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A Tale of Two Checkpoints: ATR Inhibition and PD-(L)1 Blockade

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Abstract

Innate immunity and the DNA damage response (DDR) pathway are inextricably linked. Within the DDR, ataxia telangiectasia and Rad3-related (ATR) is a key kinase responsible for sensing replication stress and facilitating DNA repair through checkpoint activation, cell cycle arrest, and promotion of fork recovery. Recent studies have shed light on the immunomodulatory role of the ATR-CHK1 pathway in the tumor microenvironment and the specific effects of ATR inhibition in stimulating an innate immune response. With several potent and selective ATR inhibitors in developmental pipelines, the combination of dual ATR and PD-(L)1 blockade has attracted increasing interest in cancer therapy. In this review, we summarize the clinical and preclinical data supporting the combined inhibition of ATR and PD-(L)1, discuss the potential challenges surrounding this approach, and highlight biomarkers relevant for selected patients who are most likely to benefit from the blockade of these two checkpoints.

THE ATR PATHWAY AND ITS TARGETING IN CANCER

The ATR Pathway

To maintain genomic integrity and cellular viability, cells rely on coordinated and sophisticated signaling pathways, collectively termed the DNA damage response (DDR), to resolve DNA damage and replication stress (1). The DDR consists of a kinase cascade that detects and processes DNA damage, both by direct DNA repair and by pausing the cell cycle to allow time for DNA repair (2). Within the DDR, ataxia telangiectasia and Rad3-related (ATR) and ataxia-telangiectasia mutated (ATM) kinases serve as key sentinels of DNA damage and orchestrate downstream DNA repair (3). Endogenous or exogenous sources of DNA damage lead to the formation of single-stranded DNA (ssDNA) ends at stressed replication forks or areas of resected DNA double-strand breaks (DSBs) (4), which are rapidly coated by replication protein A (RPA). In turn, RPA recruits ATR/ATR-interacting protein complexes to damage sites (5). ATR phosphorylates and activates downstream checkpoint kinase 1 (CHK1), which interrupts the gap 2/mitosis (G2/M) checkpoints by concurrent inactivation of cell division cycle (CDC)25A/CDC25C and activation of wee-like protein kinase 1, respectively (6). Inactivation of CDC25A by CHK1 also suppresses global replication origin firing, increasing cell cycle arrest and promoting replication fork slowing, thus allowing time for DNA damage resolution. In addition, ATR and CHK1 act to stabilize and protect stalled replication forks by mediating fork remodeling in cooperation with RAD51, ZRANB3 (zinc-finger RANBP2-type containing 3), and SMARCAL1 (SWI/SNF-related, matrix-associated, actin dependent regulator of chromatin, subfamily-A-like 1) (7). Recent studies have shown that the ATR-CHK1 pathway is similarly triggered by high levels of RNA R-loops (8), which are RNA-DNA hybrid transcription intermediates consisting of displaced ssDNA (9). Not only does ATR-CHK1 activation induce G2/M arrest and promote replication fork recovery, but also ATR prevents excessive digestion of reversed forks by MUS81, suppressing R-loop-induced double-stranded DNA production (8). Upon resolution of replication stress, ATR and CHK1 promote fork reversal and restart through various mechanisms, such as by facilitating homologous recombination through the recruitment of BRCA2 and RAD51 to DNA damage foci (10, 11). By initiating cell cycle arrest, suppressing replication origin firing, and stabilizing stalled replication forks, ATR acts as a major regulator of the DDR, preventing cells harboring DNA damage from entering mitosis (12).

Targeting the ATR Pathway in Cancer

In cancer cells, acquired DDR deficiencies promote cellular proliferation, oncogenesis, and tumor progression despite the accumulation of genomic errors and instability (13). Resistance to cancer treatment has also been associated with increased DDR and ATR pathway signaling in various cancer types (14). Furthermore, oncogenic activation provoking uncontrolled cellular proliferation, as well as the loss of gap 1 (G1) checkpoint regulation, causes cells to become more reliant on the remaining DDR pathways and/or downstream cell cycle checkpoints in order to survive genomic insults. Therefore, targeting the remaining functional nodes within the DDR has emerged as an attractive anticancer strategy to enable selective killing of tumor cells. In particular, the significant role of ATR at the core of the DDR has led to great interest in its therapeutic targeting. Preclinical data have provided a strong rationale for synthetic lethal targeting of ATR in tumors harboring dependency on the ATR pathway, due to loss of *TP53* (15), *ARID1A* (16), or *ATM* (17), or in the context of elevated levels of replicative stress, such as from oncogenic *KRAS* activation (18), *CCNE1* amplification (19), or *c-MYC* amplification (20). The relevance of the ATR-CHK1 pathway in safeguarding the genome against R-loops has also led to interest in targeting ATR in cells harboring high levels of R-loops (21). Spliceosome hot-spot mutations such as *SF3B1*, *U2AF1*,

and *SRSF2* are prevalent in hematological malignancies such as myelodysplastic syndrome and acute myeloid leukemia (22) and induce R-loop accumulation (23). A preclinical study has demonstrated ATR inhibitor sensitivity in *U2AF1*-expressing cells (24) on the basis of this rationale, and early-phase trials selecting patients on the basis of these genomic biomarkers are enrolling (**Table 1**).

Several ATR inhibitors have entered developmental pipelines; these include ART0380 (Artios Pharma), berzosertib (M6620, VX-970; Merck Serono), ceralasertib (AZD6738, AstraZeneca), elimusertib (BAY1895344; Bayer), M4344 (VX-803, Merck Serono), and RP-3500 (Repare Therapeutics) (**Table 1**). To date, limited clinical activity has been described for berzosertib and ceralasertib as monotherapies in phase I trials of unselected advanced solid tumor patients [objective response rate (ORR), 9% and 7%, respectively] (25, 26) (**Table 1**). Combinations of ATR inhibitors with different types of cytotoxic chemotherapy, such as platinum salts, taxanes, and antimetabolites, are also under investigation (**Table 1**). Berzosertib displayed synergy when combined with cisplatin in non-small-cell lung cancer (NSCLC) cell lines in vitro, as well as in patient-derived xenograft models that were previously resistant to cisplatin or ATR inhibitor monotherapy (27). In colorectal cancer models, the combination of berzosertib and oxaliplatin displayed similar synergy in oxaliplatin-resistant cell lines and syngeneic mice by potentiating oxaliplatin-induced immunogenic cell death (28). ATR inhibitors have also been combined with antimetabolite chemotherapies such as gemcitabine, which target ribonucleotide reductase and thus increase replicative stress (29). In pancreatic cancer cell lines, the addition of low doses of gemcitabine increased replicative stress above the required threshold to resensitize previously ceralasertib-resistant cells to ceralasertib treatment (30). Recently, a randomized phase II trial demonstrated improved progression-free survival (PFS) for platinum-resistant ovarian cancer patients treated with gemcitabine plus berzosertib compared with gemcitabine alone [median PFS, 22.9 weeks versus 14.7 weeks; hazard ratio, 0.57; 90% confidence interval (CI), 0.33–0.98; $p = 0.04$] (31) (**Table 1**).

