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Harnessing R-loop dynamics: Challenging cancer therapy resistance

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ABSTRACT

R-loops are atypical three-stranded nucleic acid structures composed of a stretch of DNA:RNA hybrids that displace the unpaired, single DNA strand, resulting in the formation of a characteristic loop structure. When properly regulated, R-loops have been demonstrated to control crucial processes related to RNA metabolism, epigenetic gene regulation, DNA damage repair, homologous recombination, and DNA replication. However, unscheduled R-loops can induce DNA damage, thus compromising genome stability. In line with these central features, cancer cells frequently exhibit deregulated R-loop metabolism. The action of oncogenes or mutant tumor suppressor genes is associated with alterations in R-loop levels, which in turn can disrupt physiological processes or drive cancer genome instability. A panel of antineoplastic drugs that interfere with R-loop prevention, resolution or processing has been shown to exacerbate R-loop-mediated genome instability, modulate immunity pathways and mediate cell death. Mechanisms of resistance to these drugs are expected to include the activation of pathways that counteract R-loop-mediated genome instability. In this review, we will discuss key regulators of R-loops in cancer cells, therapeutic strategies that promote R-loop formation and the relevance of R-loops for cancer therapy resistance.

1. Introduction

RNA:DNA hybrids are atypical nucleic acid structures that predominantly form during the transcription of RNA, resulting in the displacement of the non-template strand and the formation of a three-stranded nucleic acid structure, commonly called "R-loop"[1]. However R-loops were also shown to form *in trans* by invasion of RNA into complementary regions, as demonstrated for telomeres or the pairing of guide RNAs to DNA target sites, a defining feature of type I and type II CRISPR-Cas systems [2–6]. In addition, Okazaki fragment synthesis during DNA replication leads to RNA:DNA hybrid formation without single-stranded DNA (ssDNA) displacement [7].

R-loops predominantly form behind progressive RNA polymerases (RNPAs), but have also been demonstrated to build up during RNA polymerase backtracking [8]. R-loop levels are dynamic and are modulated by growth conditions and developmental status and have been reported to range from 60 nucleotides to several kilobases. Sequencing-based techniques have revealed that R-loops occupy approximately 5–10 % of the genome, with enrichment at sequences with GC-skew. Consequently, DNA:RNA hybrids frequently promote G-quadruplex (G4) structures in the unpaired ssDNA loop with a corresponding impact on genome stability and gene expression [9–15]. Given the strong link to transcription, R-loops are prevalently genic and were recently subdivided into promoter-paused (Class I) and elongation-associated (Class II) R-loops [16]. R-loops were found to be enriched in proximity to sites of transcriptional initiation, but also at termination sites where RNA:DNA hybrid formation promotes the disengagement of RNA polymerase II (RNAPII) [13,17]. However, R-loops have also been mapped to enhancers, tRNA genes, rDNA arrays, and repeat-rich regions such as (peri)-centromeres, (sub)-telomeres, and transposable elements [17–21]. In total, vertebrate cells are estimated to process approximately 27,000 RNA:DNA hybrid structures per day, anticipating the existence of efficient management machineries that keep R-loop levels in check [22–25].

2. Classic factors controlling R-loop metabolism in vertebrate cells

Multiple studies report a central role of R-loop structures in normal cell physiology. R-loops were shown to control immunoglobulin class switch recombination (CSR), the initiation of DNA replication in the ColE1 plasmid, T4 bacteriophage, and mitochondrial DNA, the control of DNA methylation at gene promoters, the regulation of chromatin structure, termination of transcription, telomere stability, mitotic

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stability, but also DNA damage repair [17,26–32]. In addition, R-loops impact on epigenetic gene regulation and chromosome segregation [21, 33]. Together, this indicates that R-loop formation and resolution represent an integral part of cell physiology that is controlled by multiple mechanisms. Due to the involvement of R-loops in multiple molecular pathways, a growing list of factors have been demonstrated to control R-loop levels in vertebrate cells [34]. Classic R-loop management machineries include central players of RNA maturation such as the THO complex and SRSF1 [21,35]. Ribonucleases RNAseH1 and RNaseH2 degrade RNA components of R-loops [36]. ATP-dependent helicases such as the Bloom's syndrome helicase (BLM), Aquarius (AQR), FANCM, members of the DEAD and DExD/H-box RNA helicases, as well as chromatin regulatory factors such as the FACT complex; INO80 and SWI/SNF chromatin remodelling complexes and DAXX/ATRX histone chaperones prevent the formation of unscheduled R-loops [37–41]. Finally, factors that control replication fork stability, such as DNA Repair Associated 1 and 2 (BRCA1 and 2), Fanconi family proteins, topoisomerases, or DNA damage signalling factors such as Ataxia telangiectasia and Rad3 related (ATR), prevent an uncontrolled accumulation of R-loops [42–47]. Mutations and alterations in the expression of R-loop regulatory factors lead to the accumulation of unscheduled R-loops that impact on normal cell physiology and drive genome stability in human disease, including cancer and neurodegeneration [48, 49].

3. Unscheduled R-loops as source for genome instability

3.1. R-loops are hot spots for genome instability

R-loop structures are hotspots for mutations that occur via classic mutagenesis but also by inducing transcription-replication conflicts [1] (Fig. 1a, b). The exposed, ssDNA present in R-loop represents a prime site for mutagenesis by deaminases such as Activation-Induced cytidine Deaminase (AID) that converts cytosine to uracil that is followed by processing to DNA nicks by base excision repair factors (Fig. 1c, d) [50, 51]. Alternatively, single stranded DNA can also be cleaved by the FEN1, XPF or XPG endonucleases resulting single or double strand breaks [52, 53]. Reactive oxygen species (ROS) can induce DNA nicks that promote R-loop formation and DNA damage at telomeres [54,55] (Fig. 1c). Finally, exposed ssDNA can fold into secondary structures such as G-quadruplexes (G4) that cause conflicts with DNA replication and gene regulation [56]. Importantly, the major source of genome instability caused by unscheduled or pathological R-loops arises from conflicts with transcription and DNA replication. In this context, ectopic expression of RNaseH1 can resolve R-loops by digesting the RNA component of RNA: DNA hybrids, alleviating genome instability phenotypes [21]. (Fig. 2)

3.2. R-loops drive transcription stress

Unscheduled R-loops can drive transcription stress, a condition caused by excessive pausing, arrest, or backtracking of RNA Polymerases (RNAPs) [57–60] (Fig. 1a). On the other hand, persistent pausing of RNAPs has been linked with an increase in local R-loop levels [61,62]. This indicates that R-loop formation is tightly connected with

