Exploiting DNA Replication Stress for Cancer Treatment
Tajinder Ubhi1,2 and Grant W. Brown1,2

Abstract
Complete and accurate DNA replication is fundamental to cellular proliferation and genome stability. Obstacles that delay, prevent, or terminate DNA replication cause the phenomena termed DNA replication stress. Cancer cells exhibit chronic replication stress due to the losses of proteins that protect or repair stressed replication forks and due to the continuous proliferative signaling, providing an exploitable therapeutic vulnerability in tumors. Here, we outline current and pending therapeutic approaches leveraging tumor-specific replication stress as a target, in addition to the challenges associated with such therapies. We discuss how replication stress modulates the cell-intrinsic innate immune response and highlight the integration of replication stress with immunotherapies. Together, exploiting replication stress for cancer treatment seems to be a promising strategy as it provides a selective means of eliminating tumors, and with continuous advances in our knowledge of the replication stress response and lessons learned from current therapies in use, we are moving toward honing the potential of targeting replication stress in the clinic.

Introduction
The DNA replication machinery successfully carries out accurate genome duplication in the face of numerous obstacles, many of which cause DNA replication stress. Replication stress, defined as any hindrance to DNA replication that either stalls, blocks, or terminates DNA polymerization, activates a highly conserved replication stress response pathway mediated by a network of repair proteins to resolve the damage. Efficient removal of obstacles to replication progression safeguards genome integrity by ensuring timely completion of DNA replication and by limiting susceptibility to mutagenesis. However, failure to remove replication stressors due to the loss of replication stress response and repair proteins and sustained proliferative signaling leads to chronic replication stress, and is a prominent feature of tumor cells. In particular, whole-genome sequencing efforts of tumor samples have indicated the important role of replication stress in tumor progression and maintenance, complementing early findings from the groups of Halazonetis and Bartek who demonstrated that replication stress accumulates upon cellular transformation, and is rarely observed in even the most proliferative tissues (1, 2). Thus, due to the selective nature of replication stress as a therapeutic target, the dependency of tumor cells on the replication stress response for cell survival, and the ever-expanding network of replication stress response proteins that can be targeted, replication stress is beginning to be explored as an attractive target in the clinic. In contrast to normal cells, cancer cells can be pushed toward cell death by introducing further DNA damage in a catastrophic manner or by promoting their entrance into cell cycle stages where unresolved replication stress is detrimental. In this review, we provide a summary of the therapies centered on enhancing both endogenous and drug-induced replication stress and discuss the rationales associated with them. We also highlight the potential of using replication stress to stimulate the cell-intrinsic innate immune response to improve immunotherapy effectiveness.

DNA Replication Stress: Causes, Consequences, and the Cellular Response
Eukaryotic DNA replication is a tightly regulated and complex process that requires the orchestrated functions of several hundred proteins during the S phase of each cell cycle (reviewed in refs. 3, 4). Each time a cell divides, billions of nucleotides of DNA must be accurately polymerized, and ensuring this process occurs prior to the next cell cycle is essential for cellular homeostasis. However, there are numerous hindrances to DNA replication that can prevent progression of the replication machinery and cause replication forks to stall. Obstacles to replication fork progression can arise from several sources, either endogenous or exogenous, ranging from the depletion of nucleotide pools available for DNA synthesis to transcription-replication machinery collisions, RNA–DNA hybrids, oncogene-induced increases in replication origin firing, and inherently hard-to-replicate regions (Fig. 1A; reviewed in ref. 5). The type of stress challenging replication fork progression dictates the cellular response and network of repair proteins activated in response to the replication stress. For example, ribonucleotides that are misincorporated into replicating DNA can cause replication stress, and are recognized and removed by ribonucleotide excision repair, whereas interstrand crosslinks are mainly repaired by the Fanconi anemia pathway, a specialized branch of the DNA damage response. Stalled replication forks are often transient, with cells able to resolve them and continue replication. However, when left unresolved, replication stress can lead to small-scale and large-scale genome alterations such as the mutations and chromosomal rearrangements found frequently in human cancers and developmental disorders.

