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The G-quadruplex ligand CX-5461: an innovative candidate for disease treatment

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Abstract

The ribosomal DNA (rDNA) plays a vital role in regulating protein synthesis by ribosome biogenesis, essential for maintaining cellular growth, metabolism, and more. Cancer cells show a high dependence on ribosome biogenesis and exhibit elevated rDNA transcriptional activity. CX-5461, also known as Pidnarulex, is a First-in-Class anticancer drug that has received 'Fast Track Designation' approval from the FDA. Initially reported to inhibit Pol I-driven rDNA transcription, CX-5461 was recently identified as a G-quadruplex structure (G4) stabilizer and is currently completed or undergoing multiple Phase I clinical trials in patients with breast and ovarian cancers harboring BRCA1/2, PALB2, or other DNA repair deficiencies. Additionally, preclinical studies have confirmed that CX-5461 demonstrates promising therapeutic effects against multifarious non-cancer diseases, including viral infections, and autoimmune diseases. This review summarizes the mechanisms of CX-5461, including its transcriptional inhibition of rDNA, binding to G4, and toxicity towards topoisomerase, along with its research status and therapeutic effects across various diseases. Lastly, this review highlights the targeted therapy strategy of CX-5461 based on nanomedicine delivery, particularly the drug delivery utilizing the nucleic acid aptamer AS1411, which contains a G4 motif to specifically target the highly expressed nucleolin on the surface of tumor cell membranes; It also anticipates the strategy of coupling CX-5461 with peptide nucleic acids and locked nucleic acids to achieve dual targeting, thereby realizing individualized G4-targeting by CX-5461. This review aims to provide a general overview of the progress of CX-5461 in recent years and suggest potential strategies for disease treatment involving ribosomal RNA synthesis, G4, and topoisomerase.

Keywords CX-5461, Ribosomal RNA, Ribosome biogenesis, G-quadruplex, Cancer, Targeted therapy

Introduction

CX-5461, commonly known as Pidnarulex, is derived from the fluoroquinolone A-62176, a topoisomerase (TOP) II poison [1]. Initially described in 2011, CX-5461 was noted for its ability to selectively inhibit RNA polymerase I (Pol I)-mediated transcription by disrupting the initiation stage of ribosomal RNA (rRNA) synthesis. Moreover, it induces senescence and autophagy through a p53-independent process in solid tumor cell lines [2]. Additionally, CX-5461 can induce apoptosis in tumor cells by triggering DNA damage response pathways and apoptosis signaling pathways [3–5]. In recent years, CX-5461 has

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demonstrated substantial anti-tumor activity across a range of tumor types, particularly those characterized by DNA replication or DNA damage repair deficiency, including prevalent cancers such as breast cancer, ovarian cancer, gastric cancer, and prostate cancer. Currently, CX-5461 has been granted 'Fast Track Designation' by the FDA, and is completed and undergoing several phase I clinical trials, underscoring its promising therapeutic potential.

A crucial study in 2017 confirmed that CX-5461 acts as a DNA G-quadruplex (G4) stabilizer [6], specifically targeting telomeric G4 s [7] and promoter G4 s associated with numerous oncogenes, such as *c-KIT1* and *c-MYC* [8], therefore inducing DNA breaks and genomic instability, disrupting replication forks, impairing DNA repair mechanisms, and modulating the expression of pivotal genes [9]. Subsequent studies revealed that CX-5461 exerts its cytotoxic effects in cancer therapy through TOP poisoning [10], with a particular targeting TOP IIα, and TOP IIβ. Compared to conventional chemotherapy agents, CX-5461 exhibits multiple unique mechanisms of action, enabling it to overcome the drug resistance exhibited by tumor cells to other treatments. Moreover, it represents a promising combination strategy of CX-5461 and immunotherapy, offering new avenues for treating refractory tumors. Additionally, CX-5461 has demonstrated efficacy in treating various non-neoplastic diseases, including viral infections, immune disorders, and cardiovascular conditions, highlighting its broad therapeutic potential.

In summary, the research progress in various diseases, especially in the field of cancer, confirms that CX-5461 is a promising disease treatment candidate. This review summarized the mechanism of action of CX-5461, emphasizing its function of RNA Pol I inhibition, G4 stabilization, and TOP poison; and then formulated the research status and therapeutic effects of CX-5461 in different diseases; Furthermore, given the ubiquitous presence of G4 s and TOP in a diverse array of tissues and cells, the non-specificity associated with the utilization of C-5461 in disease treatment may result in unforeseen adverse effects, the targeted therapy strategies of CX-5461 based on nano-drug delivery and the potential individualized G4-targeting strategies are emphasized in this review; finally, it will elaborate on the challenges and prospects of applying CX-5461 in clinical practice. This review endeavors to furnish a theoretical foundation for the advancement of further research and clinical trials involving CX-5461, as well as other rRNA synthesis inhibitors, G4 stabilizers, topoisomerase poisons, and tumor immunotherapy.

Mechanism of action of CX-5461 in tumor treatment

Inhibition of rRNA synthesis

Ribosomes serve as the cellular sites for protein synthesis. The biosynthesis of ribosomes guarantees that cells can effectively produce the necessary proteins, a process comprising several stages: transcription, processing, assembly, and maturation [11]. During transcription, RNA polymerase I (Pol I) executes transcribing rRNA genes to produce 47S precursor rRNA, a rate-limiting step in ribosome biogenesis, and transcription factors such as TOP I, upstream binding factor (UBF), transcription initiation factor RRN3, transcription initiation factor IA (TIF-IA) and selective factor SL1, serve essential auxiliary and regulatory functions [12]. Following this, the processing stage involves a series of precise cleavage and modification steps that convert the 47S precursor rRNA into mature 18S, 5.8S, and 28S rRNA. Subsequently, in the assembly stage, the mature rRNAs combine with ribosomal proteins to form the two subunits of the ribosome: the small and large subunits [13]. Lastly, during the maturation stage, these two subunits are further assembled into complete ribosomes within the cytoplasm, rendering them ready for protein synthesis [14].