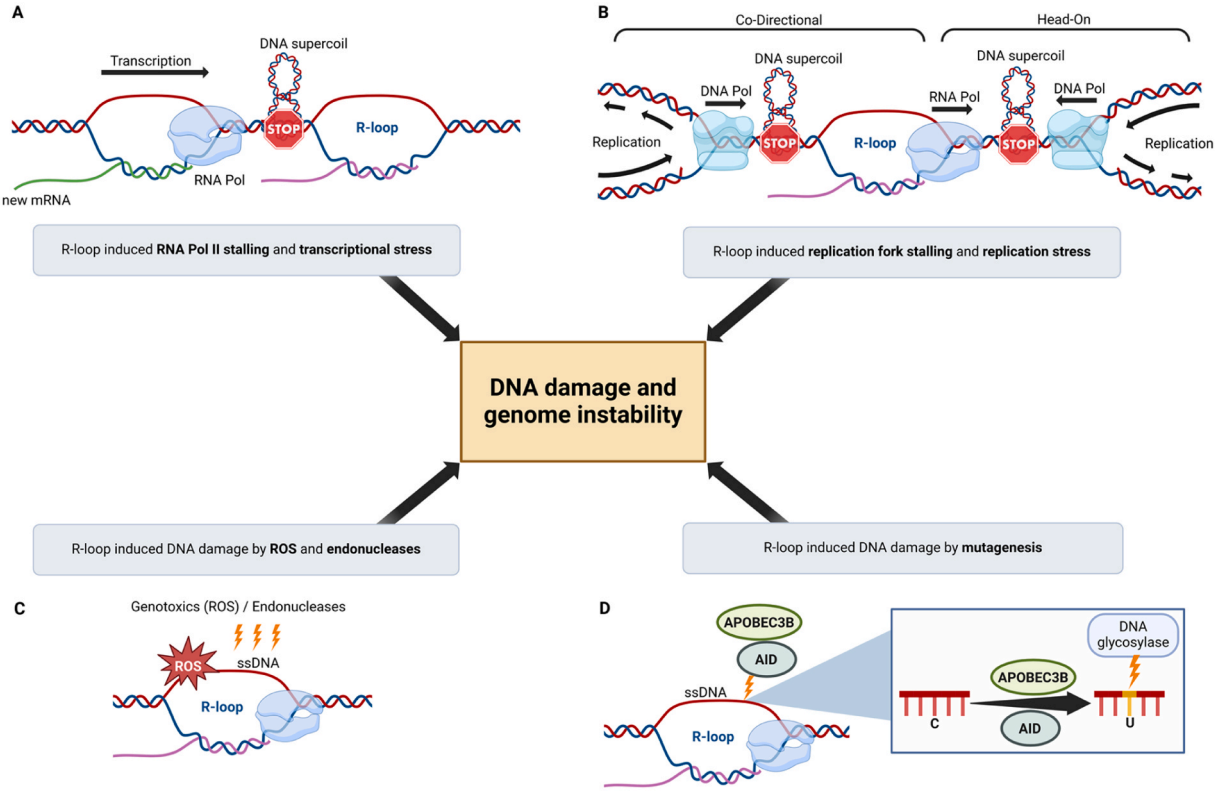

Fig. 1. Mechanisms of R-loop mediated genome instability. A. The presence of R-loops in front of RNA polymerases leads to transcription stress provoking RNA polymerase stalling. B. Head-on or co-directional transcription-replication conflicts. R-loop accumulation induce replication stress and genome instability by forming supercoiled DNA regions causing the stalling of the replication fork. C. R-loop as source of DNA damage through reactive oxygen species (ROS) and nucleases. The displaced ssDNA of R-loop structures is more exposed to genotoxic agents and endonucleases, thereby increasing susceptibility to DNA damage and genome instability. D. Mutagenesis by cytidine deaminases. The displaced ssDNA of the R-loop structure is more exposed to the action of deaminases such as AID and APOBEC3B, that convert cytosine residues into uracil. The uracil base is then processed by DNA glycosylases, leading to the formation of an abasic site, which ultimately results in the creation of a single-strand break.

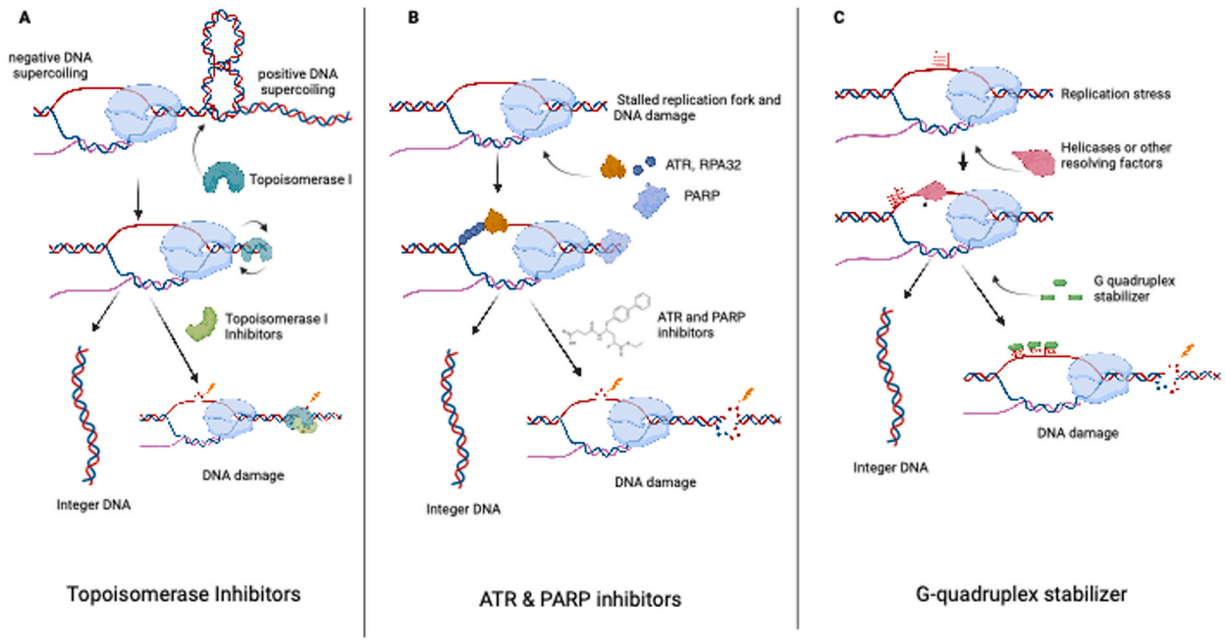

Fig. 2. Mechanisms of drug-induced, R-loop mediated genome instability: A. Topoisomerase I inhibitors prevent the resolution of positive and negative supercoils generated by progressing RNA polymerases. The accumulation of R-loops drives transcription-replication stress and genome instability. B. Inhibition of enzymes that directly respond to R-loops such as ATR and PARP inhibitors block R-loop resolution or processing, leading to genome instability. C. G-quadruplex stabilizers provoke G-loop formation and allow the formation of extended DNA:RNA hybrids that induce replication stress leading to genome instability.

transcription stress. However, R-loops can also support transcription by modulating promoter activity and ensuring transcriptional termination by mediating transcript release via the recruitment of RNA-DNA helicases SETX and DHX9 [11,26,63–65]. R-loop-mediated transcription stress driven by deregulated R-loops can be resolved by the nucleotide excision repair endonucleases XPG and XPF, which produce an ssDNA gap that progresses to a double-strand break. This engages downstream DNA damage repair in both cycling and non-cycling cells [66].

3.3. *R-loops promote replication stress*

The central source of DNA damage resulting from R-loops stems from conflicts between DNA replication and RNA transcription during S-phase of the cell cycle (Fig. 1b). The appearance of replication stress is a central outcome of experimentally increased R-loop levels [67–70]. Depending on the direction of processivity of the involved DNA and RNA polymerases, replication forks can encounter R-loops in head-on (HO) or codirectional (CD) orientations. Resulting transcription-replication conflicts (TRC) provoke the stalling of replication forks, which are either processed by DNA nucleases or collapse [11,23,71–73] (Fig. 1b). CD-TRCs are more tolerated in experimental cells, due to to Cdc45-MCM-GINS (CMG) helicase activity allocated at the progressing replisome, that holds DNA:RNA hybrid resolution activity. Alternatively, the replisome may also translocate to the 3'-OH of the involved RNA molecule, perform an extension synthesis, and rescue fork progression [23,74]. CD-TRCs specifically activate Ataxia telangiectasia mutated (ATM), which contrasts with HO-TRCs that are defined by the activation of ATR. Activation of ATM due to CD-TRC was observed in the context of WRN helicase mutations and depend on processing of R-loops by the NER pathway [52]. HO-TRCs are reported to be more detrimental, presumably due to the build-up of positive supercoils ahead of the progressing DNA and RNA polymerases, provoking fork stalling and replication stress [75–77]. At the same time, negative supercoiling behind the replication fork and the colliding transcription bubble promotes R-loop formation [78–80]. In line with this, several studies highlight the relevance of topoisomerases in suppressing R-loop formation and replication stress by releasing torsional stress that antagonizes RNA:DNA hybrid formation [81] (Fig. 1b). The appearance of replication stress induced by R-loop-mediated HO-TRCs leads to exposure of ssDNA, resulting in the specific activation of ATR as a central step in the processing of R-loops [72,82–84]. Activation of ATR by ectopic R-loops requires the structure-sensitive endonuclease MUS81 to process R-loops [85]. Once activated, ATR limits TCR frequency, promotes replication fork recovery, and induces G2/M arrest to allow full replication. Remarkably, activated ATR also limits MUS81 activity to protect replication forks from excessive cleavage [86]. ATR has also been shown to recruit R-loop resolving activities such as the RNA helicases DDX19 and DHX9 [83,84]. Commonly, replication stress resulting from R-loop mediated TRC leads to DNA damage and genome instability that is typically manifested by the appearance of cytoplasmatic DNA and micronuclei [23,87]