The Role of ATR in the Tumor Immune Microenvironment

Recent research has revealed intrinsic links between the ATR-CHK1 pathway and innate immune signaling networks (32) (**Figure 1**). Increased ATR pathway signaling triggered by genotoxic stress and stalled DNA replication upregulates immunosuppressive PD-L1 on tumor cells mediated by signal transducer and activator of transcription (STAT)1– and STAT3–interferon (IFN) regulatory factor 1 (IRF1)-related pathways, which were further enhanced by BRCA2 or Ku70/80 depletion (33). ATR pathway signaling also upregulates the expression of natural killer (NK) group 2D (NKG2D) cell surface ligands (NKG2DLs) (32), which bind to NKG2D receptors on NK cells and activated $CD8^+$ T cells, triggering degranulation and pro-inflammatory cytokine production. Pharmacological inhibition of ATR can suppress NKG2DL upregulation (32). Furthermore, *ATR* mutations modulate the tumor immune microenvironment in melanoma models. When compared with *ATR* wild-type tumors, homozygous *ATR* mutated melanoma tumors showed reduced numbers of infiltrating $CD3^+$ T cells but a significant increase in infiltrating macrophages and B cells compared with *ATR* wild-type or hemizygous *ATR* mutated tumors, as demonstrated on both flow cytometry and immunohistochemistry (IHC) (34). This ATR deficient state was associated with increased PD-L1, CD206, and Arginase1 expression, along with downregulation of butyrophilin expression, suggesting a T cell–suppressed immune environment (34). A phase I study that recruited 58 patients with advanced solid tumors investigated the ATR inhibitor ceralasertib and paclitaxel; intriguingly, 11 patients with metastatic melanoma who were previously resistant to programmed cell death protein 1 (PD-1) inhibitors achieved durable responses (35).

Table 1 Key trials investigating ATR inhibitors

ATR inhibitor	Phase	Study population	Selective biomarker	Treatment	Safety	Efficacy	Reference or clinical trial
Berzosertib (M6620, VX-970)	I	Advanced solid tumors; $n = 40$	NA	Berzosertib once or twice weekly Berzosertib (D2, D9) + carboplatin AUC 5 (D1) q21 days	Grade ≥3 AE: neutropenia (21.7%), anemia (4.3%), and thrombocytopenia (4.3%) for combination therapy	RP2D for once- or twice-weekly administration: 240 mg/m^2 RP2D for combination with carboplatin: 90 mg/m^2	26
	II	Platinum-resistant ovarian cancers; $n = 88$	NA	Gemcitabine 1,000 mg/m^2 (D1, D8) ± berzosertib 210 mg/m^2 (D2, D9) q21 days	Grade ≥3 AE: neutropenia (47%), thrombocytopenia (24%) for gemcitabine + berzosertib	PFS: 22.9 weeks versus 14.7 weeks (HR, 0.57; 90% CI, 0.33–0.98; $p = 0.044$)	31
	I	Advanced, unresectable solid tumors; $n = 51$	NA	Irinotecan + berzosertib (D1, D15) q28 days for 12 cycles	NA	Recruitment ongoing	NCT02595931
	I/II	SCLCs and extrapulmonary small-cell cancers; $n = 70$	NA	Topotecan (D1–5) + berzosertib (D5 or D2 and D5 at escalating doses) q21 days	NA	Recruitment ongoing	NCT02487095
	I/II	Platinum-sensitive ovarian cancers; $n = 31$	Platinum-sensitive disease	Gemcitabine 480–800 mg/m^2 (D1, D8) + carboplatin AUC 4 (D1) + berzosertib 90–120 mg/m^2 (D2, D9) q21 days	NA	Recruitment ongoing	NCT02627443
	I/II	SCLCs and HRD cancers resistant to PARPi; $n = 70$	Pathogenic mutations in *BRCA1*, *BRCA2*, *ATM*, *BRIP1*, *BARD1*, *CDK12*, *CHEK1*, *CHEK2*, *FANCL*, *PALB2*, *PPP2R2A*, *RAD51B*, *RAD51C*, *RAD51D*, or *RAD54L*	Sacituzumab govitecan (D1, D8) + berzosertib (escalating doses) q21 days	NA	Recruitment ongoing	NCT04826341
	II	Metastatic castration-resistant prostate cancers; $n = 130$	NA	Docetaxel 60 mg/m^2 (D1) + carboplatin AUC 4 (D1) q21 days versus Berzosertib 90 mg/m^2 (D2, D9) + carboplatin AUC 5 (D1) q21 days	NA	Active, not recruiting	NCT03517969
	II	Leiomyosarcomas; $n = 72$	NA	Gemcitabine 1,000 mg/m^2 (D1, D8) ± berzosertib 210 mg/m^2 (D2, D9) q21 days	NA	Not yet recruiting	NCT04807816
	II	Metastatic or unresectable gastric or gastroesophageal junction cancers; $n = 28$	*TP53* mutation	Irinotecan + berzosertib (D1, D15) q28 days	NA	Recruitment ongoing	NCT03641313

(*Continued*)

Table 1 (*Continued*)

