Targeting the Double-Strand DNA Break Repair Pathway as a Therapeutic Strategy

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Abstract DNA repair pathways are crucial for the maintenance of genome integrity. The pathway that repairs DNA double-strand breaks (DSB) has components involved in both signaling and repairing DNA damage. Impairing DSB repair using specific inhibitors of signaling or repair might, in principle, sensitize tumor cells to particular DNA-damaging agents. Moreover, the existence of specific defects in DNA repair pathways in tumors provides the rationale for the use of “synthetic lethal” approaches targeting this cellular “Achilles’ heel.” Here, we discuss the mechanisms involved in DSB repair and detail potential therapeutic approaches based on targeting this pathway.

The human genome must resist relentless attacks on its integrity, and as a consequence, eukaryotic cells possess a range of distinct mechanisms to cope with the various forms of DNA damage that may occur (1). DNA double-strand breaks (DSB) can be particularly problematic; modest numbers of DSBs caused by ionizing radiation can be lethal. Furthermore, the inaccurate repair of DSBs can result in chromosomal rearrangements and aneuploidy, both candidate mechanisms for initiating carcinogenesis (2).

DSBs arise via diverse mechanisms, and these differ in the complexity of damage caused (3). An exogenous source, such as ionizing radiation, can create multiple clusters of DSBs, whereas endogenous damage, such as that produced by reactive oxygen species (which arise metabolically), cause fewer and less complex but nevertheless lethal lesions. DSBs may also occur from closely spaced single-strand breaks (SSB) or when a replication fork encounters a single-strand lesion, such as a break or reactive oxygen species—induced damage. Additionally, endonucleases, such as those involved in processes, such as meiosis and V(D)J recombination, also generate site-specific DSBs as part of the normal functioning of the cell (3).

Mammalian cells mount a coordinated response to DSBs with the aim of appropriately repairing DNA damage so that a minimal mutational load is passed to daughter cells (1). Not only does this response encompass the core machinery that physically repairs the DNA damage but also pathways that arrest the cell cycle at appropriate points (checkpoints), while repair occurs. In addition, the DSB response can also stimulate cell death or senescence if the damage caused is assessed to be too great for the cell to deal with. Conventionally, this response has been divided into those components that transduce or signal the response (transducer molecules) and those that perform the repair of DSBs (effector molecules). Now, however, it seems likely that this artificial subdivision is too simplistic, and that many proteins involved in the DSB response have both signaling and effecter functions (4).

Signaling the Cellular Response to DSBs

Two kinases from the phosphatidylinositol 3-kinase–like kinase family, ataxia-telangiectasia mutated (ATM), and ATM and RAD3-related (ATR) are at the heart of the cellular response to DSBs. ATM is activated when it is recruited to sites of DSB damage by the MRE11-RAD50-NBS1 (MRN) complex (5–7). Conversely, ATR is activated and recruited to the sites of DSBs by the binding of replication protein A (RPA) to ssDNA that accumulates at these sites (5, 6), although recent work suggests a novel mechanism of ATR activation that is independent of its localization to the DSB (8). It is generally perceived that ATR activation is restricted to DSBs formed as the result of stalled replication forks, whereas ATM responds to DSBs resulting from other types of DNA damage (3). However, this distinction is likely to be too rigid as ATR and ATM activation have both been shown to be involved in the response to ionizing radiation exposure, with ATR acting downstream of ATM activation, emphasizing the potential crosstalk between the activities of these two kinases (9).

When activated, ATM and ATR phosphorylate a multitude of proteins, which initiate a cascade inducing cell cycle arrest and facilitating DNA repair (4, 10). Substrates of activated ATM include P53, the kinases CHK1 and CHK2, the Fanconi anemia protein FANCD2, SMC1 (part of the cohesin multi-protein complex involved in sister chromatid cohesion), the DNA helicase BLM1, BRCA1, the histone H2AX, the replication fork-associated MCM proteins, and the MRN complex itself. Similarly, ATR phosphorylates CHK1, BRCA1, and BLM. These proteins act as “transducers,” affecting apoptosis (P53 and CHK2), DNA repair (BLM, BRCA1, H2AX, CHK1, CHK2,
MRN, and SMC1), cell cycle arrest (P53, FANCD2, SMC1, CHK1, and CHK2), and DNA replication (MCM; reviewed by refs. 4, 10).

