non-homologous end-joining, however it is also known that if damage accumulates at S-phase during replication that stalled replication forks can be produced leading to the indirect production of dsb (Lobrich and Jeggo, 2007).

The other key pathway used for repairing ionising radiation damage is the base excision repair pathway. This can repair damaged bases and also ssb using the undamaged strand of the DNA helix as a template.

2. Therapeutic application of small molecular inhibitors of DNA repair and cell cycle control proteins

DNA damage repair and cell cycle control proteins have recently attracted attention as molecular drug targets for the treatment of malignant tumours in combination with chemotherapy and radiotherapy. The overarching rationale is the induction of DNA damage by chemotherapeutic agents or ionising radiation in conjunction with the inhibition of DNA damage repair. Malignant tumours frequently consist of rapidly proliferating cells where the combination of DNA damage induction and interference with its adequate repair will subsequently result in increased cell death.

A number of small molecular inhibitors of proteins involved in DNA repair and cell cycle control are currently being developed including small molecular inhibitors of poly-adenosine diphosphate-ribose polymerase -1 (PARP-1), DNA -dependent protein kinase (DNA-PK), ataxia-telangiectasia mutated (ATM) protein kinase and Chk1.

The pre-clinical evaluation of PARP-1 inhibitors for therapeutic use, like AG14361, AG14447, AG014699, and CEP-6800 (Curtin et al., 2004; Miknyoczki et al., 2003; Thomas et al., 2007), is most advanced, and selected drugs have already entered clinical trials (Plummer, 2006; Plummer et al., 2008). Although mechanisms of PARP-1 inhibitor interactions with DNA damage repair processes are not entirely understood yet, pre-clinical evaluation has provided evidence for their use to potentate both chemotherapeutic agents and radiotherapy (Albert et al., 2007; Calabrese et al., 2004; Drew and Calvert, 2008), and

also as single agents in BRCA related cancers (Jones and Plummer, 2008; Lord and Ashworth, 2008). Several studies have contributed to the appreciation of the role of PARP-1 in DNA repair processes (reviewed in (Helleday et al., 2005)). PARP-1 binds to DNA ssb and dsb and attracts ssb repair proteins. Notably, PARP-1 is involved in a backup dsb repair pathway but is not required for homologous recombination (HR). PARP-1 is important in maintaining genomic stability and increased sister chromatid exchange was observed in the absence of PARP-1 activity. This was explained by remaining unrepaired endogenous ssb leading to the collapse of replication forks which triggers recombination repair. In normal cells, the collapsed forks do not result in genetic instability as homologous recombination is an error-free repair pathway, but cells with defects in homologous recombination, e.g. BRCA mutant cells, are susceptible to PARP-1 inhibition (Farmer et al., 2005; Lord and Ashworth, 2008; McCabe et al., 2006), and PARP-1 inhibitors have been proposed as single or combination treatment in BRCA1/2 mutant tumours like breast and ovarian cancer (Drew and Calvert, 2008; Farmer et al., 2005). A most recent phase 1 trial with the PARP-1 inhibitor Olaparib (AZD2281) confirmed antitumor activity in cancer associated with the BRCA1 or BRCA2 mutation (Fong et al., 2009).

Pharmacological inhibitors of the ATM protein kinase have also been proposed as radiosensitising agents since the inherited ataxia-telangiectasia (A-T) syndrome results in a profound hypersensitivity to ionising radiation (Cowell et al., 2005; Hickson et al., 2004; Hollick et al., 2007; Rainey et al., 2008; Sarkaria and Eshleman, 2001). KU-55933 is a novel, specific, and potent inhibitor of the ATM kinase which significantly sensitises cells to the cytotoxic effects of ionising radiation and to DNA dsb-inducing chemotherapeutic agents in pre-clinical evaluation (Hickson et al., 2004). The suitability of another non-toxic, specific and rapidly reversible ATM inhibitor CP466722 for the purpose of radiosensitisation has been reported most recently (Rainey et al., 2008).