ATR inhibitor	Phase	Study population	Selective biomarker	Treatment	Safety	Efficacy	Reference or clinical trial
Ceralasertib (AZD6738)	I	Advanced solid tumors; $n = 46$	NA	Escalating doses of ceralasertib followed by dose expansion	Grade ≥3 TRAEs in 67% and 20% of patients receiving continuous and intermittent dosing, respectively Frequent TRAEs were fatigue, anemia, nausea, and thrombocytopenia	PR in 7% of patients, SD in 48% 21% of patients in dose escalation and 25% in dose expansion had SD ≥ 16 weeks	59
	I	Advanced solid tumors; $n = 58$	NA	Paclitaxel 80 mg/m^2 (D1, D8, D15) + ceralasertib at escalating doses 40 mg OD–240 mg BD q28 days	1 DLT of neutropenic fever occurred in each cohort of patients dosed at 160 mg BD and 240 mg BD D1–14	MTD: ceralasertib 240 mg BD (D1–14) Of 51 evaluable patients, 1 CR (1.9%), 12 PR (23.5%), 18 SD (35.3%); ORR 25.5%	35
	I	Advanced cancers; $n = 55$	NA	Gemcitabine at escalating doses (500–1,000 mg/m^2, D3, D10, D17) + ceralasertib at escalating doses (40–120 mg, D1–21), q28 days Gemcitabine at escalating doses (500–1,000 mg/m^2, D3, D10, D17) + ceralasertib at escalating doses (40–120 mg, for up to 12 days), q28 days	NA	Recruitment ongoing	NCT03669601
	I	Advanced solid tumors with HER2 expression; $n = 15$	Positive HER2 expression by IHC (1+ to 3+) or HER2 amplification by FISH or NGS	Trastuzumab deruxtecan (D1) + ceralasertib BD (D1–7)	NA	Not yet recruiting	NCT04704661
	Ib	MDSs or CMMLs showing progression on frontline therapy; $n = 52$	*SF3B1* mutations: E622, Y623, R625, N626, H662, T663, K666, K700, V701, I704, G740, K741, G742, A774, and D781 *U2AF1* mutations: S34, R156, Q157 *SRSF2* mutations: P95 deletion, including amino acid P95 *ZRSR2* mutations: any frameshift or nonsense mutation	Ceralasertib monotherapy q28 days	NA	Recruitment ongoing	NCT03770429
	II	Advanced solid tumors; $n = 52$	Target 60% of participants to have ATM expression (by IHC) ≤ 5%	Ceralasertib monotherapy	NA	Recruitment ongoing	NCT04564027

(*Continued*)

Table 1 (*Continued*)

ATR inhibitor	Phase	Study population	Selective biomarker	Treatment	Safety	Efficacy	Reference or clinical trial
Elimusertib (BAY1895344)	I/Ib	Advanced solid tumors; $n = 241$	*ATM* deleterious mutation or ATM loss for expansion cohorts	Escalating doses of elimusertib monotherapy	Grade ≥4 TRAE: neutropenia (33% in 80 mg cohort, 17% in 60 mg cohort), thrombocytopenia (17% in 80 mg cohort)	ORR: 30.7% in patients treated at ≥40 mg BD	55
	I	Advanced gastrointestinal cancers; $n = 90$	NA	Elimusertib BD (D1, D2, D15, D16) + FOLFIRI (D1, D15) q28 days	NA	Not yet recruiting	NCT04535401
	I	Advanced solid tumors, pancreas and ovarian cancers; $n = 54$	NA	Elimusertib BD (D1–3, D8–10) + gemcitabine (D1, D8) q21 days	NA	Not yet recruiting	NCT04616534
	I	Advanced solid tumors, urothelial cancers; $n = 68$	NA	Elimusertib BD (D2, D9) + cisplatin (D1) q21 days Elimusertib BD (D2, D9) + gemcitabine (D1, D8) + cisplatin (D1) q21 days	NA	Not yet recruiting	NCT04491942
	I	Advanced solid tumors, SCLCs, neuroendocrine tumors; $n = 87$	NA	Elimusertib BD (D1–2) + irinotecan (D1) q14 days Elimusertib BD (D2, D5) + topotecan (D1–5) q21 days	NA	Not yet recruiting	NCT04514497
ART0380	I/II	Advanced solid tumors; $n = 180$	Target 40 participants to have loss of ATM expression	ART0380 intermittently OD (3 days on, 4 days off) or continuously ± gemcitabine 1,000 mg/m^2 (D1, D8) q21 days	NA	Recruitment ongoing	NCT04657068
RP-3500	I/II	Advanced solid tumors; $n = 239$	NA	RP3500 ± talazoparib	NA	Recruitment ongoing	NCT04497116

Abbreviations: AE, adverse event; AUC, area under the curve; CI, confidence interval; CMML, chronic myelomonocytic leukemia; CR, complete response; D, day; DLT, dose-limiting toxicity; FISH, fluorescence in situ hybridization; FOLFIRI, folinic acid, fluorouracil, irinotecan; HR, hazard ratio; HRD, homologous recombination deficient; IHC, immunohistochemistry; MDS, myelodysplastic syndrome; MTD, maximum tolerated dose; NA, not applicable; NGS, next-generation sequencing; ORR, objective response rate; PARPi, poly(ADP)-ribose polymerase inhibitors; PFS, progression-free survival; PR, partial response; RP2D, recommended phase II dose; SCLC, small-cell lung cancer; SD, stable disease; TRAE, treatment-related adverse events.