It is vital that cell cycle progression is arrested, while DSB repair occurs, and two of the most notable mediators of this process are the kinases CHK1 and CHK2 (10–12). As a substrate of both ATM and ATR, CHK1 is a key player in the DSB repair pathway, and its major function is in arresting cell cycle at the G2/M and S phase checkpoints. After activation by phosphorylation at Ser345 and Ser317, CHK1 is able to phosphorylate the CDC25A phosphatase (Fig. 1). This modification results in CDC25A ubiquitination and subsequent degradation. In its nonubiquitinated state, CDC25A would otherwise dephosphorylate and activate the cyclin-dependent kinases CDK1 and CDK2. This would allow progression through the S phase checkpoint and on towards mitosis, which in a cell harboring a DSB could have catastrophic consequences. CHK1 also phosphorylates CDC25C at Ser216, leading to its cytoplasmic sequestration by the 14-3-3 protein and preventing this phosphatase from acting on CDK1. This results in arrest at the G2/M checkpoint. CHK1 may also have a bearing on other aspects of DSB repair as CHK1 function is essential for the maintenance of stalled replication forks, structures that can often degenerate into DSBs (10–12).

Like CHK1, CHK2 is a multifunctional serine/threonine kinase, which is activated in response DNA damage. In this case, however, activation is through ATM-mediated phosphorylation of Thr68 on CHK2. This leads to oligomerization of CHK2 monomers and trans-phosphorylation at Thr383 and Thr387, resulting in kinase activation. CHK2 has a number of substrates, which when phosphorylated mediate both cell cycle arrest and apoptotic responses to DNA damage. Like CHK1, CHK2 can phosphorylate both CDC25A and CDC25C, leading to both S and G2-M phase arrests. In addition, CHK2 can regulate apoptosis in response to DNA damage through phosphorylation of PML, P53, and E2F-1 (10, 13).

**Mechanisms of DSB Repair**

Coincident with cell cycle arrest, the core machinery of the DSB repair pathway is activated. In mammalian cells, two principal pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR) are involved in processing DSBs (ref. 1; Fig. 1). NHEJ is the major route for DSB repair in the G0/G1 phases of the cell cycle and involves the alignment and ligation of DSB termini. In this process, the KU70 and KU80 proteins form a heterodimer that binds to the ends of a DSB and then recruits DNA-dependent protein kinase (DNA-PKcs), another member of the phosphatidylinositol 3-kinase-like kinase family. DNA-PKcs, in turn, recruits and activates another protein (Artemis) that is responsible for processing DNA ends before XRCC4 and DNA ligase IV along with the newly identified component XLF/Cernunnos (14–16) facilitate the final ligation step. DNA residues lost at the site of the DSB are generally not restored and consequently NHEJ can be mutagenic. HR, which is favored during S phase and G2, can be further subdivided into two distinct mechanisms, gene conversion (GC), which is conservative, and single-strand annealing (SSA), which is nonconservative. Gene conversion uses an identical sequence, normally the sister chromatid, as a template to copy and replace damaged DNA. In contrast, during SSA, homologous sequences on either side of the DSB are aligned...
followed by the deletion of the intermediate noncomplemental sequence (Fig. 1). In complex genomes, these homologous sequences are likely to be repetitive elements. Both GC and SSA are dependent on BRCA1 function (17), and this protein apparently serves multiple functions, including recruitment of additional DNA repair proteins to the DSB, regulation of DNA resection by the MRN complex, and also control of cell cycle checkpoint regulatory proteins (18).

In the process of gene conversion, single-stranded 3’ overhangs are generated at the DSB by the MRN complex. This ssDNA is then bound by RPA, which is later displaced by RAD51. To enable this displacement, RAD51 is localized to the 3’ overhang by the BRCA2 protein, which binds RAD51 via eight evolutionarily conserved binding domains, known as the BRC repeats. The RAD51 nucleo-protein filament then catalyzes the search for homologous target sequences, invades the sister chromatid at the site of homology, and then initiates DNA synthesis, using the sister chromatid as template. The final gaps at the end of the newly synthesized sequence are then ligated, and the process is completed by resolvases, which remove the links between sister chromatids (19).