It has affirmed that ribosomal biosynthesis is intimately linked to tumorigenesis [15], primarily evidenced by the aberrant regulation of ribosomal biosynthesis in tumor cells, which includes the heightened activity of RNA Pol I, the overexpression of rRNA, and increased production of ribosomal proteins. These alterations result in an elevated number of ribosomes and boosted protein synthesis capabilities within tumor cells, thereby facilitating the energy metabolism and biosynthetic pathway, and supporting the rapid proliferation and growth of tumor cells. Given its pivotal role in tumorigenesis, ribosomal biosynthesis has emerged as a promising target for cancer therapy. By targeting the activity of RNA Pol I, disrupting the assembly process of ribosomes, or impairing ribosomal function, it becomes feasible to suppress the protein synthesis capacity of tumor cells, ultimately hindering their proliferation and growth [16]. To date, a range of inhibitors targeting ribosomal biosynthesis have been developed, including RNA polymerase I inhibitors such as BMH-21 [17], rRNA processing inhibitors like Actinomycin D and Oxaliplatin [18], ribosomal subunit assembly inhibitors including Shiga toxin and Shiga-like toxin [19], as well as ribosome function inhibitors comprising tetracyclines and macrolide antibiotics [20, 21], which all have exhibited encouraging anti-tumor effects in both in vitro and in vivo studies.

In 2011, CX-5461 was initially reported as a potent and selective inhibitor capable of suppressing rRNA

synthesis mediated by RNA Pol I in cancer cells, without affecting the synthesis of DNA, mRNA, or proteins [2], and exhibited robust in vivo anti-tumor activity in mouse xenograft models of human solid tumors with favorable safety profile; Mechanistically, CX-5461 disrupted the interaction between the transcription factor SL1 within the RNA Pol I complex and the rDNA promoter and resulted in an unproductive mode of RNA Pol I by impeding the release of the RNA Pol I-Rrn3 complex from the rDNA promoter (Fig. 1) [22], therefore inducing autophagy, DNA damage, and senescence in cancer cells through a p53-independent pathway, rather than apoptosis, while leaving normal cells unaffected [2]. Subsequently, in a range of preclinical cancer models, CX-5461 exhibited excellent anti-cancer effects both as a monotherapy and combination therapy, encompassing acute myeloid leukemia (AML) [3, 23–27], lymphoma [5, 28–30], breast cancer [31, 32], prostate cancer [33–35], and ovarian cancer [36–40]. In the completed phase I clinical trials for hematological and solid cancers [41, 42], CX-5461 demonstrated excellent biosafety and showed promising therapeutic activity.

CX-5461 shows anti-proliferative activity with different p53 status. The cellular response to rRNA synthesis inhibition mediated by CX-5461 involves both p53-dependent and p53-independent pathways. In the