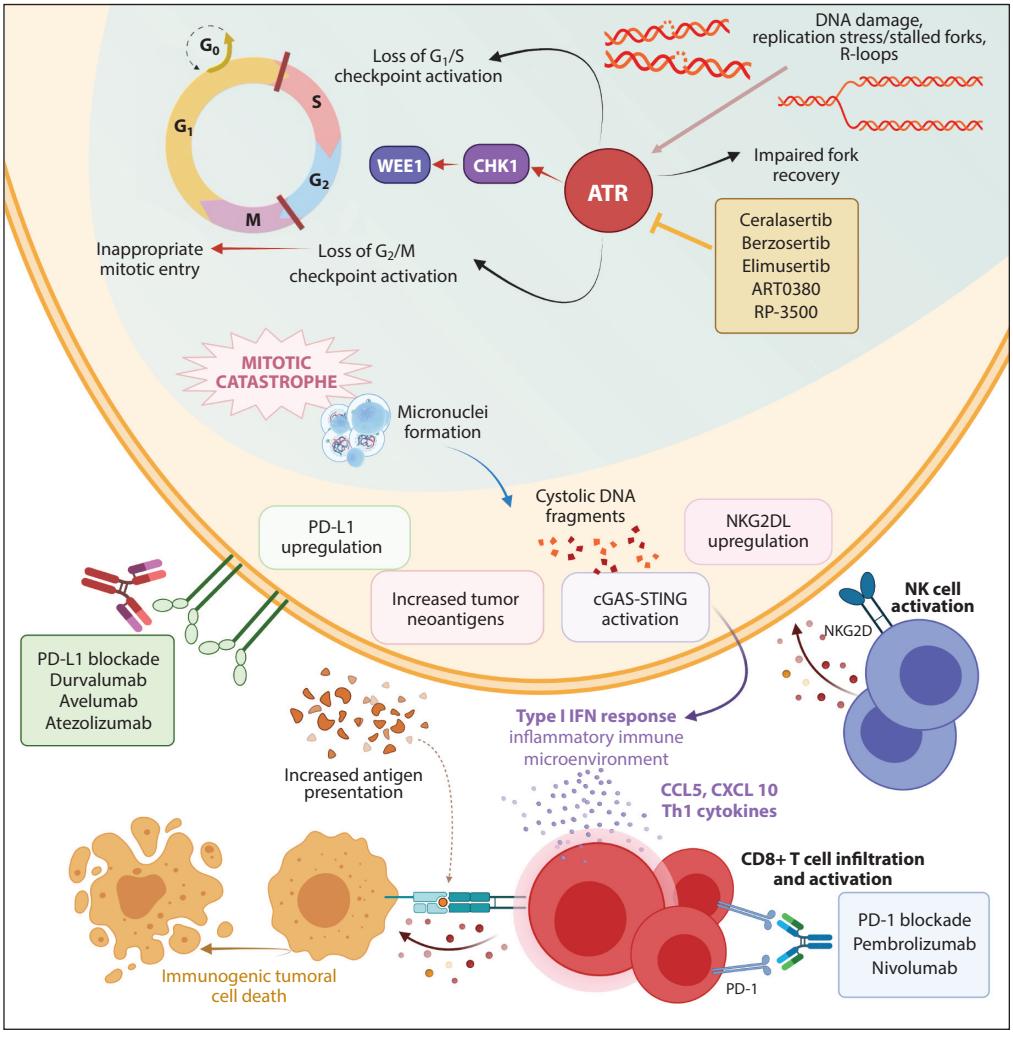

Figure 1

The ATR axis modulates antitumor immunity. ATR is activated in response to replication stress, single-stranded DNA, and increased R-loops, triggering a kinase cascade involving CHK1 and WEE1 that, in turn, leads to checkpoint activation and cell cycle arrest for DNA repair. ATR inhibition disrupts these functions of ATR, allowing inappropriate mitotic entry and culminating in mitotic catastrophe. The cytosolic DNA thus released may activate the cGAS-STING pathway and a type I IFN response. DDR may also be a means of increasing tumor mutational burden and therefore the generation of neoantigens. Abbreviations: ATR, ataxia telangiectasia and Rad3-related; CCL5, chemokine ligand 5; cGAS-STING, cyclic GMP-AMP synthase–stimulator of IFN genes; CHK1, checkpoint kinase 1; CXCL10, C-X-C motif chemokine ligand 10; DDR, DNA damage response; IFN, interferon; PD-L1, programmed death ligand 1; PD-1, programmed cell death protein 1; NKG2DL, natural killer group 2D cell surface ligand; Th1, T helper type 1; WEE1, wee-like protein kinase 1. Figure adapted from image created with BioRender.com.

Translational studies from this trial detected cyclical fluctuations in serum interleukin (IL)-12 levels in patients benefiting from this combination, indicating potential immunological mechanisms of action from this combination (35). Thus, the emerging role of ATR in the tumor immune microenvironment has led to great interest in combining ATR inhibitors with immunotherapy as a potential anticancer strategy.

ADVANCES IN ANTI-PD-(L)1 IMMUNE CHECKPOINT BLOCKADE IN THE CLINIC

The dawn of cancer immunotherapy has dramatically revolutionized our approach to cancer treatment. By harnessing the immune system to recognize and kill tumor cells, monoclonal antibodies targeting PD-1, programmed death ligand 1 (PD-L1), and cytotoxic T lymphocyte–associated protein 4 (CTLA-4) have become a backbone of therapy across several tumor types (36–43) and have afforded many patients the possibility of durable remission and even cure. The PD-1/PD-L1 axis promotes immune evasion and escape by inhibiting the survival and activation of cytotoxic T cells. PD-1 is constitutively expressed on most activated T cells, B cells, NK cells, and on many macrophages and dendritic cells (44, 45), while PD-L1 is expressed on tumor cells and on most myeloid cells. Interactions between PD-1 and PD-L1 induce and maintain immune tolerance within the tumor microenvironment by suppressing T cell coactivation signals that are usually triggered by T cell receptor binding with CD28, thus dampening immune activation. PD-1 interactions with PD-L2 in the tumor microenvironment similarly inhibit T cell function (46).

Despite the rapidly growing list of approved indications for immune checkpoint blockade, the current reality is that immune checkpoint blockade responses are variable because most patients harbor immunologically cold tumors due to multiple layers of immune suppression, such as suppressive cytokine release, lack of immune checkpoint protein expression, reduced tumor antigen presentation, and/or unfavorable metabolic states within the tumor immune microenvironment (47). Overcoming these obstacles will require new strategies to improve the efficacy of current immune checkpoint blockade therapies, analytically validated predictive biomarkers of response to personalize immunotherapy combinations, and even multipronged immune interventions to "warm up" the immunologically cold tumor milieu, in order to expand the accessibility of immunotherapy agents to wider patient populations.

COMBINING ATR INHIBITION WITH ANTI-PD-(L)1 THERAPY

Rationale, Preclinical and Clinical Data

ATR inhibition plays a potent immunomodulatory role in the tumor microenvironment through cyclic GMP-AMP synthase–stimulator of IFN genes (cGAS-STING) activation. Originally characterized as a pathway through which cells detect and sense microbial infection and autoimmune inflammation, the STING pathway has recently been recognized as the primary innate immune sensing pathway for tumor detection (48–50). Cytosolic DNA fragments arising through unrepaired DNA damage interact with cGAS-STING, thereby triggering tank-binding kinase 1 (TBK1) and subsequently an IRF3/nuclear factor κB–dependent transcriptional pathway, leading to increased type I IFN gene transcription (51, 52) and PD-L1 upregulation (50). In breast cancer, tumors harboring a 44-gene DDR-deficiency signature associated with loss of S-phase DDR contained increased cytosolic DNA and constitutive PD-L1 expression as a result of cGAS-STING upregulation (53). Increased IFN-related gene expression and $CD4^+$/$CD8^+$ T cell infiltration were observed in the microenvironment of these tumors (53). In advanced prostate cancer mouse models, features of S-phase DNA damage and cGAS-STING activation were observed in response to ATR inhibitor treatment, together with upregulation of C-X-C motif chemokine ligand 10 (CXCL10) and chemokine ligand 5 (CCL5), which are known transcriptional targets of IRF3, indicating activation of innate immunity (54). In support of these data, in a phase I clinical trial of elimusertib in advanced solid cancers, paired tumor samples indicated an upregulation of PD-L1 expression among a subset of patients with PD-L1-positive tumors after treatment with

elimusertib (55). Collectively, these data suggest that pharmacological inhibition of ATR and resultant DNA damage may contribute to cGAS-STING-mediated antitumor immunity and may prime tumors for immune checkpoint blockade. In colorectal cancer mouse models, the PD-L1 inhibitor avelumab led to improved tumor reduction and survival when added to berzosertib plus cisplatin or carboplatin, in comparison to berzosertib plus platinum chemotherapy alone (56). Significantly, mice who achieved a complete response to avelumab–berzosertib–platinum triple therapy were refractory to attempts at reinoculation with further MC38 colorectal cancer cells, suggesting the development of antitumor immunogenic memory (56).