It is vital that the core activities of cell cycle arrest and physical DNA repair are integrated when DSBs are processed. Recently, a model has been proposed by Jazayeri et al. (9), describing the crosstalk between ATM and ATR activation depending on the stage of the cell cycle, which may direct the type of DNA repair used and the stage at which cell cycle arrest occurs. When DSBs are detected in the S and G2 phases of the cell cycle, ATM is activated, which in turn activates the exonuclease activity of the MRN complex. This activity processes DNA at the DSB such that a stretch of ssDNA is produced. RPA coats this ssDNA, which in turn leads to ATR recruitment and activation. Activated ATR phosphorylates CHK1, which in turn phosphorylates and activates the recombinase protein RAD51 (20). RAD51 is directly involved in the gene conversion pathway that uses RPA-coated ssDNA as a substrate to initiate DSB repair. At the same time, ATM phosphorylates CHK2, which along with activated CHK1 causes cause cell cycle arrest as described above. Conversely in the G1 phase of the cell cycle, ATM is still activated but has minimal effect on the MRN complex. As minimal exonuclease processing of the DSB occurs, ATR is not activated, nor CHK1 and the absence of RAD51 phosphorylation and MRN processing means that gene conversion is not possible, leaving the way open for NHEJ to predominate as the method of DSB repair. In this phase of the cell cycle, ATM still phosphorylates CHK2, which stalls the cell cycle by causing cell cycle arrest (9).

An additional CHK2 substrate is the BRCA1 protein. BRCA1 is phosphorylated on Ser988 in response to DNA damage, in a CHK2-dependent manner. Mutation of this residue to alanine in the G1 phase of the cell cycle, ATM is still activated but has significant effect on ATM activity (23). At micromolar concentrations, KU55933 is able to inhibit ATM (IC50 = 12.9 nmol/L), reduce the phosphorylation of a range of ATM substrates (P53, NBS1, H2AX, and SMC1), and radiosensitize and chemosensitize cells to ionizing radiation, etoposide, doxorubicin, and camptothecin. The specificity and efficacy of KU55933 illustrates the potential of ATM inhibition, which was only previously showed using nonspecific phosphatidylinositol 3-kinase–like kinase inhibitors, such as wortmannin and caffeine, which are both too toxic for clinical exploitation at doses with significant effect on ATM activity (23). As another member of the phosphatidylinositol 3-kinase–like kinase family, DNA-PKcs has also been proposed as a therapeutic target, primarily based on its role in NHEJ and with a similar rationale to ATM inhibition; cells deficient in DNA-PKcs are radiosensitive, hypersensitive to DNA cross-linking agents, and defective in DSB repair. Several small-molecule inhibitors of DNA-PKcs have been developed, including Vanillin, Salvicin, OK-1035, LY294002, NU7026, NU7441, IC87102, and IC87361 (24, 25). These compounds cause radiosensitization and chemosensitization in both in vitro cell models and tumor xenograft in vivo models.

### CHK1 and CHK2 Kinases as Drug Targets

Both biochemical and genetic evidence suggest that CHK1 is a key mediator of both S and G2/M checkpoints. In P53-deficient cells, incapable of sustained G1 arrest, loss of CHK1 function has also been shown to sensitize to DNA damage. This has led to the therapeutic rationale that inhibition of CHK1 in P53-deficient cancer cells will sensitize these cells to genotoxic cancer agents, and indeed, this seems to be the case. The emerging role of CHK1 in the repair of replication-induced DSBs (20) suggests a second therapeutic strategy for the use of CHK1 inhibitors. In this context, loss of CHK1 function in combination with genotoxic therapeutic agents would allow the generation of lethal DNA lesions because of compromised HR repair.

A number of CHK1 inhibitors have been reported, many of which are structurally related to the multi-kinase inhibitor UCN-01, which has been shown to abrogate a G2/M checkpoint. These compounds include Go6976, isoguanulamide, and SB-218078 (26). However, their selectivity for CHK1 versus CHK2 is unclear. Recently, however, there have been a number of reports of CHK1 selective inhibitors. The first of these (CEP-3891) is a potent (IC50 = 4 nmol/L) CHK1 inhibitor. This compound can rescue DNA damage–induced degradation of CDC25A and cause premature mitotic entry in response to ionizing radiation (27, 28). The other two new structural classes of CHK1 inhibitor are ureas and indolinoles, both of which possess potent inhibitory activity (IC50 ≤ 10 nmol/L) towards the CHK1 enzyme (29, 30). The ureas in particular are of interest as an example of this structural class will abrogate G2/M arrest and sensitize tumor cells to treatment with camptothecin and doxorubicin (30).