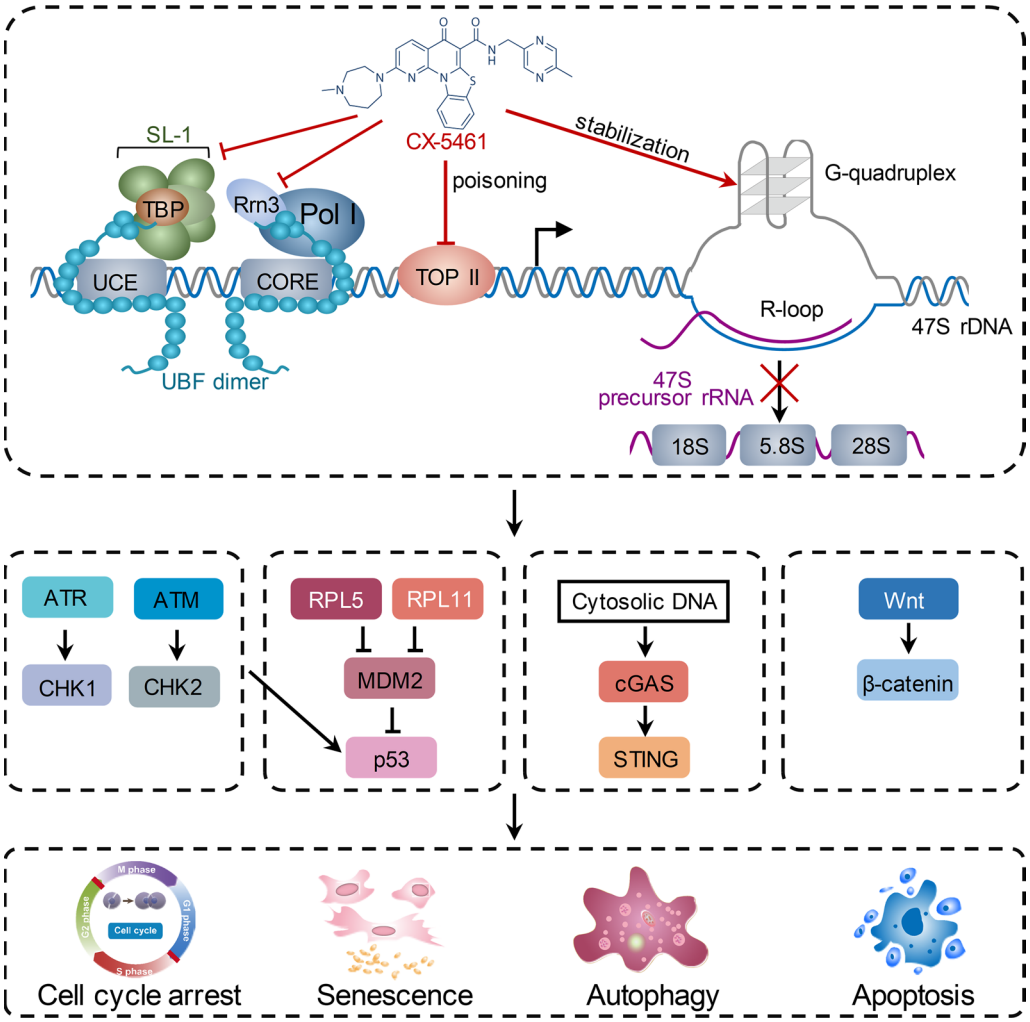

Fig. 1 The molecular mechanism of CX-5461 in inhibiting rDNA transcription and ribosome biogenesis in cancer and its potential impact on tumor cells. CX-5461 can disrupt the interaction between the SL1 complex within the RNA Pol I complex and the rDNA promoter, resulting in an unproductive mode of RNA Pol I by impeding the release of the RNA Pol I-Rrn3 complex from the rDNA promoter, poison TOP II, stabilize G4 s in the rDNA promoter, and induce R-loop formation. This regulation affects multiple pathways, including the ATR/ATM signal, RPL5/RPL11-MDM2-p53 signal, cGAS-STING pathway, and Wnt/β-catenin pathway, ultimately leading to cell cycle arrest, cell senescence, autophagy, and apoptosis

p53-independent manner, CX-5461 can prevent Pol I from loading onto the rDNA, leading to the presence of abnormal, open, and accessible rDNA devoid of Pol I, which triggers the ATM/ATR signaling pathway (Fig. 1) and induces p53-independent cell cycle checkpoints, effectively targeting aggressive Tp53-null ($Tp53^{-/-}$) *MYC*-driven lymphomas in vivo [43]. Similarly, in acute lymphoblastic leukemia cells with different cytogenetic abnormalities, the ATM/ATR pathway has also been activated by CX-5461, resulting in G2 phase arrest and p53 status-independent apoptosis [3]. The p53-independent manner of antitumor activity by CX-5461 was further confirmed in the mutant myeloma models in a TP53 KO xenograft model [44].

Meanwhile, in a p53-dependent manner, CX-5461 can disrupt the nucleolus and activate apoptotic signaling by RPL5/RPL11-MDM2-p53, selectively eliminating B-lymphoma cells in vivo while preserving a viable wild-type B cell population [5]. Homoplastically, the RNA Pol I complex inhibited by CX-5461 can also lead to the activation of the ATR-CHK1-p53 pathway, subsequently inducing cell cycle arrest, apoptosis activation, and a DNA damage response during erythropoiesis [45]. Moreover, in the osteosarcoma model, CX-5461 was reported to increase the accumulation and stabilization of p53 and the mRNA level of its target genes, including p21, MDM2, and Sestrin1/2, induced p53-dependent autophagy [46]. Apart from tumor cells, CX-5461 can also activate p53 in T cells [47], cardiac fibroblasts [48], pulmonary arterial smooth muscle cells [49], aortic medial smooth muscle cells [50, 51], and so on.

Meanwhile, the activation of p53 by CX-5461 can directly restrain ribosome biogenesis. Firstly, it is a consensus that p53 can prevent the interaction between SL1 and upstream binding factor 1 (UBF), thereby directly interfering with the assembly of the Pol I complex [52]. Furthermore, as a transcription factor, p53 can regulate the expression of components in the Pol I transcription initiation complex, such as eIF4E [53], as well as suppress the expression of *MYC*, the key oncogene facilitating Pol I transcription [54]. Apart from p53, CX-5461 can impair ribosome biogenesis, which in turn leads to the activation of other canonical pathways in response to nuclear stress, such as the Wnt/β-Catenin pathway [55]; However, combination therapies involving CX-5461 and inhibition of the Wnt/β-Catenin pathway for targeting cancer or other diseases is devoid.

G-quadruplex stabilization

The G-quadruplex (G4) is a class of nonclassical higher-order structures of nucleotide sequences formed by the interaction of four guanine bases through hydrogen bonds to create a planar four-strand structure known as a G-quartet [56]; multiple G-quartets stack together and are connected by three loops, assembling into a complex G4 structure. G4 was initially identified in telomeric sequences, with subsequent genomic analyses revealing their enrichment in functional regulatory elements including gene promoters, untranslated regions (UTRs), non-coding RNAs, and mitochondrial/ribosomal DNA loci [57]. G4 structures display topology-dependent structural plasticity dependent on the sequence and solution environment, with canonical parallel, antiparallel, or hybrid configurations, coexisting with multiple noncanonical configurations characterized by long-loops, bulges, guanine vacancies, or hybrid stem-loop architectures, which were reviewed previously [9, 58].