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Replication stress triggers the activation of a signaling cascade that promotes resolution of the stress and resumption of replication fork progression, collectively known as the S phase checkpoint (Fig. 1B). Stalled replication forks are characterized by stretches of single-stranded DNA (ssDNA), larger than those found during lagging strand DNA synthesis, that activate the S phase checkpoint. It is thought that the ssDNA gaps are caused mainly by polymerase and helicase uncoupling (6), but ssDNA can also be generated by nucleolytic processing of DNA at reversed replication fork structures (7). The ssDNA is bound with high affinity by replication protein A (RPA), which serves as a platform for the recruitment of numerous sensor proteins, including ataxia telangiectasia and Rad3-related (ATR)-interacting protein (ATRIP), the 9-1-1 DNA clamp complex (RAD9-RAD1-HUS1), topoisomerase II binding protein 1 (TOPBP1), and Ewing tumor-associated antigen 1 (ETAA1), which recruit and activate the central replication stress response kinase ATR (reviewed in 8). Upon activation, ATR orchestrates a multifaceted response at stalled replication forks by phosphorylating several downstream targets, including its main effector kinase, checkpoint kinase 1 (CHK1). The ATR–CHK1 signaling pathway leads to cell cycle arrest, stabilization of stalled replication forks, and the inhibition of late origin firing to prevent excess ssDNA formation (9). In doing so, the S phase checkpoint ultimately promotes replication fork repair and restart, or resumption of replication from an adjacent origin (10, 11), to ensure complete replication of the affected genomic loci.

In instances where cells endure chronic replication stress, such as in the early stages of tumorigenesis, or during extended periods of antineoplastic drug treatment, replication forks can collapse (12–14). It is thought that ssDNA stretches found at stressed replication forks are converted to DNA double-strand breaks during replication fork collapse and that the replication fork loses its capacity to synthesize DNA. Although collapsed replication forks can likely be rescued through homologous recombination (15) or mitotic DNA synthesis (16), inability to resume DNA replication in these regions promotes mutagenesis and genome instability. Cells recruit error-prone translesion DNA polymerases that inaccurately synthesize DNA as a means to complete bulk genome replication, failure of which leads to incompletely replicated DNA persisting into mitosis. The presence of under-replicated genomic loci in mitosis can result in chromosomal entanglements between sister chromatids (17) or lagging chromosomes that fail to segregate with the remaining genetic material, leading to the formation of micronuclei (18). These events not only increase the risk of genome instability and promote transformation, but they also perpetuate the existing genome instability through global missegregation of chromosomes, thus providing a route of evolution for cancer cells (19). Unresolved replication stress following mitosis leads to the formation of nuclear bodies marked by the DNA damage response protein p53 binding protein 1 (53BP1) in the subsequent G1 phase of the cell cycle in daughter cells, which may act to protect or promote resolution of the replication stress (20).

Exploiting DNA Replication Stress–Centered Vulnerabilities in Cancer

As it is well documented that tumor cells have persistent replication stress (21), and with replication stress now acknowledged as an enabling characteristic of cancer (22), it is not surprising that replication stress is beginning to be rationally leveraged in cancer therapies. The elevated replication stress in cancer cells has been largely attributed to the loss of cell cycle checkpoint activators, often tumor suppressor genes, and to the overexpression or constitutive expression of oncogenes (21, 23). These alterations modify replication fork structures, increase replication initiation or origin firing, and promote premature entry into S phase (2, 13, 24). Strategies aiming to exploit replication stress fall within two major categories; they either function to increase the endogenous replication stress present within tumor cells to induce cell death or they produce tumor cell-specific replication stress that can be further enhanced by additional therapeutics.