3.4. *R-loops as cause or consequence of DNA damage*

In normal conditions, cells avoid replication stress by spatial and temporal separation of transcription and replication, the use of replication barriers and preferential codirectional replication and transcription by positioning origins of replication upstream of transcription initiation sites [88]. In addition to genome organization that reduces the risk of TRCs, multiple factors resolve or antagonize R-loops explaining the observation that only 20 % of detectable R-loops are associated with replication stress markers [89]. The fact that mutations in genes that control replication, transcription or RNA:DNA hybrid management factors drive exacerbated R-loop mediated TRCs underlines the relevance of R-loops in molecular pathology [90]. However, also alteration of pathways that are not directly linked to the process of transcription or the rate of DNA replication can indirectly increase R-loop levels. In particular knock-down or inactivation of ATR/CHK1, ATM/CHK2 that signal HO-TCRs and CD-TCRs result in increased RNA:DNA hybrid levels [91]. In line with this, loss of function of other DNA damage checkpoint (DDC) genes such as BRCA1, BRCA2 or other Fanconi Anaemia proteins and also post-replicative repair (PRP) factors result in increased R-loop levels [42,92]. This suggests that accumulation of RNA:DNA hybrids due to impaired processing of an intermediate product rather than impaired

DNA damage response is causative for R-loop formation. Finally, RNA:DNA hybrids were also demonstrate to form at the position of DNA double strand breaks (DSBs) [93]. Thus, alteration of R-loop levels can be causative for genome instability but can also a consequence of DNA damage and genome instability.

3.5. RNA:DNA hybrids at double strand breaks modulate DNA damage repair

R-loops are a defining event for the induction of replication stress and DSB formation. However RNA:DNA hybrids are also reported to form at sites of DSBs, previously induced by anti-neoplastic drugs or radiation. In this context, hybrid structures can modulate downstream DNA damage repair processes [93–95]. Break-induced RNA:DNA hybrids (BIRDHs) are thought to be formed by RNA molecules that are already positioned at the future DSB position, either by local transcription or insertion into the double-stranded DNA *in-trans* [96–98]. Interestingly, also damage-induced long non-coding RNAs (dlincRNAs), that are produced by recruitment of RNAPII to DSB and subsequently processed to small RNAs to enhance DNA damage signalling, can engage in local RNA:DNA hybrid formation and support homologous recombination (HR) by recruiting BRCA1 and BRCA2 [99–101].Regulators of RNA:DNA hybrid abundance such as RNaseH1 or RNA helicases DDX1, DDX5, DHX9 and SETX were shown to limit BIRDHs levels at DSB resulting the activation of HR pathways [94,95,97,102–107]. The functional intersection HR with BIRDH helicases or RNases, exemplified by BRCA1-SETX, BRCA2-RNaseH2 and BRCA2-DDX5 complexes, suggest that controlled DNA:RNA hybrid resolution at DSBs may regulate the activation of HR dependent DNA damage repair [108–112]. Accordingly, aberrant accumulation of BIRDS was shown to lead towards non-homologous end joining (NHEJ) [97,103,113]. In addition to the role of BIRDHs at DSB, RNA:DNA hybrids formed at recombination donor DNA were shown to promote HR via a R to D-loop switch [114]. In this model RNAs produced at the site of DNA damage can invade into donor sites for HR, creating an R-loop. This facilitates pairing of the 3'overhang of the resected DSB with the exposed ssDNA of the R-loop at the donor site. The resulting DR-loop will be subsequently processed by removing the RNA component and processed by the HR machinery [115].

4. Cancer cells are under R-loop stress

The mutational and epigenetic setup, rapid proliferation and high transcriptional activity renders cancer cells vulnerable to R-loop mediated genome instability. Altered R-loop levels have been observed in solid and non-solid tumors and an increasing number of cell based studies demonstrate that mutations or altered expression of tumor suppressor genes and oncogenes causes the formation of unscheduled R-loops [48,116–119]. Cancer cells may take advantage of altered R-loop regulation by shaping gene expression, transcript maturation but also HR dependent maintenance of telomeres [48]. However, an excess of R-loops can mediate cancer genome instability that can promote tumor progression but also increase the vulnerability to anti-neoplastic therapies [48].

4.1. Oncogenes that drive R-loop Formation

Ectopic expression of the oncogene HRASV12 in immortalized human fibroblasts mediates elevated transcription by upregulating the TATA-box-binding protein TBP; this is paralleled by increased R-loop formation, replication stress, and genome instability [120]. Cyclin E overexpression drives the initiation of DNA replication, fuelling replication stress [121]. Human cancer or mouse cancer models confirm the relevance of oncogenes in driving R-loop formation. In Ewin Sarcoma, a paediatric bone and soft tissue tumor, the oncogenic EWSR-FL1 fusion protein mediates alterations in gene transcription and R-loop formation, driving replication stress and consequently an increased sensitivity to etoposide, ATR and PARP inhibitors [122]. SS18–SSX gene fusions in synovial sarcoma alter the composition of the BAF chromatin–remodelling complex, leading to elevated R-loop formation, replication stress, and increased sensitivity to ATR and PARP inhibitors [123]. The MYCN oncogene, a main driver of neuroblastoma, activates the histone H3 serine 10 specific Aurora kinase A (AURKA), which also ensures H3.3 deposition, finally leading to R-loop-mediated replication stress. Accordingly, this effect is paralleled by increased sensitivity to Aurora kinase A and ATR inhibitors [124]. In addition to oncogenic mutations, central signalling pathways in breast and prostate cancer increase R-loop levels. Estrogen exposure results in an elevated risk of breast carcinogenesis but also drives R-loop-mediated DNA damage in estrogen receptor-positive breast cancer cells [125]. Human prostate cancer is driven by the activation of androgen receptor (AR) signalling, sustaining growth and pathogenesis. In particular, the AR coactivator AR-interacting protein 4 (ARIP4) helicase collaborates with DNA Topoisomerase II β (TOP2B) to resolve R-loops at promoter-proximal regions, allowing RNAPII evasion [126]. Multiple studies connect R-loop management by DExH-Box Helicase 9 (DHX9) with cancer aggressiveness. DHX9 has been shown to directly interact with the AKT Serine/Threonine Kinase 1 (AKT1) to suppress R-loop-mediated replication stress, mediating poor patient outcomes in high-grade serous ovarian cancer (HG-SOC) [127]. In small cell lung cancer (SCLC) cells, DHX9 depletion drives R-loop-mediated genome instability that results in the formation of cytoplasmic DNA and activation of anti-tumor immune pathways, increasing the efficacy of anti-PD1 immunotherapy in mouse xenografts. Remarkably, DHX9 and immune signatures show a negative correlation in SCLC patients [128].