Advanced sequencing methodologies have revolutionized G4 mapping and functional characterization. Genome-wide profiling through polymerase arrest-coupled next-generation sequencing identified 716,310 putative G4 motifs, revealing significant proportions of noncanonical long-loop G4 s (21.5% in K^+) and bulge-containing G4 s (21.6%) under physiological conditions [59]. RNA G4 (rG4) landscapes were similarly mapped via reverse transcriptase stalling assays, detecting 3,383 rG4 s in HeLa cells with predominant 3'UTR localization (61.7% vs 16.0% in 5'UTRs) [60]; mechanistic studies established rG4-mediated post-transcriptional regulation through interactions with RNA helicases like DHX36 [61, 62], and translation initiation factors like eIF4 A [63]. The GC-rich mitochondrial genome (16.6 kb) was confirmed to contain 209 G4-forming sequences through integrated bioinformatics analysis and in vitro validation [64]; persistent G4 accumulation at respiratory chain complexes I and IV loci could repress mitochondrial genome transcription, impair mitochondrial respiration, cause senescence-associated mitochondrial dysfunction [65], and could even serve as a biomarker for mitophagy detection [66]. As for ribosomal RNA (rRNA), it reveals conserved G-rich motifs in the human 28S rRNA expansion segment 7, featuring ten contiguous G-tracts in tentacle a, and four in tentacle b [67]. In vivo studies indicate that 5% of cytoplasmic ribosomes contain G4-forming sequences, accounting for 83% of extranuclear G4 structures [68]. The locations of G4 structures suggest their various critical biological functions (Fig. 2) [9], including: (1) G4 s located in regulatory regions of genes participate in the regulation of gene expression, including transcription and translation; (2) G4 s can protect the ends of chromosomes, maintaining chromosomal stability and integrity; (3) The formation of G4 s may create obstacles during DNA replication and repair processes, leading to

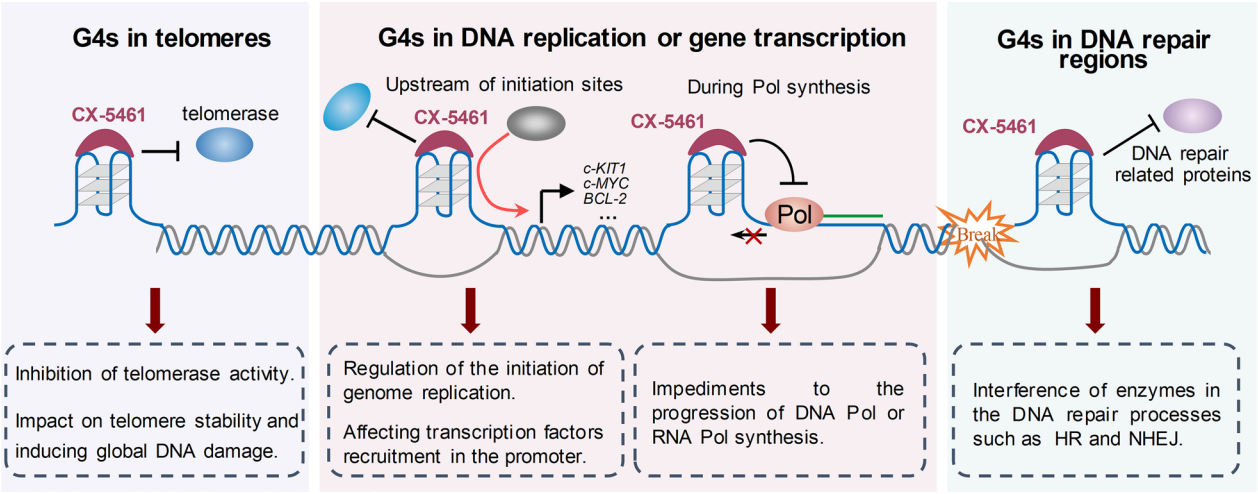

Fig. 2 The impact of CX-5461 on the DNA transaction processes by binding and stabilizing G4 in the genome

the accumulation of DNA damage and cell apoptosis; (4) G4 s are involved in chromatin remodeling; (5) G4 s can directly affect mitochondrial metabolism by regulating mitochondrial gene expression; (6) G4 s may directly regulate ribosome activity.

In addition to their role in normal physiological processes, G4 s also play significant roles in tumorigenesis and development. In certain cases, the formation of G4 s in gene expression regulatory regions can influence the recruitment and interaction of different transcription factors, regulating the assembly and progression of transcription complexes, and thereby controlling gene expression. This may involve the regulation of tumor suppressor genes, promoting the proliferation and survival of tumor cells. Beyond telomere ends, biologically functional G4 structures have been identified in the promoter regions of various oncogenes, such as *c-MYC*, *c-KIT*, *BCL2*, *KRAS*, and others [9, 69]. Furthermore, the formation of G4 s may be associated with chromosomal instability, as G4 formation can induce structural instability during chromosome replication and repair, leading to chromosomal breaks and rearrangements, thereby facilitating tumor development. As a specific structural target, G4 has become a potential target for cancer therapy. Numerous studies are exploring anti-tumor treatment strategies that target G4 s, with current G4 stabilizers including BRACO-19, Pyridostatin, Phen-DC3, RHPS4, TM-6089, and others [70].