DNA-PK was identified as another promising target for radiosensitisation. The DNA-PK inhibitor NU7441 has been evaluated in pre-clinical studies which demonstrate sufficient proof of principle through *in vitro* and *in vivo* chemosensitisation and radiosensitisation to justify further development of DNA-PK inhibitors for clinical use (Nutley et al., 2005; Veuger et al., 2003; Veuger et al., 2004; Willmore et al., 2004; Zhao et al., 2006).

The cell cycle checkpoint kinase Chk1 is another promising molecular target to enhance the cytotoxic effects of radiotherapy and chemotherapy in the treatment of certain cancers. Chk1 plays a major role in mediating S- and G2-arrest in response to DNA-damage. Inhibition of Chk1 enhances the cytotoxicity of DNA-damaging agents like ionising radiation through abrogation of these cell-cycle checkpoints. Convincing preclinical studies demonstrating radio- and chemosensitisation (Mack et al., 2004; Ree et al., 2004) have fuelled the development of a range of pharmacological Chk1 inhibitors (reviewed in (Tse et al., 2007)). UCN01 is a non-selective Chk1 inhibitor that has already been applied in clinical trials (Phase I/II) (Hotte et al., 2006; Welch et al., 2007), but pharmacokinetic data was unfavourable (Lara et al., 2005). Novel compounds have been developed since and entered first clinical trials, e.g. 17AAG (Phase I) and XL884 (Phase I) (Tse et al., 2007). CEP-3891 (Syljuasen et al., 2004), PF-00477736 (Blasina et al., 2008), isogranulatimide (Jiang et al., 2004) and AZD7762 (Zabludoff et al., 2008) are further examples of recently developed Chk1 inhibitors that undergo preclinical tests.

3. Understanding DNA damage and repair processes in bystander cells

In contrast to DNA damage induced by direct irradiation, bystander cell DNA damage is still poorly understood. The concept of signalling networks between irradiated cells and neighbouring non-irradiated cells resulting in the induction of DNA and chromosomal

damage in non-irradiated cells, the so-called bystander effect, is relatively new and its potential role in cancer therapy has recently been discussed (Prise and O'Sullivan, 2009). Early events of the radiation induced bystander effect are rapid calcium fluxes and generation of reactive oxygen species in bystander cells (Azzam et al., 2002; Shao et al., 2006). Mitochondrial functions and mitochondria-dependent signalling seem to play a central role in bystander signalling (Chen et al., 2008; Tartier et al., 2007) and the relation between mitochondrial calcium signalling and reactive oxygen species production has previously been reviewed (Camello-Almaraz et al., 2006).

Several endpoints of studies on radiation-induced bystander effects are indicators of DNA and chromosomal damage in non-targeted cells. The induction of micronuclei (Azzam et al., 2002; Prise et al., 1998; Shao et al., 2005), γ H2AX foci (Burdak-Rothkamm et al., 2007; Hu et al., 2006; Smilenov et al., 2006; Sokolov et al., 2005) and sister chromatid exchange (SCE) (Nagasawa et al., 2002; Nagasawa et al., 2005) in bystander cells clearly demonstrates the induction of DNA damage although the exact type of the initial damage is still a focus of investigation.

Bystander studies in double strand break repair (non-homologous end-joining) deficient cells (CHO xrs-5) showed marked induction of micronuclei (Kashino et al., 2004) and HPRT mutations (Nagasawa et al., 2003). Similar studies in p53 wild-type (TK6), p53 null (NH32), and p53 mutant (WTK1) lymphoblastoid cells using siRNA to knock down DNA PKcs confirmed a role of non-homologous end-joining in processing damage leading to increased mutation induction at the thymidine kinase locus in bystander cells. In contrast, knockdown of Rad54, a component of homologous recombination, had no impact on the mutation yield in bystander cells (Zhang et al., 2008). Homologous recombination is essential for the induction of sister chromatid exchanges in bystander cells, presumably through the contribution of Brca2 and the Rad51 paralogs to DNA damage repair processes induced in bystander cells which is thought to be via oxidative damage repair in S-phase cells (Nagasawa et al., 2008). However, homologous recombination is unable to repair the DNA damage induced in non-homologous end-joining -deficient bystander cells that leads to either sister chromatid exchanges or chromosomal aberrations (Nagasawa et al., 2005). From the study of repair deficient cell lines it is concluded that the repair phenotype of the cell receiving the bystander signal determines the overall response rather than that of the cell producing the bystander signal (Kashino et al., 2007).