4.2. Tumor suppressor genes and R-loops

BRCA1 and BRCA2 are tumor suppressor genes that play crucial roles in DNA repair, particularly through HR. BRCA1 is a pleiotropic protein involved in both checkpoint activation and DNA repair, while BRCA2 acts as a mediator of HR. Both proteins were shown to antagonize the formation of unscheduled R-loops [129]. BRCA1 recruits SETX to resolve R-loops during transcriptional termination permitting the release of RNAPII and nascent RNAs and suppressing R-loop-mediated centromere instability [41,130]. In contrast, BRCA2 resolves RNAPII stalling by coordinating the ubiquitination of histone H2B by the RNF20/40 E3 ubiquitin ligase complex [60]. Loss of BRCA1 or BRCA2 causes R-loop accumulation, replication stress, DNA damage and defects in chromosome segregation that lead to micronuclei formation. BRCA1 and BRCA2 were also shown to ensure the stability of telomere repeats, classic hotspots for R-loop formation. BRCA1 and BRCA2 belong to the family of Fanconi Anaemia (FA) proteins. Consistent with this, other FA family proteins such as FANCD2, FANCA, FANCI, and FANCM suppress R-loop formation, and carriers of mutations in these genes are predisposed to various types of cancer [42,131–134].

The ribonuclease Dicer is reported as haploinsufficient tumor suppressor that also holds cleavage activity towards the RNA component of R-loops [135,136]. In line with this, DICER germline mutations in embryonal tumors with multi-layered rosettes (ETMRs) display elevated R-loop levels that match mapped breakpoints or detected single-nucleotide variants (SNV) [116]. Alterations in the spliceosome complex components SRSF2 and U2AF1 result transcripts carrying splicing defects that promote myelodysplastic syndromes (MDSs). SRSF2 mutations provoke RNAPII stalling, promoting R-loop formation, replication stress, and DNA damage [137]. Myeloproliferative neoplasms (MPNs) have been recently linked with altered R-loop regulation. In chronic myeloid leukemia (CML), the RNA exonuclease 5 (REXO5) was shown to use its RNA recognition motif (RRM) to selectively bind R-loops and degrade the associated RNA component; in line with this, REXO5 loss of function leads to R-loop accumulation and replication stress [138]. In familial myelodysplastic syndrome (MDS), mutations in

the DEAD box helicase 41 (DDX41) elevate levels of m6A RNA modification in R-loops, leading to impaired DNA damage response and genome instability [139,140]. ATRX (alpha-thalassemia mental retardation X-linked) is a chromatin remodelling factor that regulates gene expression and chromatin structure in an ATP-dependent manner [37]. ATRX mutations give rise to α-thalassemia and intellectual disability but are also frequently found in glioma, neuroblastoma, osteosarcoma, and pancreatic neuroendocrine tumors (PanNETs). A critical role of ATRX is to interact with the chaperone protein DAXX (death-domain-associated protein) to deposit H3.3 and suppress R-loop formation in telomeres, pericentric heterochromatin, and other DNA repeat sites to ensure genome stability [112–115].

In telomerase-negative tumors, ATRX mutations result in the accumulation of R-loops at telomeres, replication stress, and the formation of recombinogenic substrates that engage in homologous recombination, mediating the activation of the alternative lengthening of telomere pathway [141–144].

5. Pharmacological induction of R-loops in cancer cells

A series of studies demonstrated that depletion or mutating central R-loop regulators increases R-loop levels. However, also

Table 1
Drugs reported to induce R-loops in cancer cell models.

Drug Class	Drug	Drug target	R-loop modulation	Molecular Features	Model System	Reference
Topoisomerase I inhibitors	Irinotecan, SN−38, Topotecan, Camptothecin	Topoisomerase I	Accumulation of topological tension in DNA, favouring the formation and stabilization of R-loops.	DNA double-strand breaks, replication stress, mitotic instability, micronuclei, activation of innate immunity	HeLa, GBM, U−2 OS, A−673, WI38	https://doi.org/10.1093/narcan/zcad013 https://doi.org/10.1038/s41467–023–40243–8 10.1093/noajnl/vdab180 https://www.biorxiv.org/content/10.1101/2023.05.30.542894v2 10.1016/j.celrep.2019.08.041
Topoisomerase II inhibitors	Etoposide	Topoisomerase II	Unresolved DNA tangles during transcription, facilitating R-loop accumulation.	Chromosomal translocations, DNA damage, apoptosis.	TC−32, MR90, RMG1 cells	10.1038/nature25748 https://doi.org/10.1371/journal.pgen.1009238
ATR inhibitors	VE−821, AZD6738 (Ceralasertib), (VX−970) Berzosertib	ATR kinase	ATR is essential for preventing DNA damage in cells with elevated R-loop levels, results in the accumulation of unresolved R-loops.	DNA damage, replication stress, cell death, mitotic instability.	HeLa, U−2 OS	10.1016/j.molcel.2019.10.010 https://doi.org/10.1158/0008-5472.CAN-17-3970 https://doi.org/10.1038/s41467-024-50428-4
BET inhibitors	JQ1, ARV−825, YK−4–279	Bromodomain and Extra-Terminal domain (BET) family proteins	Deregulation of transcription, R-loop accumulation due to impaired RNA processing and RNAP stalling.	Transcriptional repression, DNA damage, apoptosis.	Hela, U−2 OS	http://www.genesdev.org/cgi/doi/10.1101/gad.331231.119. https://doi.org/10.1038/s41467–020–17503-y
HDAC inhibitors	Vorinostat (SAHA), Romidepsin	Histone deacetylases	Hyperacetylation of histones, resulting in open chromatin that can facilitate R-loop formation during transcription.	Altered gene expression, DNA damage, apoptosis.	BM CD34 + , LOXIMVI	https://doi.org/10.1038/s41467–024–46547–7 10.1158/1541–7786.MCR−20–0833
PARP inhibitors	Olaparib, Talazoparib, Veliparib	PARP enzymes (Poly (ADP-ribose) polymerases)	R-loop accumulation due to impaired DNA repair mechanisms; impaired R-loop resolution	Synthetic lethality in BRCA-mutant cells, DNA damage, cell death, mitotic instability, micronuclei.	Hela, U−2 OS	https://doi.org/10.1038/s41586–024–07217–2
Splicing inhibitors	Pladeinolide B (Pla-B), E7107,	SF3B1, a subunit of the spliceosome complex	Altered splicing can modify the structure of nascent RNA promoting stable R-loops; deregulation of pTEFb promotes RNAP stalling and R-loops	Hinders DNA replication; reduces the DNA damage response, DNA damage and increased instability of the genome	Hela, U−2 OS	10.1038/s41467–018–06677–1 10.1126/sciadv.abj8357 https://doi.org/10.1101/2021.03.19.436079
Transcription inhibitors	Trabectedin, Lurbinectedin	DNA	Formation of DNA adducts and blocking access of transcription factors, leading to increased R-loop formation,	Enhanced transcription-dependent genome instability, replication stress, DNA damage	Hela,	10.1158/1541–7786.MCR−18–0575
RNA helicase inhibitor	ATX968	DHX9	Block of R-loop resolution DHX9, alters the transcription process	Replication stress, DNA damage; especially in tumor cells with specific genetic alterations, such as mutations in the BRCA1/2 genes or microsatellite instability.	HCT116, LS411N	https://doi.org/10.1158/0008–5472.CAN-24–0397
G-quadruplex Stabilizers	Pyridostatin (PDS), FG, Braco−19, Quarfloxin (CX−35461)	G-quadruplex structures	G4 stabilization of promotes R-loop formation by hindering DNA replication and transcription processes; extended DNA:RNA hybrids	Telomere dysfunction, replication fork stalling, genome instability, micronuclei, activation of innate immunity.	U−2 OS, HeyA8, OVCAR8,	https://doi.org/10.1073/pnas.1810409116 https://hdl.handle.net/11585/834066 https://doi.org/10.3390/cancers13205056 10.1038/s41467–020–16393–4
Alkylating agents and Crosslinking agents	Cisplatin, Temozolomide (TMZ), benzo[α] pyrene, Mitomycin-C Simple aldehydes	DNA, DNA:RNA hybrids	Alkylating agents form DNA adducts, causing transcriptional stress and R-loop accumulation/ stabilization. Inter/Intrastrand crosslinking, stabilization of DNA:RNA hybrids	Replication stress, DNA damage, mitotic instability. DNA damage induced by crosslinking agents particularly affects cells with defects in the FA pathway.	HEK293T Hela U−2 OS	10.1093/nar/gkae845 10.1016/j.celrep.2024.115142 https://doi.org/10.1101/2024.02.22.581374 https://doi.org/10.1371/journal.pgen.1005674 10.1016/j.molcel.2015.09.012

pharmacological treatment with anti-neoplastic compounds can efficiently induce R-loop formation by: i) altering DNA topology, ii) blocking R-loop signalling or resolution, iii) modifying transcription, or iv) stabilizing non-B DNA structures (Table 1). This demonstrates that induction of R-loop mediated genome instability has an important contribution to the efficacy of cancer therapy.