As for CX-5461, it was primordially developed from the fluoroquinolone A-62176, which was a Top2 poison, and then A-62176 subsequently generated QQ58 which was designed to bind to G4 s [71]; However, CX-5461 was proposed to be RNA Pol 1 transcription inhibitors in cancer treatment and neglected the premier intention that this quinolone derivative was designed to be a G4-targeting compound after 2011[2]. In 2017, it was discovered that CX-5461 could bind and stabilize G4 DNA structures in vitro, dispute the progression of DNA replication result in the increase of G4 structures in vivo, and exhibit specific toxicity against BRCA deficiencies in cancer cells and polyclonal patient-derived xenograft models, including tumors resistant to PARP inhibition, which reflourish the G4-targeting ability of CX-5461 and strengthen the concept of G4-targeting as an anti-tumor strategy, specifically for targeting homologous recombination (HR) and non-homologous end joining (NHEJ) deficient cancers and other tumors deficient for involved in DNA replication or DNA damage repair (Fig. 2) [6].

In vitro, the molecular dynamics of CX-5461 binding to multiple G4 s were quantified using a Förster resonance energy transfer (FRET) melting temperature assay. The results indicated that the stabilizing effect conferred by CX-5461 binding was most pronounced in human telomeric G4, followed by *c-KIT1* G4 and *c-MYC* G4, with the least effect on DNA duplex. This suggests a substrate preference for CX-5461 [7]. It is noteworthy that CX-5461 can nonselectively intercalate into dsDNA with high affinity, exhibiting a dissociation constant (Kd) of 0.47 μM and a saturation γ of 0.312 ligand/bp [72], similar to the typical dsDNA intercalator ethidium bromide (EB). This finding suggests that the nonselective binding of CX-5461 to genomic dsDNA at micromolar concentrations may also induce cytotoxicity and impact biological processes, particularly DNA replication and transcription. Recent single-molecule magnetic tweezer experiments have demonstrated that CX-5461

primarily functions as a *c-MYC* G4 stabilizer, reducing the unfolding rate of the *c-MYC* G4 [8]; this mechanism is distinctly different from that of PDS and 360 A, both of which act as G4 chaperones, accelerating the folding rates of the *c-MYC* G4. Furthermore, CX-5461 can bind at the 5'-end of the *c-MYC* G4 corresponding to the 1:1 (ligand: G4) complex and occupy both the 5'and 3'-ends of the *c-MYC* G4 when the complex is in a 2:1 (ligand: G4) ratio [73], which provides valuable insights for the development of G4-targeted ligands to treat *c-MYC*-driven cancers. Although the three-dimensional structure of CX-5461 binding to G4 is not yet available, previous structural studies have indicated that G4 ligands bind to G4 s through π-π overlap interactions between the ligands'hetero-aromatic moieties and the planar G-quartets [74, 75]. It can be speculated that π-π stacking between CX-5461 and G4 s is essential, but this requires further verification.

The antitumor efficacy of CX-5461 through its action on G4 s in vivo is also striking. Firstly, CX-5461 will induce an overall increase in the stability of G4 in the genome, which may subsequently lead to global DNA damage in tumor cells. The classical example is that α-thalassaemia/mental retardation X-linked (ATRX) deficiency can lead to abnormal G4 formation, DNA damage, and disease-relevant copy number alterations in isogenic normal human astrocytes [76, 77], CX-5461 treatment in patient-derived glioma stem cells (GSCs) and flank and intracranial murine xenografts in vivo has shown significant G4 induction both alone and in combination with ionizing radiation [78], paving the way for its clinical translation in ATRX-deficient malignant glioma. Meanwhile, the continuous formation of G4 stabilized by CX-5461, especially during the rDNA transcription, DNA replication, or HR process, is also the cause of R-loop's production in the genome, the stabilized G4 and R-loop jointly triggers DNA damage and can also induce the release of DNA fragments into the cytoplasm, inducing the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) signaling and innate immune pathway (Fig. 1) [37, 79]. Additionally, there are reports of CX-5461 directly targeting promoter G4 to modulate gene expression in vivo (Fig. 2). A prototypical example is the fibroblast growth factor receptor (FGFR), a crucial molecule in cellular plasticity. CX-5461 can stabilize G4 structures in the proximal promoter region of FGFR, resulting in reduced FGFR1 expression and effective inhibition of metastatic breast cancer [31]. Although numerous studies have documented the interaction of CX-5461 with promoter G4 s in vitro, research on the direct targeting of promoter G4 s by CX-5461 to influence gene expression in vivo remains relatively uncommon and deserves further exploration. Moreover, it is well-established that G4 are component of telomeric ends and can directly influence telomerase activity, thereby affecting tumor cell proliferation. It is plausible to speculate that in vivo, CX-5461 could also inhibit telomerase activity and impede tumorigenesis by stabilizing the telomeric G4 s (Fig. 2); however, to date, aside from the known effect of CX-5461 on alternative splicing of human telomerase reverse transcriptase in gliomas [80], there have been no reports on this aspect of in vivo research.

Additionally, CX-5461 can influence the function of DNA damage repair-related proteins (Fig. 2), especially those primarily responsible for equilibrating G4 stability [81]. Fanconi anemia group J protein (FANCJ), is an interaction partner of breast cancer type 1 susceptibility protein (BRCA1), and biallelic mutations in FANCJ can cause Fanconi anemia, a disorder characterized by chromosomal instability and a predisposition to cancer. It has been reported that FANCJ can unwind parallel G4 structures and facilitate the replication of G4 structures by DNA polymerase delta, showing increased sensitivity to both Pyridostatin and CX-5461 [82]. HERC2, a HECT ubiquitin ligase, has been shown to promote the RecQ family members BLM and WRN helicases to suppress G4 structures in conjunction with RPA [83]. Conversely, the inactivation of HERC2 leads to G4 accumulation, hinders the nucleolar localization of these two helicases, and inhibits the relocalization of BLM to replication stress-induced nuclear RPA foci, thereby enhancing the effects of CX-5461 [83, 84]. However, whether CX-5461 can directly impact the G4-remodeling activity of the RecQ family or other G4-related helicases such as Pif1 and DHX36 requires further investigation.