### Targeting ATM and DNA-PKcs

Inhibitors of ATM DNA-PKcs are being developed as potential therapeutics for the treatment of cancer. The rationale behind the clinical use of ATM inhibitors assumes that ATM signaling is dysfunctional in tumor cells, and that inhibition would create a therapeutic index by hyper-sensitizing tumor cells to agents that cause DSBs. Recently, a small-molecule inhibitor of ATM (KU55933) has been described (23). At micromolar concentrations, KU55933 is able to inhibit ATM (IC50 = 12.9 nmol/L), reduce the phosphorylation of a range of ATM substrates (P53, NBS1, H2AX, and SMC1), and radiosensitize and chemosensitize cells to ionizing radiation, etoposide, doxorubicin, and camptothecin. The specificity and efficacy of KU55933 illustrates the potential of ATM inhibition, which was only previously showed using nonspecific phosphatidylinositol 3-kinase–like kinase inhibitors, such as wortmannin and caffeine, which are both too toxic for clinical exploitation at doses with significant effect on ATM activity (23). As another member of the phosphatidylinositol 3-kinase–like kinase family, DNA-PKcs has also been proposed as a therapeutic target, primarily based on its role in NHEJ and with a similar rationale to ATM inhibition; cells deficient in DNA-PKcs are radiosensitive, hypersensitive to DNA cross-linking agents, and defective in DSB repair. Several small-molecule inhibitors of DNA-PKcs have been developed, including Vanillin, Salvicin, OK-1035, LY294002, NU7026, NU7441, IC87102, and IC87361 (24, 25). These compounds cause radiosensitization and chemosensitization in both in vitro cell models and tumor xenograft in vivo models.
Like CHK1, CHK2 can mediate S and G2/M phase arrest and also regulate the apoptotic response via PML, p53, and E2F-1 (10). This has led to the therapeutic rational that CHK2 inhibition may both sensitize P53-deficient tumor cells to genotoxic agents while transiently protecting normal cells through blocking their apoptotic response. Inhibition of CHK2 may also sensitize tumor cells to genotoxins through inhibition of error-free HR while allowing error-prone NHEJ, providing a second strategy for CHK2 inhibition.

The first selective inhibitor of CHK2 reported was CEP-6367 (IC50 = 20 nmol/L), although the structure of this molecule is not currently available (27). A synthetic analogue of the marine sponge metabolite hymenialdisine has also been identified as a potent inhibitor of CHK2 (IC50 = 8 nmol/L), but no cellular studies were reported with this compound (31). Most recently, high-throughput screening of recombinant human CHK2 has identified a series of 2-arylbenzimidazoles as potent and selective CHK2 inhibitors (32). Furthermore, one of these compounds (IC50 = 13 nmol/L) showed dose-dependent radioprotection of human T cells from apoptosis due to ionizing radiation. This supports the concept that CHK2 inhibitors may provide protection for normal cells against genotoxic insult. To date, the only known small-molecule inhibitor of CHK1 or CHK2 to enter clinical trial is XL844 (33), which inhibits both CHK1 and CHK2.

### Synthetic Lethal Approaches to Targeting DSB Repair Defects

**Targeting BRCA1 and BRCA2 mutation.** Individuals with heterozygous, deleterious, germline mutations in either the BRCA1 or BRCA2 genes exhibit high lifetime risks of developing breast and other cancers. Tumors arising in mutation carriers have generally lost the wild-type allele and do not express functional BRCA1 or BRCA2 proteins. Therefore, loss of BRCA1 or BRCA2 function leads to a tumor-specific dysfunction in the repair of DSBs by HR. Although still an area of active investigation, haploinsufficiency in normal tissues of gene carriers has not been convincingly shown (34). Therefore, there is potentially a significant difference in the ability to repair DSBs by HR between tumor and normal tissue, providing a putatively large therapeutic window. Agents that cause an increase in DSBs normally repaired by HR should selectively affect BRCA-deficient cells, with much less effect on normal cells (35).

This effect is graphically shown by the observation that cells defective in BRCA1 or BRCA2 are hypersensitive to drugs such as mitomycin C and the platinum analogues cisplatin and carboplatin. These agents cause covalent cross-links between bases on the two opposing strands of DNA, which are known as interstrand DNA cross-links. It seems likely that partially processed cross-links cause replication fork stalling when encountered by the DNA replication machinery during S phase. Stalled replication forks may degenerate into DSBs and would normally be repaired, at least in part, by HR in the presence of functional BRCA1 and BRCA2. Therefore, it seems likely that the selective lethality seen in BRCA-deficient cells treated with agents causing interstrand DNA cross-links is caused either by an inability to repair the replication fork blockage or by the use of an inappropriate nonconservative pathway of DSB repair, such as NHEJ or single-strand annealing. This induces genomic instability and would ultimately lead to cell death (36). Based on this in vitro data, a clinical study has now commenced, randomizing BRCA1 or BRCA2 mutation carriers with metastatic breast cancers to either the DNA cross-linking agent carboplatin or docetaxel, the existing gold-standard treatment (refs. 35, 37; http://www.brcatrial.org).