5.1. Topoisomerase inhibitors

Toposiomerase 1 (TOP1) is efficient in removing negative supercoils behind progressing RNA polymerases to eliminate a DNA topology that favours R-loop formation. TOP1 also acts in front of RNA polymerases to allow efficient transcription by removal of positive supercoils. TOP1 action is coupled with RNAPs and splicing complexes during transcription, can remove the torsional stress generated by R-loop formation *in trans* an guides R-loop removal by helicases or RNaseH1 [145]. Camptothecin and its related derivatives including Irinotecan, Topotecan, Belotecan or SN38 are efficient TOP1 inhibitors that trap TOP1 cleavage complexes (TOP1cc), thus reducing the pool of active TOP1. This leads to an accumulation of topological DNA alterations that promote R-loop formation. In addition, covalent TOP1-DNA crosslinks cause RNA polymerase stalling, backtracking and R-loop formation [145,146]. In analogy to TOP1, also the class II toposiomerases TOP2A and TOP2B have been reported to resolve positive and negative supercoils, thus counteracting R-loop formations [147]. Interestingly, TOP2A also interacts with the E3 ubiquitin ligase RNF168 that ubiquitylates DHX9, a potent R-loop resolving helicase [127,148]. Accordingly, also the TOP2 inhibitor etoposide that has TOP2A as main target, has been reported to efficiently drive R-loop formation [38,122].

5.2. ATR inhibitors

As reported in Section 3.3 of this review, ATR represents a central player in sensing and processing R-loops, as well as controlling the activity of R-loop resolving mechanisms. Consequently, ATR inhibitors such as VE-821, VE-822, Ceralasertib or AZD6738 drive an accumulation of R-loops in cancer cells [43,149,150]. Excessive R-loop accumulation can induce genomic stress and DNA damage, contributing to cell death.

5.3. BET inhibitors

BET (Bromodomain and Extra-Terminal domain) inhibitors (BETi) block the interaction between Bromo domains of BET proteins and acetylated histones, thereby impairing transcription and enhancer function [151,152]. BET inhibitors also drive the formation of R-loops by induction of RNAPII stalling [153]. However, loss of the BET family protein BRD4 was also linked to reduced expression of key R-loop suppressors including RNaseH1, RNaseH2, TOP1, TOP2, and ATR [154]. Consequently, BET inhibitors drive R-loop formation via multiple pathways.

5.4. Histone deacetylase (HDAC) inhibitors

Histone deacetylase inhibitors (HDACs) mediate elevated levels of histone acetylation, generating an epigenetic code that promotes the activation of transcription. HDAC inhibitors impact on multiple pathways, resulting in overall antineoplastic action [155]. HDAC inhibitors such as Romidepsin or Vorinostat were shown to increase R-loop levels and single-strand DNA (ssDNA) breaks [156]. The impact of histone acetylation status on R-loop formation is also supported by the finding that Sin3A, a core component of the Sin3–HDAC complex, prevents R-loop accumulation and R-loop–mediated genomic instability [157]. Suppression of R-loops by the histone acetyltransferase (HAT) inhibitor, anacardic acid (AA), levels confirms the relevance of histone acetylation in the control of R-loop levels [157].

5.5. Poly (ADP-ribose) polymerase (PARP) Inhibitors

Binding of PARP1 to DSBs triggers its activation and the recruitment of multiple DNA repair proteins to the site of the damage. PARP inhibitors are particularly effective in tumor cells with defective homologous recombination, best exemplified by mutations in the R-loop suppressor BRCA1 and BRCA2 [158,159]. PARP1 is also a central factor in the repair and reactivation of stalled replication forks and has been shown to directly bind R-loops, leading to the activation of ADP-ribosylation activity [160]. Additionally, PARP1 interacts with multiple proteins that have R-loop resolution activity, such as DDX18 and DHX9 [161]. Consequently, genetic depletion or inhibition of PARP1 using Olaparib, Talazoparib or Veliparib drives the accumulation of unresolved R-loops, replication-transcription conflicts, and genome instability [160,162]

5.6. Splicing inhibitors

Mutations in splicing factors that cause myelodysplastic syndromes and acute myeloid leukemia have been reported to mediate R-loop-dependent activation of ATR [149]. Relevant mutations have been identified in the genes encoding the spliceosome complex components U2AF1, SF3B1, ZRSR2, and SRSF2 [163]. Altered splicing function is associated with the impairment of transcription elongation factor pTEFb, leading to defects in transcription from gene promoters, R-loop formation and TRCs [163]. In line with this, Pladeinolide B or Pladienolides A-G (E7107), that both target SF3B1, drive R-loop-mediated genome instability.

5.7. Inhibitors of transcription: trabectedin; lurbinectedin

Trabectedin and Lurbinectedin bind to the minor groove of DNA with a preference for specific DNA triplets. The action of these drugs impairs the recruitment of transcription factors and mediates the degradation of RNAPII. Treatment of experimental cells with these drugs has been shown to drive DNA:RNA hybrid-dependent obstacles to DNA replication [164].

5.8. G-quadruplex stabilizers

Guanine-rich sequences can promote the formation of G-quadruplexes (G4s) located in the single-stranded DNA loop of R-loop structures [165]. These structures, also known as G-loops, are stabilized by G4 stabilizers such as Pyridostatin (PDS) or Quarfloxin (CX-35461), resulting in the formation of extended stretches of RNA:DNA hybrids and genome instability [166–168].

5.9. Alkylating and crosslinking agents

Alkylating agents interfere with transcription and replication, causing replication stress and stalling of RNA polymerases. Both conditions favour R-loop formation [169]. Crosslinking agents, such as Mitomycin and Cisplatin drive R-loop formation, in particular in cells with defects in the Fanconi Anaemia pathway [170].

6. Connecting R-loops with resistance to cancer therapy

In recent decades, significant improvements have been achieved in cancer care; however, resistance to cancer therapy remains a major obstacle to successful treatment [171,172]. Therapy resistance can be categorized into intrinsic and acquired resistance. Intrinsic resistance is governed by the genetic, epigenetic, or metabolic setup of cancer cells prior to treatment [173,174]. In contrast, adaptive resistance occurs during cancer therapy and is mediated by a selection process that enriches for favourable genetic or non-genetic alterations in initially sensitive cancer cells [175,176]. Resistance to therapy is mediated by

multiple mechanisms that are not mutually exclusive. These include the amplification of oncogenes, mutations in drug targets, alterations in DNA damage repair pathways, the acquisition of stem cell properties, metabolic inactivation of drugs, enhanced drug efflux, drug compartmentalization, and modifications to the tumor microenvironment [177,178]. In addition, cancer stem cells play an important role in therapy resistance [179]. The observation that R-loop-mediated genome instability significantly contributes to the anti-neoplastic effects of multiple drugs also suggests that improved suppression or resolution of R-loops may contribute to adaptive or intrinsic therapy resistance.

6.1. Targeting R-loop metabolism to overcome therapy resistance

Therapy resistance has been documented for several drugs that drive R-loop-mediated genome instability, such as ATR inhibitors and topoisomerase inhibitors [180,181]. Accordingly, several studies report that impairing factors that prevent, signal or resolve R-loops increase sensitivity to anti-neoplastic drugs or challenge therapy resistance.