Furthermore, RPA and apurinic/apyrimidinic endonuclease (APE, a key enzyme of the base excision repair pathway) showed enhanced expression in bystander cells which was attributed to a combination of DNA strand breaks and oxidized base lesions in the genomic DNA of bystander cells (Balajee et al., 2004).

Our recent studies into DNA damage signalling in cells receiving bystander signals have revealed a central role for the ATM- and Rad3-related (ATR) protein kinase. The induction of γ H2AX foci in bystander cells depended on ATR function and bystander γ H2AX foci predominantly occurred in S-phase cells (Burdak-Rothkamm et al., 2007). Similar results were obtained for bystander 53BP1 foci induction. Furthermore, ATM was activated in an ATR-dependent manner and was essential for the decrease in clonogenic survival of bystander cells. Either ATR or ATM deficiency led to the abrogation of bystander cell killing although the radiosensitivity of directly irradiated cells was increased. In contrast, DNA-PK function is not essential for bystander cell killing or bystander γ H2AX foci induction. These observations provide a rationale for the differential modulation of targeted and non-targeted effects of radiation which could be exploited for the benefit of radiotherapy treatment regimes (Burdak-Rothkamm et al., 2008).

4. Differential modulation of targeted and non-targeted effects of ionising radiation by DNA repair inhibitors

As reviewed above, inhibitors of PARP-1, ATM, DNA-PK and Chk1 enhance the cytotoxic effect of ionising radiation. Previous *in vitro* studies focused on directly targeted cells where radiosensitisation was demonstrated, e.g. (Hickson et al., 2004; Mack et al., 2004; Noel et al., 2006; Veuger et al., 2003). Although targeted effects are probably most important for acute cytotoxicity of ionising radiation, a growing number of studies could demonstrate DNA and chromosome damage and reduced cell survival in non-irradiated bystander cells. Interestingly, the DNA damage in bystander cells seems to persist for a prolonged time (Burdak-Rothkamm et al., 2007) whereas DNA damage induced by direct irradiation is repaired completely within several hours depending on the radiation dose. Taking into account recent reports of the involvement of cytokines in the propagation of bystander effects (Shao et al., 2008; Shareef et al., 2007), it seems plausible that the radiation-induced bystander effect could trigger a systemic response affecting tissues away from the irradiated field, which was confirmed in recent studies (Koturbash et al., 2007; Koturbash et al., 2006). Therefore, bystander effects may be important for normal tissue reaction in radiotherapy treatment. Furthermore, DNA repair processes in bystander cells appear to be complex and may be triggered in S-phase when DNA damage interferes with replication fork progression (Burdak-Rothkamm et al., 2007; Nagasawa et al., 2008). The recent observations of the dependency of bystander γ H2AX foci induction on ATR function but not on ATM or DNA-PK function (Burdak-Rothkamm et al., 2007) and dependency of bystander cell killing on ATR/ATM function (Burdak-Rothkamm et al., 2008) suggest potential targets for the modulation of bystander DNA damage. As it is desirable to protect bystander normal tissue in radiotherapy treatment, it is also important to further investigate the fate of cells rescued from bystander cell killing by ATR/ATM inhibition. The initial bystander DNA damage presumably is present in the rescued cells and may lead to genomic instability with an increased risk of (secondary) cancer (Morgan, 2003; Mothersill and Seymour, 2003). In contrast, the DNA-PK inhibitor NU7021 did not abrogate bystander cell killing (Burdak-Rothkamm et al., 2008) which is in keeping with previous observations of an increased bystander responses in non-homologous end-joining deficient xrs-5 CHO cells (Nagasawa and Little, 2002). Therefore, a DNA-PK inhibitor has the potential to radiosensitise both targeted and non-targeted cells.