Furthermore, to identify additional genetic defects that could sensitize tumors to CX-5461, synthetic lethality screens were conducted for 480 DNA repair and genome maintenance genes concerning CX-5461 [85]; the screen identified numerous components of the HR-efficiency, Fanconi Anemia pathway, and POLQ, a polymerase containing a helicase domain involved in G4 resolving. These findings highlight novel genetic vulnerabilities to CX-5461, which may have significant implications for patient selection in forthcoming clinical trials.

Topoisomerase poison

Topoisomerase (TOP) enzymes coordinate nucleic acid metabolism by resolving topological issues that emerge during various cellular processes, such as DNA replication, transcription, recombination, and chromosome condensation. TOPs are extensively expressed across eukaryotic tissues and are categorized into TOP I, TOP II, and TOP III based on the nature of the breaks they create in the DNA molecule [86]. TOP I

is a prominent expression in the lymphoid tissue and has tissue specificity for endothelial cells; its mitochondrial variant is preferentially expressed in skeletal muscle, heart, brain, and fetal liver. TOP II contains two isoforms, TOP IIα, mainly expressed in proliferation zones including the thymus, spleen, and bone marrow, and TOP IIβ, detected in a broader range of cell populations, such as the spleen, bone marrow, uterus, ovary, lymph nodes, adrenal gland, eye, bladder, and heart. For the TOP III isoforms, it showed three transcripts of TOP IIIα and two transcripts of TOP IIIβ, detected in the testis, heart, skeletal muscle and pancreas, thymus, testis, ovary, small intestine, heart, and skeletal muscle [10]. Under physiological conditions, TOPs perform transient DNA cleavages; the rapid relegation phase is well-tolerated by cells. However, in continuously proliferating cancer cells, excessive DNA breakage by TOPs occurs, inducing permanent alterations and impeding subsequent processes [87], which makes TOP inhibitors a promising strategy for cancer therapeutics. Right now, multiple TOP inhibitors are in ongoing clinical development [10], including the TOP I inhibitor Indimitecan, Gimatecan, Indotecan, and the TOP II inhibitor Vosaroxin, Aldoxorubicin, and Sabarubicin.

TOP poisoning constitutes one mechanism of action for TOP inhibitors, whereby the inhibitor is capable of blocking the nucleic acid-TOP cleavable complex formed during the operation of topoisomerase, resulting in irreversible DNA damage and consequently leading to cell cycle arrest and apoptosis. Given that CX-5461 is derived from fluoroquinolone A-62176, a recognized TOP poison [88], it is conceivable that CX-5461 serves as a TOP poison. Indeed, CX-5461 was initially reported to exert its primary cytotoxic activity through