6.1.1. Drug sensitization by targeting R-loop prevention

Loss of members of the THO complex that has a critical role in RNA metabolism was shown to increase R-loop meditated genome instability, and promoted cancer cell sensitivity to cisplatin treatment in hepatocellular carcinoma and HEK-293 cells [182,183]. Clear cell ovarian cancer is characterized by frequent mutation of ARID1A, a member of the SWI/SNF family. ARID1A interacts with TOP2 and ATR, and its depletion drives R-loop formation, increasing sensitivity to etoposide [38].

6.1.2. Drug sensitization by impairing R-loop helicase activity

R-loop resolving helicases and RNaseH enzymes have a fundamental role in controlling R-loop levels under normal physiological condition but also modulate the sensitivity of cancer cells to anti-neoplastic treatment. In Human papillomavirus (HPV)-associated head and neck squamous cell carcinoma, cisplatin-resistant HPV-positive and HPV-negative cells exhibit elevated R-loop levels. Interestingly, the R-loop resolving helicase SETX is exclusively upregulated in HPV-positive, resistant cells. SETX depletion has a more pronounced effect on HPV-positive cells. This suggests an increased vulnerability to alterations in R-loop processing in HPV-positive cells [184]. Additional R-loop regulators such as DDX1, DDX5 and in particular DHX9 alter drug sensitivity of cancer cells. In particular, DDX5 promotes therapy resistance in prostate cancer cells, and its downregulation restores treatment sensitivity in castration-resistant prostate cancer [185,186]. The Zinc finger and BTB domain-containing protein 11 (ZBTB11) was shown to be expressed at high levels in bladder cancer. ZBTB11 drives DDX1 expression to suppress R-loops resulting in increased resistance to cisplatin and poor patient survival [187]. Recently, the DExH-box RNA helicase DHX9 has been demonstrated to act as potent R-loop resolution factor in different types of cancer cells. In Ewing sarcoma, the EWS:FLI1 fusion protein hijacks DHX9, preventing R-loop resolution and resulting in replication stress, thus sensitizing cells to genotoxic agents such as topoisomerase or ATR inhibitors [122,188]. Targeting the R-loop resolution helicase DHX9 has been shown to sensitize lymphomas to the Bcl2 inhibitor ABT-737 [189]. A drug-sensitizing effect by impairing DHX9 function was confirmed by the action of the C1orf109L protein, which competes with PARP1 to bind DHX9. Preventing PARP1-DHX9 interaction leads to enhanced R-loop formation that increases the sensitivity of cancer cell lines to camptothecin [190]. Also lncRNAs have recently been demonstrated to connect to DHX9. The taurine upregulated gene 1 (TUG1) lncRNA is highly expressed in various tumors and modulates central aspects of cancer biology, including therapy resistance [191]. Remarkably, under conditions of replication stress, TUG1 is upregulated through the ATR-CHK1 pathway, interacts with RPA and DHX9, and promotes the resolution of R-loops in microsatellite repeats. Targeting TUG1 improves the antineoplastic effect of the alkylating agent temozolomide (TMZ) by driving exacerbated R-loop levels in glioblastoma cells [192].

6.1.3. Drug sensitization by altering R-loop ribonucleases

In line with their action in resolving R-loops, ribonucleases were linked to drug sensitivity of cancer cells. In glioblastoma, cisplatin and the alkylating agent Temozolomide (TMZ) induce translocation of transketolase (TKT) from the cytoplasm to the nucleus, and together with 5′-3′ exoribonuclease 2 (XRN2), promotes R-loop removal and cell viability under genotoxic stress. Conversely, TKT depletion results in R-loop accumulation and increased sensitivity to Cisplatin and Temozolomide (TMZ) [193,194]. In melanoma, the single-stranded, DNA cytidine to uracil deaminase APOBEC3B drives R-loop-mediated replication stress that sensitizes cancer cells to ATR/Chk1 inhibitors. This effect is rescued by improved R-loop resolution via the ectopic expression of RNaseH1. Remarkably, the combination of high APOBEC3B and low RNaseH1 levels in patients may also serve as a predictive biomarker for ATR inhibitor sensitivity across various tumor types [195].

6.1.4. Drug sensitization by targeting DNA damage repair factors

A protein of the meiotic synaptonemal complex, SYCP2, is aberrantly expressed in breast and ovarian cancer, potentiating transcription-coupled homologous recombination. SYCP2 promotes R-loops at DSBs and enhances the recruitment of RAD51. This promotes transcription coupled homologous recombination (TC-HR). This mechanism supports resistance to treatment with TOP1 or PARP inhibitors; moreover high SYCP2 expression in patients is correlated with resistance to PARP or TOP1 inhibitors [196]. In hepatoma cells, PARP and the Tonicity-responsive enhancer-binding protein (TonEBP, also called NFAT5) interact and locate to R-loops and mediate their resolution in the context of pharmacological treatment with the TOP1 inhibitor Camptothecin (CPT). Accordingly, inhibiting TonEBP or PARP increases CPT sensitivity [197]

6.2. A complex network: R-loops, signalling pathways, and drug resistance in cancer

Genetic and epigenetic alterations of signalling pathways represent a central element in cancer formation, progression, metastasis, and resistance to therapy [198]. Recent studies provide indications that signalling pathways connect to R-loop management, thereby impacting on cancer cell biology and therapy response [199].

6.2.1. R-loops and the epidermal growth factor receptor (EGFR) signalling pathway

The EGFR signalling pathway supports survival, uncontrolled proliferation, migratory functions, and therapy resistance of cancer cells [200]. A recent *in silico* study on lung adenocarcinoma (LUAD) reports on a strong correlation between EGFR mutations, R-loop levels, and chemoresistance [201]. Low R-loop levels were found to be associated with poor prognosis, resistance to EGFR-targeting therapies, chemotherapy, and a limited response to immunotherapy in advanced tumors harbouring KRAS and TP53 mutations. Conversely, chemotherapy appeared to increase R-loop levels [201]. This suggests that EGFR may influence therapy response by modulating R-loop dynamics.

6.2.2. R-loops and the hypoxia signalling

A hypoxic tumor microenvironment (TME) supports cancer progression, metastasis and is strongly associated with the failure of anti-neoplastic therapy [202]. However, hypoxia has also been demonstrated to drive R-loop formation, which is limited by the concurrent upregulation of SETX via the PERK/ATF4 branch of the unfolded protein response (UPR). Accordingly, PERK inhibition under hypoxic conditions drives R-loop-mediated genome instability [203]. Hypoxia was also shown to induce the expression of the replication fork stabilizing protein Retinol Saturase (RESAT). RESAT interaction with R-loop resolving

DDX38 supports replication fork stabilization, leading to increased resistance to gemcitabine in pancreatic ductal adenocarcinoma (PDAC) [204].

6.2.3. R-loops and the PI3K/AKT/mTOR pathway

In PARP inhibitor-resistant, high-grade serous ovarian cancer (HG-SOC), a synergistic connection between the PI3K/AKT pathway and ATR was shown to control R-loop levels. In PARP inhibitor-resistant cells, DHX9 was found to interact with AKT1 to resolve R-loops, resulting in poor patient prognosis. Notably, the combined use of AKT and ATR inhibitors mediated near-complete tumor regression in HG-SOC xenograft models [127]. This suggests that the enhancement of R-loop formation in the context of impaired R-loop signalling using ATR inhibitors may represent a promising therapeutic strategy. In hepatocellular carcinoma (HCC) cells, the PI3K/AKT/mTOR pathway was linked to Aurora Kinase B (AURKB) and R-loop metabolism. RNAi-mediated depletion of AURKB increased R-loop levels, leading to reduced PI3K/AKT/mTOR pathway activity, thereby limiting cancer cell aggressiveness in mouse xenograft experiments. Mechanistically, AURKB interacts with DHX9 allowing to dampen R-loop levels. As expected, ectopic DHX9 expression rescued PI3K/AKT/mTOR pathway activity in AURKB-depleted HCC cells, connecting R-loop metabolism with the PI3K/AKT/mTOR pathway [205]. Remarkably, DHX9 loss of function has been shown to sensitize lymphomas to the Bcl2 inhibitor ABT-737, providing a link between R-loops and apoptosis [190].