Other preclinical studies have illuminated the synergistic role of ATR inhibitors with radioimmunotherapy (57, 58). In immunocompetent hepatocellular carcinoma mouse xenografts, the addition of ceralasertib to combined radiation therapy with PD-L1 inhibition led to increased $CD8^+$ T cell infiltration while reducing immunosuppressive T regulatory cell (Treg) infiltration, T cell immunoglobulin and mucin domain-containing protein 3 expression, and T cell exhaustion, leading to a more favorable tumor immune microenvironment in comparison to mice treated with radioimmunotherapy alone (57). Increased cGAS, phosphor (p)-STING, and p-TBK1 levels were observed, supporting cGAS-STING pathway activation. Importantly, improved survival was observed following the addition of ATR inhibition to radioimmunotherapy in vivo (57). In a second study of immunocompetent mouse models of human papillomavirus–driven cancer, the effect of combining ceralasertib and radiation on tumors was investigated using gene expression analysis, cytokine quantification, and flow cytometry (59). The ceralasertib and radiation combination was associated with a gene expression signature matching a type I/II IFN response, and it led to the upregulation of genes that play a role in nucleic acid sensing, including *Ddx58/RIG-I*, *Ifih1/MDA5*, *Zbp1/DAI*, and *Ddx60*. Furthermore, increased major histocompatibility complex class I levels were observed with increased antigen processing and presentation, as well as modulation of cytokine gene expression, especially CCL3, CCL5, and CXCL10, after combined ceralasertib and radiation exposure (59). A third preclinical study, conducted by Vendetti et al. (58), showed that the combination of ceralasertib and radiotherapy led to $CD8^+$ T cell–dependent antitumor responses in syngeneic mouse models as well as in mouse models of *KRAS* mutant cancer, with a trend toward greater tumor inhibition with the combination of ceralasertib and radiation in comparison to radiation alone. Durable antitumor responses were associated with the potentiation of $CD8^+$ T cell activity and attenuation of $CD8^+$ T cell exhaustion in the tumor microenvironment following combined ATR inhibitor and radiation exposure. Consistent with the prior report of ATR inhibitor mediated immune potentiation, Sheng and colleagues (57) found that ceralasertib reverted radiation-induced tumor PD-L1 upregulation and dramatically reduced the infiltration of Tregs, while modestly increasing the intra-tumoral density of IFN-γ-competent and proliferating ($Ki67^+$) $CD8^+$ T cells early after radiation exposure. This was followed by an even more pronounced increase in infiltrating $CD8^+$ T cells, reduced T cell exhaustion marker expression, and increased IFN-γ and tumor necrosis factor α (TNF-α) coproduction at days 9–12, signifying that the combination of ceralasertib and radiation promoted increased functional tumor-infiltrating $CD8^+$ T cells at later time points. Unexpectedly, this combination also generated immunologic memory in complete-responder syngeneic mice (58). Taken together, these findings suggest that an IFN response triggers antigen presentation and innate immunity following treatment with ATR inhibitor and radiation combination therapy and support the use of this rational combination.

A recent study by Chen et al. (60) has shed light on the presence of additional damage-associated molecular patterns triggered upon ATR inhibitor therapy, apart from cGAS-STING activation. Interestingly, loss of cGAS or STING did not completely eliminate inflammatory signaling upon ATR inhibitor therapy in cell lines (61), and the cytosolic RNA sensor retinoic

acid–inducible gene I was observed to be an additional component of this inflammatory response (60). Furthermore, ATR inhibition may play a potential role in increasing the tumor mutational burden (TMB) and neoantigen repertoire. Preclinical studies have explored the role of DDR inhibition as a means of increasing the TMB and therefore the generation of neoantigens (62), which may, in turn, increase sensitivity to immune checkpoint blockade by increased antigen presentation. An analysis of data from The Cancer Genome Atlas and The Cancer Immunome Atlas showed that samples harboring mutations in DNA damage signaling genes, including *ATR*, exhibited high neoantigen levels (62), enhancing the rationale for combining ATR inhibitors with anti-PD-(L)1 immune checkpoint blockade.

In the clinic, early-phase trials are actively studying combinations of anti-PD-(L)1 and ATR inhibitors (**Table 2**). The HUDSON multiarm clinical trial, which is investigating novel combinations for NSCLC patients who have progression on prior anti-PD-(L)1 immunotherapy agents and prior platinum-based chemotherapy, has reported preliminary data for the durvalumab-plus-ceralasertib combination (63). Among 44 non-biomarker-matched patients [24 and 22 of whom, respectively, had acquired (progression after 6 months) and primary (progression within 6 months) resistance to anti-PD-(L)1 immunotherapy agents], ORR was 8.7–11.1%, 6-month PFS was 37.0–53.8%, and 6-month overall survival (OS) was 74.8–77.3%. Among 18 biomarker-matched patients with *ATM* mutations or loss of expression, ORR was 13.3%, 6-month PFS was 61.2%, and 6-month OS was 100% (63). No obvious overlapping toxicities between the two drug classes were reported (63). An RNA-sequencing analysis of blood samples collected after initial ceralasertib monotherapy run-in prior to durvalumab addition showed that ceralasertib treatment led to significant increases in antigen-presentation gene expression signature and reduction in T cell exhaustion and NK cell signatures (63). An analysis of on-treatment samples also revealed reduction in four different macrophage gene expression signatures after ceralasertib treatment (63). These important findings corroborate the body of preclinical evidence supporting the role of ATR inhibitors in immune activation. Other clinical trials investigating the combination of ATR inhibitors with anti-PD-(L)1 inhibitors are ongoing, and results are eagerly awaited (**Table 2**).