Traditional replication stress–inducing drugs largely resulted from serendipitous observations of tumor cell death following use of the agents (25), but increasingly drugs are designed with the specific goal of exploiting replication stress (Table 1). The increase in accessibility of deep sequencing approaches has provided remarkable insight into the catalogue of DNA repair genes mutated in cancers and offers another exciting means of targeting endogenous replication stress by identifying mutation-specific vulnerabilities (26).

Current Therapies That Harness Replication Stress

Because a key feature that distinguishes tumor cells from most normal cells is their sustained proliferation, DNA replication has been harnessed as a semi-selective target in cancer therapies for decades (25). Many traditional antineoplastic drugs act by increasing the replication stress within tumor cells, by directly damaging the DNA, depleting cellular resources required for successful cell division, or through a combination of both. Nucleoside analogues, including 5-fluorouracil, cytarabine, and gemcitabine, were among the first chemotherapeutic agents introduced to the clinic and act through diverse mechanisms to ultimately halt DNA replication. For instance, cytarabine and gemcitabine are deoxycytidine analogues that compete directly with dCTP for incorporation into newly synthesized DNA, leading to a delay in replication fork progression or even a complete termination of some replication forks (27, 28). In addition to their abilities to incorporate into DNA, these agents also reduce the size of the intracellular nucleotide pools available for DNA synthesis or limit the availability of a single nucleotide. For example, gemcitabine potentiates its cytotoxic effects by also inhibiting ribonucleotide reductase (29), whereas 5-fluorouracil functions mainly by inhibiting thymidylate synthetase, thereby reducing the amount of thymidine available for DNA replication and repair (30).

In contrast to nucleoside analogues, alkylating agents and platinum-based compounds function by directly modifying DNA through the formation of alkylation adducts on DNA bases, intranuclear or interstrand crosslinks between DNA bases, or both (31, 32). The generation of DNA adducts by alkylating agents such as dacarbazine and temozolomide poses an obstacle for the advancing DNA polymerase, leading to delayed replication fork progression. Moreover, intra- and interstrand crosslinks generated by platinum-based agents such as cisplatin and carboplatin are physical barriers to DNA replication and block the replication machinery. Intrastrand crosslinks present on the template DNA strand during replication impair proper base pairing with the nascent DNA strand, whereas interstrand crosslinks impede...
**A**

- Regulation of replication origin firing
- Oncogene-induced replication stress
- Hard-to-replicate regions

**B**

- 9-1-1 DNA clamp complex
- Late origin firing
- Stabilization of stalled replication forks

**C**

- AZD1775
- WEE1
- G2/M checkpoint
- S phase checkpoint
- G1/S checkpoint

- Prexasertib, GDC-0575, SCH 900776, and SRA737
- M6620, AZD6738, and BAY1895344

**Key**

- G1: Gap phase I
- S: Synthesis phase
- G2: Gap phase II
- M: Cell division phase

**Terms**

- Ribonucleotide incorporation
- DNA helicases
- Inter-strand crosslinks
- Fanconi Anemia pathway
- MYC
- Nucleotide pool depletion
- Oncogene-induced replication stress
- RNA-DNA hybrids and replication-transcription conflicts
- DNA sliding clamp
- DNA polymerase
- DNA helicase
- ATRIP
- ATR
- TOPBP1
- Etaa1
- CHK1
- ATR
- M6620, AZD6738, and BAY1895344
- Ubhi and Brown

**References**

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the very first step of DNA replication, that is, unwinding of the double-strand helix (33). Another class of replication stress–
inducing agents that directly damage DNA is topoisomerase inhibitors (34, 35). Topoisomerases generate transient DNA
breaks to relax DNA supercoiling that occurs during DNA replication and transcription. The intermediate state where the
topoisomerases are bound to the cleaved DNA is trapped by topoisomerase inhibitors and leads to the formation of stable
protein–DNA complexes that generate a physical barrier to ongoing replication forks, often collapsing to double-strand breaks
when encountered by a replication fork. The action of topoisomerase inhibitors resembles that of another group of replication
stress–inducing therapeutics, PARP inhibitors. PARP inhibitors generate an obstacle for the replication machinery by trapping
PARP on DNA (36), in addition to impairing single-strand break repair (37) and accelerating replication fork progression (38),
amplifying the replication stress in tumor cells.