6.2.4. Other examples for a connection of R-loops with signalling pathways

In prostate cancer, the RNA binding, Insulin-like growth factor 2 mRNA-binding proteins (IGF2BP) were shown to stabilize an N6-methyladenosine (m6A) containing RNA component of R-loops at the SEMA3F promoter, preventing DNMT1 binding. This results in increased expression of the soluble Semaphorin SEMA3F that activates the Hippo pathway. This leads to reduced proliferation and cell migration but also improves response to docetaxel [206,207]. In acute myeloid leukemia (AML), the lncRNA HOTTIP, interacts with CTCF and R-loop modulating factors including RPA1, DHX9, PARP1, SRSF3, MCM3 and SUPT16H and result the formation of R-loops at CTCF-binding sites (CBSs) in the AML genome. Thus was shown to support the formation of topologically associating domains (TADs) that increased the expression of Wnt/β-catenin target genes [208]. Reversion of this phenotype by ectopic expression of RNaseH1 confirms a role of R-loops in controlling the expression of the Wnt/β-catenin signalling pathway in AML.

Together, this indicates that R-loop metabolism is integrated into classic cancer cell signalling pathways that control cancer cell proliferation, invasiveness, and therapy resistance. Future research needs to dissect relevant pathways in more detail to understand how cancer cells may leverage these connections to survive and proliferate in the context of therapeutic challenges.

6.3. R-loops in normal and cancer stem cells

Cancer stem cells (CSCs) represent a small subpopulation of tumor cells that are characterized by their unique ability to self-renew, differentiate, initiate tumor formation, drive metastasis but also confer therapy resistance [179]. Given that CSCs share critical features with normal stem cells, current insights into the role of R-loop metabolism in normal stem cells may provide relevant indications for CSCs [209].

6.3.1. R-loops contribute to the maintenance and acquisition of embryonic stem cell self-renewal

The ectopic expression of pluripotency transcription factors OCT4, SOX2, KLF4 and C-MYC can override gene expression programs in differentiated cells and force the acquisition of induced pluripotency [210]. Interestingly, alterations in RNaseH1 expression or the ten-eleven translocation (TET) methylcytosine dioxygenases expression have been shown to impair the induction of pluripotency in mouse embryonic fibroblasts, thus affecting the acquisition of stem cell identity [211,212]. This demonstrates that stem cells and differentiated cells harbour defined programs of R-loop management that contribute to the establishment of cell identity. This concept was confirmed by genome-wide mapping of R-loops in self-renewing and differentiating embryonic stem cells (ESCs) [213]. In particular, this study has revealed a co-transcriptional formation of specific R-loop patterns. In self-renewing cells R-loop form along pluripotency genes; in differentiated cells R-loop patters shift to lineage defining genes differentiated cells. Notably, in differentiated cells a set of R-loops was shown to be allocated at repressive chromatin regions harbouring silent pluripotency genes and unrelated cells lineage specific genes [213]. On the level of R-loop management, the self-renewal transcription factor Sox2 has been shown to interact with DDX5, an R-loop resolution factor that blocks the induction of pluripotency. On the molecular level, Sox2 binds to DDX5, impairing R-loop resolution, which results in more efficient reprogramming of differentiated cells [211]. Together these reports highlight a role for R-loops in contributing to cell identify-defining gene-expression programs. Future experiments need to investigate the connection of R-loop management machineries with signalling and gene expression circuits in stem cells and differentiated cells.

6.3.2. R-loop management during stem cell differentiation

DHX9 interacts with Death Inducer-Obliterator 1 (DIDO) and RNAPII to resolve R-loops at transcriptional termination sites and enables the establishment of gene expression patterns that facilitate differentiation programs [212]. R-loops present at CpG-rich developmental regulator genes are involved in epigenetic gene regulation by recruiting Polycomb repressive complexes (PRCs) and promoting the formation of G-quadruplex (G4) structures. These structures stimulate CCCTC-binding factor (CTCF)-mediated chromatin loop formation, directing expression changes in bivalent genes that control early events in embryonic stem cell differentiation [214,215]. These data confirm results from a different study showing that R-loops and CTCF control gene expression by modulating three-dimensional chromatin conformations [216]. The zinc-finger protein ZFP281 was shown to control differentiation via different mechanisms. In stem cells ZFP281 interacts with BRCA2 and DDX5 at CpG islands to suppress R-loop-mediated blockade of S-phase progression and also allows full activation of genes that promote mouse ESC (mESC) differentiation [217]. ZFP281 also collaborates with TET1 at R-loops, impacting on DNMT3a/3b expression during early differentiation events [218]. Finally, R-loop-mediated DNA damage was also observed in early murine ESC differentiation; in this context the lncRNA Lnc530 was shown to suppress R-loop-mediated genome instability by interacting with DEAD-Box Helicase 5 (DDX5) [219,220].

6.3.3. R-loops control stem cell maintenance and quiescence

In addition to their beneficial role in shaping gene expression, unscheduled R-loops can deplete stem cell populations via the induction of genome instability. This is best exemplified in a mouse model with conditional deletion of DHX9 in mouse intestinal stem cells (ISCs), a prime model for adult stem cell biology. Loss of DHX9 triggers replication stress and genome instability, which culminated in ISC depletion and activation of innate immunity pathways [221]. Interestingly, in quiescent muscle stem cells R-loops and activated ATR are readily detectable and keep stem cells in a quiescent state. This arrest was released by conditional knockout of ATR or ectopic epression of RNaseH1 demonstrating R-loop dependence [222]. Although not directly tested, R-loop related pathways may also contribute to the role of ZFP281 in programming a dormant, mesenchymal or pluripotency-like gene expression program that prevents the acquisition of an epithelial-like proliferative program and consequent metastasis outgrowth [223].

6.3.4. Relevance for r-loops in cancer stem cell populations

To this and only limited information on R-loops in controlling cancer

stem cell biology is available. However a recent study proposes such a function in cancer stem cell populations in Acute Myeloid Leukemia (AML). In particular, PIWIL4, a germ stem cell–associated RBP belonging to the RNase H like superfamily was found to be overexpressed in AML and essential for leukemic stem cell function [224]. PIWIL4 was found to suppress R-loop accumulation, replication stress and DNA damage. Importantly, loss of PIWIL4 increases sensitivity to ATR inhibitors, suggesting a model where PIWIL4 may ensure the viability of CSC like population by suppressing R-loop mediated genome instability. This demonstrates that also cancer stem cell compartments encode specific R-loop management programs to maintain cell viability. Consequently, understanding R-loop regulation in cancer stem cell populations holds great potential to identify novel types of CSC vulnerabilities.

7. Conclusion and future outlook

In the last decade, significant progress in the molecular characterization of R-loops and their roles in cell physiology and genome instability has been achieved. Numerous factors that either prevent R-loop formation by shaping DNA topology, chemically modifying the RNA component of R-loops, or aiding the suppression, resolution or processing of RNA:DNA hybrid structures have been identified [1,19,23]. The tight link between R-loop management, oncogenes and tumor-suppressors further highlight their relevance for human cancer. Patients carrying mutations that predispose to R-loop accumulation may experience an accelerated accumulation of mutations, increasing the risk of cancer formation. In more advanced stages of cancer, increased replication conflicts need to be moderated by employing R-loop management machineries to sustain cancer cell proliferation.