ATR Versus PARP Inhibitor Combinations with Anti-PD-(L)1 Immune Checkpoint Blockade

The concept of targeting DDR pathways to modulate antitumor immunity originated from the combination of poly(ADP)-ribose polymerase (PARP) and immune checkpoint inhibitors (64). PARP inhibitors lead to the formation of DNA lesions through several mechanisms. PARP1 trapping at sites of DNA strand breaks leads to stalling and collapse of replication forks into single-strand breaks (SSBs). Unrepaired SSBs are digested into genotoxic DSBs when they are unrepaired, particularly in the presence of underlying homologous recombination deficiency, leading to synthetic lethality (65–67). PARP1 also affects replication kinetics by restricting replication fork speed (68). Several reviews have discussed in detail the rationale and reported efficacy of the combination of PARP inhibitor and immune checkpoint inhibitor therapy (64, 69). Briefly, preclinical studies have demonstrated increased intratumoral $CD8^+$ T cell infiltration and increased IFN-γ and TNF-α production after talazoparib (BMN673; Pfizer) treatment in *BRCA1* mutated ovarian cancer. The addition of anti-CTLA-4 immune checkpoint blockade to PARP inhibition further promoted T cell activation and survival with increased IFN-γ production compared with PARP inhibitor treatment alone (70). cGAS-STING-dependent PD-L1 upregulation after PARP inhibitor therapy has also been described (71). However, among reported trials, limited clinical efficacy has been demonstrated for PARP inhibitor and immune checkpoint blockade

Table 2 Trials investigating combination ATR and PD-(L)1 blockade

ATR inhibitor	PD-(L)1 blockade	Phase	Study population	Selective biomarker	Treatment	Efficacy	Reference or clinical trial
Berzosertib (M6620, VX-970)	Avelumab	I	Advanced solid tumors; $n = 36$	Gene mutations suggestive of DDR deficiency, including *ARID1A*, *ATM*, *ATR*, *ATRX*, *BAP1*, *BARD1*, *BRCA1/2*, *BRIP1*, *CDK12*, *CHEK2*, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCM*, *MRE11A*, *MSH2*, *NBN* (*NBS1*), *PALB2*, *RAD51*, *RAD51C*, *RAD51D*, *SMARCB1*, and *VHL*	Avelumab (D1, D15) + berzosertib (D1, D8, D15, D22) q28 days	Recruitment ongoing	NCT04266912
	Avelumab	II	Recurrent platinum-sensitive ovarian cancers that are resistant to PARPi; $n = 90$	NA	Part A: safety run-in of carboplatin AUC 5 (D1) + avelumab 1,600 mg (D1) + berzosertib 90 mg/m^2 (D2) q21 days Part B: chemotherapy (carboplatin + paclitaxel/gemcitabine/PLD) ± bevacizumab → bevacizumab maintenance, versus carboplatin + avelumab + berzosertib for six cycles → avelumab maintenance	Recruitment ongoing	NCT03704467
	Pembrolizumab	II	Advanced lung SCCs, chemotherapy naïve; $n = 18$	NA	Gemcitabine (D1, D8) + pembrolizumab (D1) + carboplatin (D1) ± berzosertib (D2, D9) q21 days × four cycles, followed by maintenance pembrolizumab ± berzosertib for up to 9 months, followed by maintenance pembrolizumab for up to 1 year	Recruitment ongoing	NCT04216316

(*Continued*)

Table 2 (*Continued*)

ATR inhibitor	PD-(L)1 blockade	Phase	Study population	Selective biomarker	Treatment	Efficacy	Reference or clinical trial
Ceralasertib (AZD6738)	Durvalumab	II	Advanced NSCLCs showing progression on immunotherapy; $n = 410$	Biomarker nonselected and selected populations Subjects with loss of ATM expression or *ATM* mutation were assigned to ceralasertib + durvalumab.	Novel durvalumab combinations including ceralasertib 240 mg BD (D1–14) + durvalumab 1,500 mg (D1) q28 days	Overall: ORR, 8.7–11.1%; 6-month PFS, 37.0–53.8%; 6-month OS, 74.8–77.3% Biomarker-matched patients: ORR, 13.3%; 6-month PFS, 61.2%; 6-month OS, 100%	NCT03334617
	Durvalumab	II	Advanced biliary tract cancers that have previously received immunotherapy; $n = 26$	NA	Ceralasertib 240 mg BD (D15–28) + durvalumab 1,500 mg (D1), q28 days	Recruitment ongoing	NCT04298008
	Durvalumab	II	Advanced NSCLCs showing progression on immunotherapy; $n = 120$	NA	Novel durvalumab combinations including ceralasertib 240 mg BD (D1–7 for cycle 1 and D22–28 for subsequent cycles) + durvalumab 1,500 mg (D1) q28 days Versus docetaxel chemotherapy	Recruitment ongoing	NCT03833440
	Durvalumab	I	Advanced solid tumors; $n = 330$	ATM loss, *BRCA* mutation	Ceralasertib 1–2 weeks monotherapy run-in followed by durvalumab 1,500 mg (D1) + ceralasertib 80–240 mg OD or BD for 1 (D22–28) or 2 (D15–28) weeks	Recruitment ongoing	87
	Durvalumab	II	Extensive SCLCs; $n = 30$	NA	Cisplatin/carboplatin (D1) + etoposide (D1–3) + durvalumab 1,500 mg (D1) q21 days × 4 cycles, then maintenance durvalumab 1,500 mg (D1) + ceralasertib 240 mg BD (D15–28) q28 days	Not yet recruiting	NCT04699838
Elimusertib (BAY1895344)	Pembrolizumab	Ib/II	Advanced solid tumors; $n = 110$	DDR deficiency biomarker positive, including *ATM* mutation	Elimusertib + pembrolizumab	Recruitment ongoing	NCT04095273
	Pembrolizumab	I	Recurrent HNSCCs; $n = 38$	NA	Elimusertib + pembrolizumab + RT	Not yet recruiting	NCT04576091

Abbreviations: AUC, area under the curve; D, day; DDR, DNA damage response; HNSCC, head and neck squamous cell carcinoma; NA, not applicable; NSCLC, non-small-cell lung cancer; ORR, objective response rate; OS, overall survival; PARPi, poly(ADP)-ribose polymerase inhibitors; PFS, progression-free survival; PLD, pegylated liposomal doxorubicin; RT, radiotherapy; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer.

combinations in platinum-resistant ovarian and gastric cancers, with these cohorts demonstrating ORRs of 14% (72) and 10% (73), respectively, for the durvalumab plus olaparib combination. However, an impressive 12-week disease control rate of 81% and an ORR of 63% were reported in the phase II MEDIOLA trial, which exclusively recruited germline *BRCA1/2* mutated patients, suggesting specific genomic contexts that are more likely to benefit from this strategy (74).