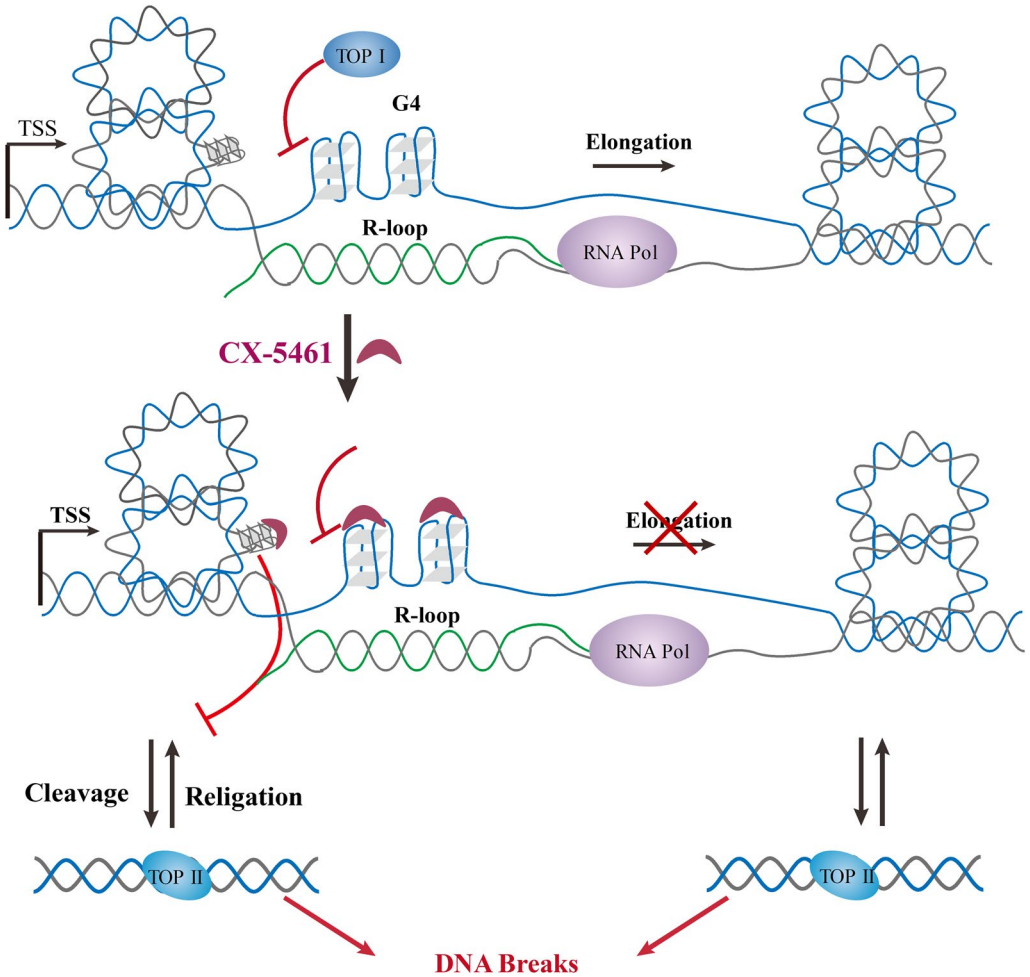

Fig. 3 CX-5461 triggers TOP2-mediated DNA DSBs at transcription sites dependent on G4. In the model, the interaction of CX-5461 with G4 is facilitated by DNA topological stress provoked by RNA-Pol-dependent transcription that can be neutralized by TOP I; G4 stabilization by CX-5461 in transcription loci would give rise to unremitting RNA-Pol stasis, mobilizing TOP to settle the topological stress, and that TOP II may be poisoned at the transcribed region bearing G4

TOP IIα poisoning (Fig. 3), as evidenced by three complementary data mining modalities with in vitro assays in melanoma and lymphoma cell lines [29], and an unbiased genetic approach in HeLa and U2OS cells [89]; Almost concurrently, a series of CRISPR screens were conducted in the retinal pigment epithelium-1 (RPE1) cell line treated with various genotoxic agents to obtain an unbiased and comprehensive view of the DNA damage response in human cells, which revealed that the cytotoxicity of the G4 ligand Pyridostatin and CX-5461 both involve trapping TOP II on DNA [90]. Mechanically, CX-5461 elicited the formation of DNA double-strand break (DSB) localized in the rDNA promoter/5'ETS in the retinal pigment epithelium-1 (RPE1) cell line treated with various genotoxic agents to obtain an unbiased and comprehensive view of the DNA damage response induction of p53 downstream targets, thereby underpinning the anticancer efficacy of CX-5461 (Fig. 1) [30]. Meanwhile, TOP IIβ, the primary oncogenic TOP II isoform in *NMyc*-driven cancers, has also been identified as a primary target of CX-5461 in high-risk neuroblastoma [91].

Furthermore, CX-5461 can also trigger TOP2-mediated DNA DSB at transcription sites dependent on G4; in the model, the interaction of CX-5461 with G4 is facilitated by DNA topological stress provoked by RNA-Pol-dependent transcription that can be neutralized by TOP I; however, G4 stabilization by CX-5461 in transcription loci would give rise to unremitting RNA-Pol stasis, mobilizing TOP to settle the topological stress, and that TOP II may be poisoned at the transcribed region bearing G4 (Fig. 3) [89]. The mode of action of CX-5461 as a TOP II poison differs from that of classical TOP II inhibitors, such as etoposide and doxorubicin [36], but the precise manner by which CX-5461 poisons TOP II remains unclear, and needs further exploration. Additionally, CX-5461 can be combined with the TOP I inhibitor topotecan to target homologous recombination-proficient high-grade serous carcinoma (HGSC) cells, enhancing the DNA damage response and replication stress, resulting in a robust G2/M cell cycle arrest, inhibition of clonogenic survival, and suppression of tumor growth in vivo [36], but the direct poisoning of CX-5461 on TOP I has not been reported yet. Additionally, TOP I/II are components of the RNA Pol I complex at the rDNA promoter. TOP poisoning represents another aspect of the molecular mechanism by which CX-5461 inhibits rRNA synthesis.

It is imperative to note that although TOP is not the sole mechanism of action for CX-5461, cardiotoxicity, and off-target events have been documented in clinically approved drugs that interact with TOP in late-emerging therapy-induced acute leukemias [91]. Therefore, the reported toxicities associated with TOP inhibitors should be strictly monitored during the subsequent clinical trials of CX-5461, although TOP II poisoning by CX-5461 is different from the classical TOP II inhibitor.

CX-5461 in clinical development

Up to now, 5 clinical trials have been registered involving CX-5461, with two completed, two recruiting, and one suspended (Table 1). The first clinical trial of CX-5461 was conducted in Australia in 2013 (registration number: ACTRN12613001061729) [41]. This trial aimed to determine the safety and tolerability of CX-5461 when administered via intravenous infusion once every 3 weeks. Additionally, it sought to assess the pharmacokinetic and pharmacodynamic profile of CX-5461, evaluate its preliminary antitumor activity, and investigate the impact of TP53 mutational status and mutations in other potential CX-5461 response factors, including members of the ATM/ATR pathway, in patients with advanced hematologic cancers. In this trial, 16 patients with advanced hematologic malignancies received CX-5461 therapy. It indicated that one patient with anaplastic large cell lymphoma achieved a prolonged partial response (PR), while five patients with myeloma and diffuse large B-cell lymphoma attained stable disease as their best response. Although the response rate in this clinical trial was not exceptionally high, it indicated that CX-5461 is safe at doses associated with clinical benefit, a dosage of 170 mg/m^2 intravenously once every 3 weeks was recommended for further study, and dose-independent dermatologic adverse events such as erythrodysesthesia and photosensitivity were manageable through preventive measures.