A significant development in the exploitation of the DNA repair defect in BRCA mutant cells has been the use of synthetic lethality approaches. A synthetic lethal interaction between two genes occurs when mutation of either alone is compatible with viability, but mutation of both leads to death (38, 39). Exploiting this concept, two groups identified the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) as a synthetic lethal partner of BRCA1 and BRCA2 (40, 41). For example, use of small-molecule inhibitors of PARP in vitro with isogenically matched BRCA deficient and wild-type cells illustrates that levels of selectivity can be obtained that are several fold higher than for conventional chemotherapies. These PARP inhibitors are able to achieve similar results in xenograft models and also in animal models of spontaneous BRCA2 loss of function (40–42). The success of this approach in experimental models has led to the initiation of clinical trials using PARP inhibitors as a monotherapy for BRCA-deficient tumors. Whereas the therapeutic use of PARP inhibitors is not novel, the use of these agents as monotherapy in BRCA-deficient tumors is novel, as previously, PARP inhibitors have only been exploited as chemosensitizing and radiosensitizing agents (43).

Why should inhibition of PARP be synthetic lethal to cells with deficiencies in either BRCA1 or BRCA2? The rationale is quite simple. PARP is a nonredundant component of the base excision repair pathway, which repairs SSBs. It is known that when PARP is inhibited, base excision repair is reduced, and SSBs that are caused during the normal cell cycle persist. It has also been established that replication forks that encounter a persistent, unrepaired SSB can degenerate to form a DSB in close proximity to a sister chromatid, the ideal template for repair by gene conversion involving BRCA1 and BRCA2. Therefore, inhibition of PARP should cause synthetic lethality along with defects in essential components of the gene conversion pathway as it would cause the persistence of lesions that would normally be processed by GC. The synthetic lethal approach uses the inhibition of an enzyme in combination with the normal levels of DNA damage that a cell is always exposed to (to cause the SSBs) as opposed to flooding cells with huge and potentially lethal levels of DNA lesions, as occurs in chemotherapy. It seems likely to be the explanation for the apparently much larger therapeutic index that PARP inhibitors have when compared with agents causing interstrand DNA cross-links (41).

**Other components of HR.** The data discussed above highlights the potential of novel therapies, such as PARP inhibitors, for the treatment BRCA1 and BRCA2 deficient tumors. Although only a very small fraction of cancers carry BRCA mutations, increasing evidence suggests that BRCA-associated pathways may be inactivated by multiple mechanisms in a substantial fraction of sporadic cancers. These may exhibit a BRCA deficiency phenocopy in the absence of germ line mutation of either BRCA gene, a phenomenon termed “BRCAlessness” (44). For example, methylation of the promoters of the
BRCA1 or FANC1 genes and amplification of the EMSY gene, which encodes a BRCA2-interacting protein, have all been hypothesized as mechanisms causing a synthetic lethality of BRCA-associated DNA repair and therefore might sensitize tumors to BRCA-directed therapies (44). In addition to the concept of BRCA2, it is also becoming apparent that sensitivity to PARP inhibitors is not the preserve of BRCA-deficient cells but is also present in cells with deficiencies in other aspects of the DSB response. By extending the rationale for synthetic lethality of PARP and BRCA1 or BRCA2, it is likely that deficiencies in any gene that is nonredundant with respect to restarting stalled replication forks and repairing the resulting DSBs should be sensitive to PARP inhibitors. Unsurprisingly, ATM deficiency has also been shown to be synthetically lethal in combination with PARP deficiency (45), and deficiencies in other known HR pathway proteins (including RAD51, DSS1, RAD54, RPA1, NBS1, ATR, CHK1, CHK2, FANCD2, FANCA, and FANCC) also show the same synthetic lethal relationship with PARP inhibition. Not only does this work give clearer insight into the mechanism of sensitivity to PARP inhibition but also widens the potential use of PARP inhibitors. For example, mutations in the ATM gene have been found in patients with T-cell prolymphocytic leukaemia and B-cell chronic lymphocytic leukaemia (46, 47) and breast cancer (48) and heterozygous CHK2 germ line mutations have been identified in Li-Fraumeni familial cancer syndrome and also in sarcomas, breast cancer, and ovarian cancer and brain tumors (49, 50). Mutations in FANCC and FANCN have been shown in pancreatic cancer (51, 52). In addition, a significant proportion of sporadic cancers have been shown to inactivate the FA pathway through methylation of the FANCN promoter, including ovarian and breast, cervical, lung, and non-small cell carcinomas (53, 54). Therefore, synthetic lethal approaches targeting DNA repair defects in tumors may be widely applicable in sporadic cancers.

References

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