ATR inhibitors may harbor immune-modulating properties different from those of PARP inhibitors when combined with immune checkpoint blockade, even though both inhibitor classes purportedly cause cGAS-STING activation as a means of promoting innate immunity. Several studies have demonstrated that mitotic progression and chromosome missegregation after genotoxic stress are necessary for activation of type I IFN signaling that is associated with cGAS localizing to cytosolic DNA within micronuclei (60, 61, 75). Unlike PARP inhibitors, ATR inhibitors do not exacerbate degradation of replication forks but instead accelerate mitotic entry, as evidenced by chromatin bridge formation and chromosome lagging on DNA fiber analysis (75). Preclinically, inhibition of CDK1 to prolong cell cycle arrest and delay mitotic entry mitigates ATR inhibitor–related mitotic aberrancy, genomic instability, and resultant inflammatory-stimulated gene expression and immune destruction of tumors. This finding emphasizes the importance of ATR in coordinating cell cycle progression after DNA damage (60, 75) and the importance of mitotic progression in activating the cGAS-STING sensing axis, which have implications for the conceptualization and development of ATR inhibitor and immune checkpoint blockade combinations (57).

Interestingly, a study has suggested potential synergy from combining both ATR inhibitors and PARP inhibitors with anti-PD-1 blockade (75). ATR inhibitors target the G2/M cell cycle checkpoint to force mitotic entry in the presence of DNA lesions that arise from PARP inhibition in appropriate genomic contexts. Schoonen et al. (75) reported that olaparib treatment alone led to dose-dependent G2 arrest in BRCA2-depleted cells and resulted in a reduced percentage of mitotic cells, indicating delayed G2/M progression in a dose-dependent fashion. Treatment with an ATR inhibitor after olaparib led to forced mitotic entry, which potentiated the cytotoxic effects of olaparib in *BRCA2* mutated and *BRCA2* knockout cancer cell line models by enhancing genomic instability. ATR inhibitor therapy further increased the numbers of cGAS positive micronuclei and the extent of inflammatory cytokine release generated by olaparib treatment in these homologous recombination–deficient models, thus providing a preclinical rationale for the triplet combination of ATR inhibitors, PARP inhibitors, and anti-PD-(L)1 blockade (75). The main challenges with such an approach will be establishing a safe and effective combination dose and determining the contribution of each component of such a triplet combination in the clinic.

Selecting Patients for Combined ATR and PD-(L)1 Targeting

No confirmed biomarkers have been determined for ATR inhibitor targeting or combination ATR inhibitor and PD-(L)1 targeting strategies. Several putative biomarkers for ATR inhibitor monotherapy have been suggested on the basis of preclinical and clinical data. Loss of ATM function is hypothesized to increase dependency on ATR signaling and to cause synthetic lethality with ATR inhibition (76). In vitro and in vivo, ceralasertib was observed to be more active in ATM-deficient NSCLC xenografts compared with wild type by inducing mitotic catastrophe (17). Furthermore, phase I studies of ATR inhibitors observed durable and deep responses in a handful of patients harboring *ATM* loss when they were treated with elimusertib, ceralasertib, or berzosertib (26, 55) (**Table 1**), and trials exploring ATR inhibitor efficacy by using ATM loss or ATM aberrations as a selective biomarker are ongoing. However, the phase I HUDSON trial investigating durvalumab plus ceralasertib in immunotherapy-resistant NSCLC observed no correlation

between ATM biomarker status (defined by loss of ATM IHC or pathogenic *ATM* mutation) and objective tumor response (63).

The importance of mitotic progression and catastrophe leading to micronucleus production, which triggers cGAS-STING upregulation, suggests that the concurrent abrogation of G1 checkpoint through p53 loss could be relevant for ATR inhibitor and immune checkpoint blockade combination therapy. Chen et al. (60) demonstrated that although abrogation of the G2/M checkpoint facilitates micronucleus formation and cGAS-STING signaling, G2/M checkpoint disruption alone was insufficient to trigger an inflammatory response in cells with prolonged residence at the G2/M boundary, due to excessive unrepaired DSBs. In this situation, disruption of both the G1/S and G2/M checkpoints was required to trigger inflammatory signaling (60). Loss of p53 in the presence of an ATR inhibitor led to enhanced formation of micronuclei, cGAS attraction, and cytoplasmic DNA accumulation (60). Previous studies have shown that ATR inhibitor–plus–radiation therapy significantly increased IFN production and immune infiltration in p53-inactivated xenografts compared with radiation therapy alone (59). These findings suggest that combined abrogation of G1/S (through p53 loss) and G2/M (through ATR inhibition) checkpoints could enhance immune infiltration and anticancer responses. Yet, despite this rationale, the wide range of *P53* mutations and diverse functional consequences in different cellular contexts may make a detected *P53* mutation difficult to interpret, adding another level of complexity to the status of *P53* mutation as a potential biomarker for treatment with combined ATR inhibitors and PD-(L)1 inhibition.

Approved biomarkers used to select patients for PD-(L)1 monotherapy in several advanced cancers include tumoral PD-L1 expression by IHC and TMB (36, 38). In NSCLC, an increased PD-L1 tumor proportion score is associated with an increased benefit from the PD-1 antibody pembrolizumab (36). However, this correlation is less marked in other tumor types, wherein a large proportion of patients with high PD-L1 expression levels do not respond to anti-PD-(L)1 treatment, although a response may still be observed even in the absence of PD-L1 expression (77). Tumors harboring an elevated TMB, defined as the number of nonsynonymous mutations in the tumor genome, have an increased likelihood of response to PD-1 or combined CTLA-4/PD-1 blockade (38), given the hypothesis that a higher mutation frequency may result in higher-level and more diversified tumor antigen presentation and increased antitumoral immunogenicity. In June 2020, the US Food and Drug Administration granted tumor-agnostic approval for pembrolizumab monotherapy treatment of patients with a TMB above 10 mutations per megabase (78). However, the HUDSON trial of durvalumab plus ceralasertib in resistant NSCLC patients found no significant correlation between TMB or PD-L1 IHC scoring and response to this combination, suggesting that there exist determinants of immune response aside from these previously recognized immunotherapy biomarkers (63).