No experimental data is available yet on the effect of PARP-1 or Chk1 inhibitors on bystander responses. The importance of PARP-1 in S-phase related DNA repair (Bryant and Helleday, 2006; Helleday et al., 2005) supports the hypothesis of a similar effect of PARP-1 inhibitors on bystander responses as ATR mutation, i.e. protection from cell death. Chk1 is a direct downstream target of ATR; its inhibition may therefore have a similar effect as ATR mutation on the bystander response. As both PARP-1 inhibitors and Chk1 inhibitors are advancing into clinical trials, it will be a key aim to establish their effect on bystander responses.

5. Conclusions

Our understanding of how radiation interacts with cells is evolving from one where direct radiation exposure of DNA within the cell nucleus drives response to one where cell to cell communication and *in vivo*, longer range bystander signalling also plays a role. Recent studies suggest that there are important differences between the DNA damage response in directly irradiated cells and non-targeted effects mediated via bystander signals. This may offer some novel routes for differential modulation of direct and bystander effects.

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Figure 1.

Depiction of the two main processes for repairing DNA dsb in mammalian cells. In NHEJ, the break is processed and rejoined with the possibility of sequence information being lost and incorrect repair. With HR an intact homolog is used as a template to ensure correct error free repair.

Table 1
Molecular inhibitors of DNA repair and cell cycle related proteins

Drug	Targeted protein	Molecular pathways	Trial phase	References
AG14361	PARP-1	S-phase related DNA repair	Pre-clinical	(Calabrese et al., 2004; Curtin et al., 2004)
AG14447	PARP-1	S-phase related DNA repair	Pre-clinical	(Thomas et al., 2007)
AG014699	PARP-1	S-phase related DNA repair	Pre-clinical	(Plummer et al., 2008)
CEP-6800	PARP-1	S-phase related DNA repair	Pre-clinical	(Miknyoczki et al., 2003)
AZD2281 (Olaparib)	PARP-1	S-phase related DNA repair	Phase 1	(Fong et al., 2009)
KU-55933	ATM	Cell cycle checkpoint signalling, phosphorylation of DNA repair proteins	Pre-clinical	(Hickson et al., 2004)
CP466722	ATM	Cell cycle checkpoint signalling, phosphorylation of DNA repair proteins	Pre-clinical	(Rainey et al., 2008)
NU7441	DNA-PK	Non-homologous end-joining	Pre-clinical	(Nutley et al., 2005; Veuger et al., 2003; Veuger et al., 2004; Willmore et al., 2004; Zhao et al., 2006)
UCN01	Chk-1	S- and G2-arrest in response to DNA-damage	Phase 1/2	(Hotte et al., 2006; Welch et al., 2007)
17AAG	Chk-1	S- and G2-arrest in response to DNA-damage	Phase 1	(Tse et al., 2007)
XL884	Chk-1	S- and G2-arrest in response to DNA-damage	Phase 1	(Tse et al., 2007)
CEP-3891	Chk-1	S- and G2-arrest in response to DNA-damage	Pre-clinical	(Syljuasen et al., 2004)
PF-00477736	Chk-1	S- and G2-arrest in response to DNA-damage	Pre-clinical	(Blasina et al., 2008)
Isogranulatimide	Chk-1	S- and G2-arrest in response to DNA-damage	Pre-clinical	(Jiang et al., 2004)
AZD7762	Chk-1	S- and G2-arrest in response to DNA-damage	Pre-clinical	(Zabludoff et al., 2008)