The replication stress–inducing effects of conventional thera-
pies have been directly visualized at the single-molecule level through DNA fiber analyses, where DNA synthesis rates of indi-
vidual DNA replication forks can be measured following drug
treatment (39–41), and through DNA pull-down techniques that identify proteins bound directly at replication forks in vivo (42–46). Of particular interest, the interactions between traditional replication stress–inducing drugs and cellular replica-
tion stress response pathways are evident in preclinical studies, where inhibition or depletion of the key replication stress
response kinases required for overcoming the replication stress present within cells, ATR and/or CHK1, sensitizes tumor cells to 5-fluorouracil, gemcitabine, cisplatin, PARP inhibitors, and temo-
zolomide, with some combinations having advanced to clinical trials (34, 35, 47–52).

Emerging Combination Therapies That Target Replication Stress: Cell Cycle Checkpoints

Although conventional replication stress–inducing agents have
been used for decades, their applicability and efficacy is limited by
their associated toxicities and the rapid emergence of resistant
tumor cells. As noted, these therapies target DNA replication, and thus have an effect on highly proliferative cells, including normal cells within the gut epithelium and bone marrow, leading to undesirable and sometimes intolerable side effects. In light of this, several promising rationally designed combination therapies that increase the specificity of these traditional therapies toward tumor cells are currently undergoing clinical trials. In addition to gener-
ating further replication stress, many of these therapies also

Figure 1.

Generation and exploitation of DNA replication stress. A, Obstacles in replication fork progression can arise from exogenous and endogenous sources and dictate the cellular response and network of repair proteins activated in response to the replication stress. Sources of replication stress include, but are not limited to, the depletion of nucleotide pools available for DNA replication and repair, oncogene-induced stress, RNA–DNA hybrids, replication–transcription
conflicts, DNA crosslinks, inherently difficult-to-replicate regions, and the misincorporation of ribonucleotides into replicating DNA. B, Replication stress activates a highly conserved signaling response called the S phase checkpoint. The S phase checkpoint is activated upon exposure of ssDNA from stressed replication forks, which leads to the recruitment and activation of the central replication stress response kinase ATR and its main effector kinase, CHK1. The ATR–
CHK1 signaling pathway promotes cell cycle arrest, stabilization of stalled replication forks, and inhibition of late origin firing to ultimately ensure complete replication of the affected genomic loci. C, Cancer cells become dependent on the S phase checkpoint for cell survival due to their high levels of intrinsic DNA replication stress. In addition to S phase checkpoint response proteins being the leading targets for cancer therapies targeting replication stress, regulators of mitotic entry are also being evaluated, as they drive cells with unresolved replication stress into catastrophic mitoses. Targets in the DNA replication stress response are shown for each cell cycle checkpoint, with inhibitors targeting those proteins currently being evaluated in clinical trials in bold.
shown promising results with an acceptable toxicity profile (67). Drugs that increase replication stress should increase the importance of the G2/M checkpoint for tumor cell survival, and accordingly there are currently greater than 30 clinical studies underway for combination therapies with WEE1 inhibitors (clinicaltrials.gov, accessed January 2019). These include combinations of gemcitabine, temozolomide, cytarabine, irinotecan, carboplatin, and cisplatin with the potent WEE1 inhibitor AZD1775 in pancreatic, blood, brain, ovarian, and colon tumors, with an emphasis on p53 deficiency as the key selection criteria. In addition to driving tumor cells into unscheduled mitosis (68), the cytotoxicity of AZD1775 could also be attributed to the effects WEE1 has on stabilizing stalled replication forks and ensuring timely origin firing, further exacerbating the replication stress in tumor cells when WEE1 is inhibited (69, 70). WEE1 inhibitors act synergistically with ATR and CHK1 inhibitors in preclinical studies (71, 72), extending the catalogue of possible combinations with other targeted inhibitors that could be implemented. Similar to ATR and CHK1 inhibitors, although AZD1775 has shown single-agent activity in preclinical studies, its efficacy is potentiated when integrated into combination regimens.