RELEVANT TRANSLATIONAL STUDIES FOR ATR AND PD-(L)1 TARGETING STRATEGIES

Assessments of Replicative Stress

Preclinical studies have suggested that ATR inhibitors selectively kill cells that are under high replication stress (79). In a proportion of early S-phase U2OS osteosarcoma and T98G glioblastoma cells, ATR inhibition induced massive ssDNA accumulation and mitotic catastrophe; however, in others, only moderate ssDNA accumulation occurred. Reduced ssDNA accumulation was linked to the triggering of backup pathways mediated by DNA-dependent protein kinase catalytic subunit and CHK1, which suppressed replication origin firing and led to a threshold of tolerable

replication stress that was not crossed by ATR inhibitor treatment (79). The investigators (79) observed that as the level of ssDNA rose, increasing fractions of ATR inhibitor–treated cells underwent mitotic catastrophe. This observation suggests that the level of cellular replication stress, as well as the amount of ATR inhibitor–induced ssDNA, may be predictive of ATR inhibitor sensitivity. Increased mitotic catastrophe and micronucleus formation after ATR inhibitor treatment in cells with elevated replication stress may increase inflammatory signaling through cGAS-STING activation, yet real-time monitoring of replicative stress in patients is likely to be difficult in clinical trials. Direct biomarkers of replicative stress, such as ssDNA, are transient and may be difficult to evaluate in tumor biopsies (6). Analysis of replication stress may be feasible using patient-derived organoids (PDOs) derived from pretreatment tumor samples and by subjecting these to ATR inhibitor treatment in order to prospectively determine replication stress levels. One study (80) successfully used PDOs as functional models of replication stress in high-grade serous ovarian cancer by performing DNA fiber assays on PDOs treated with ATR inhibitors, CHK1/2 inhibitors, and various cytotoxic chemotherapies. ATR inhibitor sensitivity was associated with replication fork instability determined through a DNA fiber assay performed on tumor PDOs (80). Such evaluations of replication stress levels may identify tumors with a higher likelihood of mitotic catastrophe resulting from ATR inhibitor therapy and inflammatory signaling for immune checkpoint blockade priming.

Multiparametric Immune Profiling

Owing to the complexity of host–tumor immune interactions across heterogeneous immune landscapes in different tumor types, it is unlikely that a single immune biomarker will be able to predict sensitivity to immune checkpoint blockade combination therapies, such as combined ATR inhibitor and PD-(L)1 immune checkpoint blockade treatment. The diversity of immune cells and the fluctuation of their pleiotropic activities between anti- and protumoral phenotypes in different tumor milieus may necessitate multiparametric assessment to better characterize tumor immune contexture and predict treatment response (81). With reference to clinical trials of PARP inhibitors plus immune checkpoint blockade combinations, translational studies have identified several potential markers associated with treatment response to these combinations by conducting multiparametric analyses of prospectively collected tissue and blood samples. During a phase II trial of olaparib plus durvalumab in prostate cancer, patients with lower baseline levels of myeloid-derived suppressor cells, increased $Ki67^+PD\text{-}L1^+CD4^+$ and $CD8^+$ T cells, and increased HLA-DR expression were significantly more likely to experience prolonged PFS (82). Increased markers of dendritic cell maturity, such as increased CD83 on $CD141^+$ dendritic cells, detected within the first 15 days of treatment, were also associated with prolonged PFS (82). The TOPACIO trial, which combined niraparib with pembrolizumab, analyzed tumor samples using immunogenomic profiling and multiplexed single-cell imaging, revealing that a positive immune score (a marker of IFN-primed exhausted effector $CD8^+$ T cells) correlated with objective response to treatment (83). Single-cell spatial imaging revealed prominent interactions between PD-L1 positive macrophages and tumor cells with exhausted $CD8^+$ T cells in treatment responders (83). With respect to circulating biomarkers, a trial of olaparib plus durvalumab in recurrent ovarian cancer reported correlations between increased systemic IFN-γ levels and improved PFS and clinical benefit (72); however, these findings were not reproduced in a phase II trial of small-cell lung cancer patients treated with the same combination (84). To date, only one clinical trial has reported a translational analysis after treatment with a combination of ATR and PD-(L)1 inhibitors (63). Among patients with advanced NSCLC treated with durvalumab plus ceralasertib, responders had greater-than-twofold-higher granzyme levels at baseline in comparison to nonresponders, while

patients who achieved disease control had a twofold reduction in peripheral IL-8 gene expression on paired blood samples in comparison to patients who had progressive disease (63).

FUTURE PERSPECTIVES ON DUAL ATR AND PD-(L)1 INHIBITION STRATEGIES

Despite promising preclinical data and early trial results suggesting the feasibility of ATR and PD-(L)1 cotargeting, many of the molecular details surrounding how ATR modulates immune signaling remain to be fully elucidated. Events downstream of ATR in the DDR pathway and changes in transcription or signaling factors will require further characterization in tumor- and genome-specific contexts in order to improve our understanding of ATR inhibitor–mediated immune modulation (85). Importantly, we need to clarify the optimal time point for dosing of ATR inhibitors with respect to anti-PD-(L)1 immune checkpoint blockade in order to achieve maximal immune activation. As described by Vendetti et al. (58), the effect of ceralasertib on PD-L1 expression appears to occur immediately after radiation therapy exposure, whereas features of immune activation and $CD8^+$ T cell infiltration seem to occur later, at days 9–12 after DNA damage exposure. Another study reported that changes in PD-L1 levels after DNA damage exposure do not persist beyond day 14 (33). Furthermore, it remains unclear whether the temporal aspects of PD-L1 expression dynamics would change among tumors with varying levels of background replicative stress (85). These aspects need to be better elucidated in order to optimally inform clinical trial design.

Finally, at present, little is understood about the potential resistance mechanisms to combined ATR and PD-L(1) inhibition. Preclinical studies have described the canonical nonhomologous end-joining pathway as a means of suppressing micronucleus formation after DNA damage, rendering cells unable to activate cGAS-STING inflammatory signaling (61). Tumor silencing of the cytosolic DNA sensing pathway may also be a mechanism of resistance (86). Improved preclinical models that can recapitulate the complexity of human immune systems are needed to help us better understand cancer response and resistance to immune checkpoint blockade combinations. Clinical studies of ATR inhibitor combinations with anti-PD-(L)1 immune checkpoint blockade are underway, and we expect that further data from these early-phase trials will help refine the future development of this rational strategy.

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AUTHOR CONTRIBUTIONS STATEMENT

T.A.Y. and N.Y.L.N. conceived of the article and curated the data. N.Y.L.N. wrote the original draft. N.Y.L.N., G.P., and T.A.Y. reviewed and edited the manuscript and prepared the visualization. T.A.Y. supervised preparation of the article. Project administration was done by N.Y.L.N.

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