Consequently, treating cancer cells with elevated R-loop levels using inhibitors that target factors that prevent or resolve R-loops, such as topoisomerase inhibitors, PARP inhibitors, or ATR inhibitors, may result in exacerbated genome instability that is incompatible with cancer cell viability [122,188,196]. Alternatively, therapeutic combinations with inhibitors of R-loop resolution and prevention may increase therapeutic efficacy (Fig. 3A). For example, combining ATR inhibitors with alkylating agents such as temozolomide resulted in synergistic effects in cancer treatment [192]. The combination of the topoisomerase inhibitor topotecan and ATR inhibitors such as berzosertib, both known to inhibit two distinct R-loop pathways, results in enhanced efficacy in the treatment of small cell lung cancer [225]. R-loop-mediated genome instability is also linked with the formation of cytoplasmic DNA species or cytoplasmic RNA:DNA hybrids that activate pathways of innate immunity via the cGAS/STING pathway [226–230]. The connection between R-loop-mediated genome instability and the formation of a pro-inflammatory tumor microenvironment may identify immunotherapies as a favourable treatment option [231]. Thus, the development of drugs that target additional R-loop resolution machineries is highly interesting in order to provide more options for integrating R-loop metabolism into antineoplastic treatment strategies [165,172–176,178,179,196] (Table 1). The newly developed DHX9 inhibitor, ATX968, is an

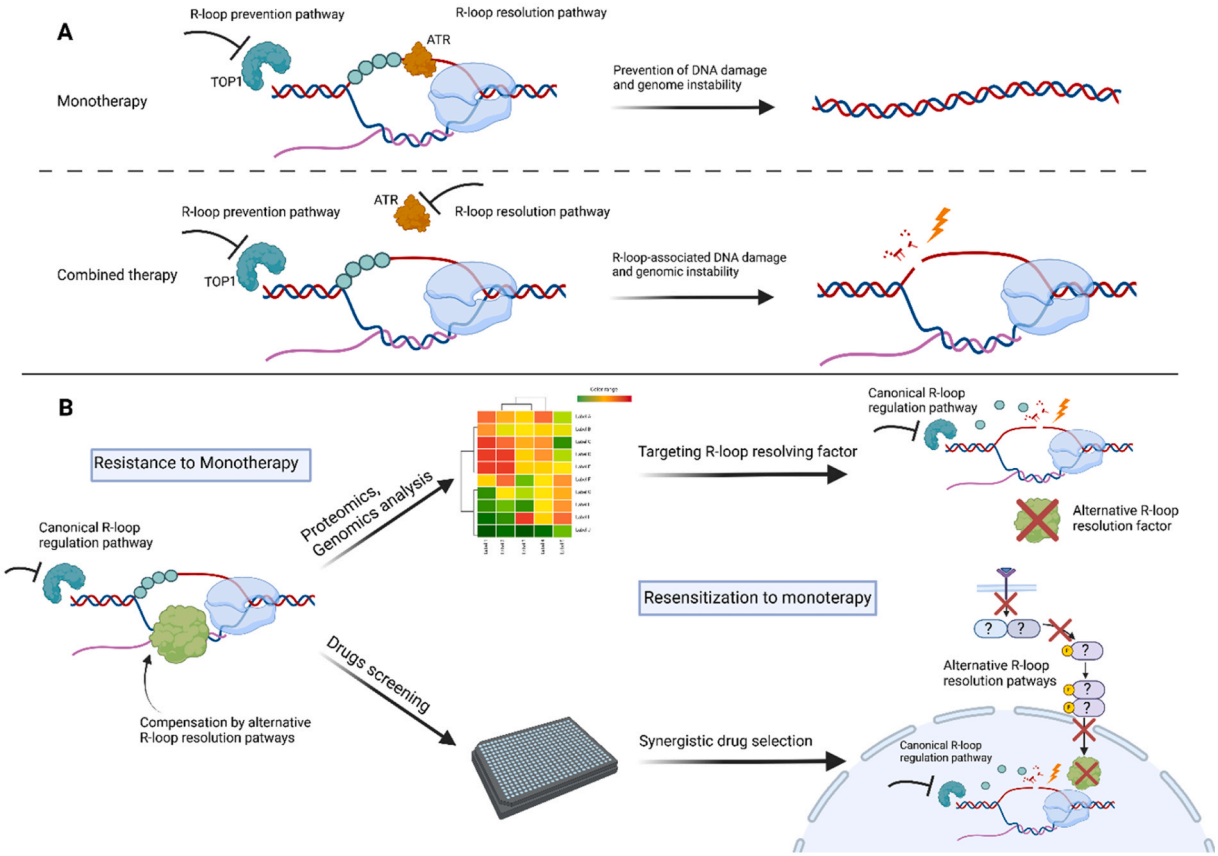

Fig. 3. Strategies to overcome therapy resistance in cancer by targeting R-loop management. A. The efficacy of R-loop driving drugs, such as TOP1 inhibitors may be limited by endogenous R-loop resolution machineries (top panel). The synergistic action of two drugs that block different R-loop resolution pathways in cancer cells can lead to an exacerbation of replication stress, causing DNA damage and genome instability levels that are incompatible with cancer cell viability (bottom). B. Upregulation of R-loop prevention or resolution machineries may have an important contribution to the establishment of cancer therapy resistance. Genomics and proteomics analysis can identify upregulated R-loop management factors in cancer cells with resistance on R-loop inducting drug (i.e. ATR inhibitor or etoposide). Targeting of such R-loop regulators may re-sensitize cancer cells to the original pharmacological treatment (top, right). Alternatively, therapy resistant cancer cells can be subjected to unbiased or directed drug-screenings to identify compounds that block R-loop resolution pathways allowing to re-sensitize resistant cancer cells to the original pharmacological treatment. An alteration of R-loop metabolism needs to be experimentally validated.

intriguing example of such a strategy [232]. However, targeting pathways that influence the expression or activity of R-loop resolution or prevention factors may also be a promising strategy. This option may enhance the genotoxic effect of classic R-loop-inducing drugs or give an opportunity to re-sensitizing therapy resistant cells. To date, the EGFR, Wnt/β-catenin and PI3K/AKT/mTOR pathways but also hypoxia have been directly or indirectly linked to R-loop management [125,189,202,203,205,208,233]. Given the central relevance of these signalling pathways for cancer cell proliferation, invasion, metastasis, and DNA damage repair, we hypothesize that the control of R-loops management may represent a new feature of these pathways in the control of cancer genome stability and eventually promote therapy resistance. Future research should aim to identify additional cancer-relevant pathways and functionally dissect signalling cascades to integrate R-loop metabolism as a central element of cancer cell physiology. Resistance to classic R-loop-driving compounds, such as topoisomerase inhibitors or ATR inhibitors, has been reported in the literature [180,234]. This suggests that intrinsic or acquired therapy resistance may be characterized by the imposition of mutational, metabolic, or epigenetic profiles that enhance the cancer cell's capacity to strengthen R-loop management machineries to mitigate genome instability. Approaches based on genomic or proteomic analysis of therapy-resistant cancer cells or patients can be useful to identify upregulated R-loop management factors that enhance R-loop prevention or resolution (Fig. 3b). Direct targeting of proteins that actively limit R-loop levels may induce sensitivity to the original therapeutic agents. The availability of several databases and reports on R-loop binding and associated factors represent valuable tools for selecting functionally relevant players [62,235–238]. Alternatively, unbiased, directed or drug-repurposing screens using therapy-resistant cells may identify compounds capable of targeting relevant cancer pathways that promote R-loop management, thereby re-sensitizing cells to the original treatment with R-loop inducing drugs such as ATR inhibitors or etoposide (Fig. 3b).

Finally, future research should also aim to conduct genome-wide mapping of R-loop dynamics in cancer patient-derived tissues, cancer stem cell populations, or patient-derived cell model systems in the context of pharmacological treatment. This may lead to the identification of loci with high susceptibility to R-loop-mediated DNA damage that may hold clinical relevance.

In the past years, research on R-loops in the context of cell physiology and genome instability has experience a rapid acceleration. Connecting R-loop management to cancer-relevant pathways and performing detailed R-loop mapping in patient-derived specimens or cell model systems will provide new insights into R-loop-mediated genome instability, unlocking novel therapeutic strategies for cancer treatment.

CRediT authorship contribution statement

Michele Giaquinto: Writing – original draft. **SCHOEFTNER Stefan:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Andrea Parlante:** Writing – original draft. **Alessandro Framarini:** Writing – original draft.

Declaration of Competing Interest

The authors declare no competing interests.

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Data availability

No data was used for the research described in the article.

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