Table 1. Chemotherapeutics that increase DNA replication stress

<table>
<thead>
<tr>
<th>Class of agents or target</th>
<th>Function</th>
<th>Compounds</th>
<th>Clinical stage</th>
<th>References</th>
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<td>Nucleoside analogues</td>
<td>Inhibition of DNA replication</td>
<td>Azacitidine</td>
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<td>Alkylating agents and</td>
<td>Direct modification of DNA</td>
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**Precision Medicine Approaches That Leverage Replication Stress**

The identification of the underlying genetic alterations across all major subtypes of cancer has provided unprecedented insight into DNA repair proteins that are altered in cancer (26) and has laid the foundation for several precision medicine approaches. Almost every tumor has at least one gene in the replication stress response altered, providing ample opportunities to exploit tumor-specific alterations without affecting normal cells. The concept of synthetic lethality (73), whereby the presence of cancer-specific mutations in specific genes renders other genes essential for cell proliferation and survival, has been fundamental for the discovery of several of these gene-targeted strategies (74). Perhaps the best example of this strategy is the successful use of PARP inhibitors for the treatment of BRCA1/BRCA2-deficient breast and ovarian cancers, as these double-strand break repair defective tumors are dependent on PARP activity for cell survival (37, 75). Considerable effort is now being expended to identify additional synthetic lethal interactions with DNA repair genes, including replication stress response genes. One of the most promising targets currently being explored is ATR. Tumors harboring mutations in...
which activate the central adaptor protein STING, inducing a transcriptional response to ultimately promote cellular senescence or elimination by the adaptive immune system. The cytosolic DNA fragments are sensed by the pattern recognition receptors, cyclic GMP-AMP synthase (cGAS) and IFN-$\beta$-inducible factor 16 (IFI16), which activate the central adaptor protein STING, inducing a transcriptional response to ultimately promote cellular senescence or elimination by the adaptive immune system.

Figure 2.
Activation of the cell-intrinsic innate immune response by DNA replication stress. In replication stress conditions, nuclear DNA is released into the cytosol, either directly from stalled replication forks in three prime repair exocuence (TREX1)-, sterile alpha motif and HD domain-containing protein 1 (SAMHD1)-, RPA- or RAD51-deficient backgrounds, or from micronuclei, which are small nuclei formed predominantly following mitotic progression in cells experiencing genome instability. The cytosolic DNA fragments are sensed by the pattern recognition receptors, cyclic GMP-AMP synthase (cGAS) and IFN-$\beta$-inducible factor 16 (IFI16), which activate the central adaptor protein STING, inducing a transcriptional response to ultimately promote cellular senescence or elimination by the adaptive immune system.
presence of cytosolic DNA derived from the nucleus or mitochondria, which engages the pattern recognition receptors that normally scan for viral infections. The mechanisms through which replication stress induces DNA release into the cytoplasm appear to be specific to the perturbation. Recent work has found micro-nuclei to be a key source of immunostimulatory DNA following mitotic progression in cells lacking RNase H2 (96, 97), whereas the release of ssDNA directly from stalled replication forks has also been demonstrated in TREX1-, SAMHD1-, RPA-, and RAD51-deficient backgrounds and following cytarabine treatment (93, 98–101). Whether these conditions could result in cytoplasmic DNA accumulation through both observed mechanisms has yet to be ruled out, and several aspects of these mechanisms, including the interplay of proteins at replication forks that promote ssDNA release, have yet to be defined.

Central to the innate immune response is the adaptor protein “stimulator of IFN genes” (STING), which couples signals from cytosolic DNA sensors to a transcriptional response from activation of both the NF-kB and type I IFN signaling axes, ultimately promoting cellular senescence or elimination by the adaptive immune system (reviewed in ref. 102). Interestingly, STING signaling is suppressed in several tumors (103) and multiple cancer cell types contain genome-derived cytosolic ssDNA (100), further affirming the presence and importance of persistent replication stress in tumors. As type I IFN production from the innate response is critical in priming the adaptive immune system, robust STING signaling has been attributed to an increased immunotherapy response. In fact, blocking T-cell inhibitory pathways using immune checkpoint inhibitors is ineffective in mice lacking TAM5173 (STING; ref. 96). However, despite current efforts to combine STING agonists with checkpoint inhibitors as antitumor therapy, therapeutic delivery of the agonists has yet to be improved for clinical settings. Combination approaches integrating replication stress–inducing agents, such as carboplatin or gemcitabine, with immunotherapies like the immune checkpoint inhibitor nivolumab, have advanced to clinical trials (NCT02944396, NCT03662074, NCT03061188, NCT02734004, NCT02849496, NCT02657889, and NCT02571725). It is tempting to speculate that the efficacy observed could be due to the engagement of STING by replication stress, and would support the use of replication stress as a predictive marker for immunotherapy efficacy. Further work is required to better understand the interplay between the host immune systems and replication stress and would provide insight into how these responses can be modulated optimally. Interestingly, somatic mutations in the genes encoding the replicative polymerases POLD1 and POLE are also being used as indicators for successful immunotherapy outcomes due to the high mutation burden present in these genetic backgrounds (NCT03375307, NCT03207347, NCT03428802, and NCT03491345). The role of the innate response in this interaction has yet to be revealed and will likely also provide additional exploitable therapeutic vulnerabilities.

Future Directions

Our knowledge of replication stress–centered vulnerabilities in tumor cells has grown dramatically in recent years and is leading to an increasingly complex view of how tumor cells deal with replication stress. Knowledge of the replication stress response continues to develop as we discover novel proteins and reveal intricate response networks, providing greater opportunities to target these proteins in therapy. In particular, advances in molecular biology tools continue to facilitate identification of novel molecular targets in the replication stress response and the interplay between the replication stress response and different cellular pathways such as metabolism (104). In addition to RNA interference screens, the advent of genome-wide screening using the CRISPR-Cas9 technology will provide a powerful tool moving forward to rapidly identify novel clinically relevant synthetic lethal interactions in cancer cells, and is already yielding promising results (105–108). As these technologies begin to be adapted in other systems, similar screens performed in more biologically appropriate settings, such as in 3D organoid cultures or in vivo mouse models, will likely yield more clinically relevant synthetic lethal interactions. Furthermore, advances in whole-genome sequencing of tumors will also assist precision medicine approaches by identifying key actionable genetic alterations for therapeutic targeting in the clinic.

Despite the unprecedented rate at which we are identifying novel replication stress response targets, there remain several obstacles to overcome for optimal treatment outcomes. One limitation of all replication stress–targeting therapies that will be difficult to diminish is achieving an acceptable therapeutic window, due to the essentiality of the replication stress response in normal cells. In contrast to conventional therapies that target all dividing cells, targeted inhibitors have shown greater precision in selectively affecting tumors, yet they continue to present adverse side effects that are sometimes intolerable. Another important limitation is the current technologies available to predict tumor backgrounds that would benefit most from certain therapies. Markers that can be effectively detected by immunohistochemistry, such as the overexpression of certain oncogenes, are starting to be implemented as a means to stratify patients for targeted treatment strategies in clinical trials. However, the dependence on immunohistochemical approaches for biomarker identification restricts the use of important replication stress markers such as RPA foci and ssDNA and the use of functional DNA repair assays as proxies for therapy response.

The notion of exploiting intrinsic replication stress has driven considerable excitement in the medical oncology field as it provides a selective means of eliminating tumors. Several approaches undergoing clinical investigation are already showing promise, and with the advent of powerful new technologies, both in academic and clinical settings, many more are likely to be revealed for therapeutic development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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