The second clinical trial of CX-5461, which included 40 patients with solid tumors across 10 dose levels (ranging from 50 to 650 mg/m^2), was conducted in Canada in 2016 (registration number: NCT02719977) [42]. In this trial, the overall disease control rate (DCR) was 20% (8/40, 95% CI 9.1–35.7%), with four patients achieving confirmed partial responses (PR)-three with breast cancer and one with ovarian cancer. Notably, all patients who experienced PR had germline DNA-repair abnormalities, including two with BRCA2 mutations, one with a PALB2 mutation, and one with a TP53 mutation (accompanied by a concurrent BRCA2 variant of uncertain significance). Additionally, 11 patients had a response of stable disease (SD), with four experiencing durable responses lasting 6 months or longer. Among these, three patients had germline or somatic BRCA2 aberrations. These findings suggest that the antitumor activity of CX-5461 is primarily observed in patients with HR-defective cancers. Furthermore, the emergence of reversion mutations in PALB2 and BRCA2 in association

Table 1 Summary of clinical trials of CX-5461 for cancer treatment

Official title	Clinical trials register ID	Phase	Status	Patient population	Intervention	Outcomes
A Phase 1, Open-Label, Dose Escalation, Safety, Pharmacokinetic, and Pharmacodynamic Study of Intravenously Administered CX-5461 in Patients with Advanced Haematologic Malignancies	ACTRN12613001061729 (Australia and New Zealand Clinical Trials Registry)	Open Label, Phase I trial	Completed	16 patients with advanced hematologic malignancies	CX-5461 was given as a 1-h intravenous infusion on day 1 of each 21-day cycle. Dose escalations were planned across 7 cohorts (25–450 mg/m^2), initially following an accelerated design, transitioning to a 3 + 3 dose-escalation schema based on predefined toxicity criteria and DLTs of CX-5461	(1) CX-5461 administered intravenously established a maximum tolerated dose (MTD) of 170 mg/m^2 (2) The dose-limiting toxicity observed was palmar-plantar erythrodysesthesia, while photosensitivity was identified as a dose-independent adverse event (AE), manageable through preventive measures (3) CX-5461 activated p53 in tumor cells from one patient who achieved a clinical response. One patient with anaplastic large cell lymphoma experienced a prolonged partial response, and five patients with myeloma and diffuse large B-cell lymphoma attained stable disease as their best response [41]
A Phase I Study of CX5461	NCT02719977	Open Label, multi-centre Phase I trial	Completed	40 patients with incurable solid malignancies	Doses were escalated using a 3 + 3 design, which allowed 3 or 4 patients to be initially enrolled in each dose level. CX-5461 was administered as a 60-min intravenous infusion on day 1 (d1) and 8 q4w in dose levels 0–6 and d1, 8, and 15 q4w for dose levels 7–9	(1) Defective HR is investigated as a predictive biomarker for response. (2) CX-5461 is generally well tolerated, with a recommended phase II dose of 475 mg/m^2 on days 1, 8, and 15 every 4 weeks, and dose-limiting phototoxicity (3) Reversion mutations in PALB2 and BRCA2 are identified upon progression following initial response in germline carriers, confirming the underlying synthetic lethal mechanism (4) In vitro characterization of UV sensitization indicates that this toxicity is associated with the CX-5461 chemotype, independent of G4 synthetic lethality [42]

Table 1 (continued)

Official title	Clinical trials register ID	Phase	Status	Patient population	Intervention	Outcomes
Phase Ib Expansion Study of CX-5461 in Patients With Solid Tumours and BRCA2 and/or PALB2 Mutation	NCT04890613	Open Label, multi-centre Phase 1b trial	Recruiting	52 solid tumor patients (estimated) with pathogenic/likely pathogenic germline BRCA2 and/or PALB2 mutation	(1) An initial 16 enrolled patients for the main cohort and 10 enrolled patients for the exploratory cohort will receive CX-5461 at 250 mg/m^2, delivered as a 60-min IV infusion on Day 1 and Day 8 of a 28-day cycle (2) Upon completion of enrollment of all patients in the initial arms, if there are no safety concerns after review of the safety data, another two arms will open to enroll an additional 16 patients for the main cohort and 10 patients for the exploratory cohort to receive CX-5461 at 325 mg/m^2, delivered as a 60-min IV infusion on Day 1 and Day 8 of a 28-day cycle	No result available
Pilot Study of Pidnarulex Pharmacodynamics in Patients With Advanced Solid Tumors	NCT06606990	Open Label Phase I trial	Recruiting	40 patients (estimated) with histologically confirmed solid tumors with metastatic disease	Patients receive Pidnarulex IV over 60 min on days 1 and 8 of each cycle. Cycles repeat every 28 days in the absence of disease progression or unacceptable toxicity	No result available
Phase 1 Trial of Pidnarulex and Talazoparib in Patients With Metastatic Castration Resistant Prostate Cancer	NCT05425862	Open Label, multi-centre Phase I trial	Suspended	48 patients with adenocarcinoma of the prostate without neuroendocrine or small cell differentiation	Pidnarulex will be given as an IV infusion on days 1 and 8 of a 28-day cycle and talazoparib will be taken once daily continuously	Enrolment suspended to further assess supplementary non-clinical study data

with acquired resistance confirms the underlying synthetic lethal mechanism.

Based on the trial NCT02719977, CX-5461 is currently undergoing a Phase Ib Expansion trial (NCT04890613), which aims to determine the tolerable dose of the CX-5461 in patients with specific solid tumors carrying BRCA2, PALB2, and other HR-deficiency associated somatic mutations, and inform the design of future Phase II clinical trials. Meanwhile, another clinical trial of CX-5461 has begun recruiting participants recently (NCT06606990), which objective is to assess whether CX-5461 induces the Rad51 response and evaluate other DNA damage and repair signaling markers including Top2, G4 stabilization, RPA32, pSer33-RPA32, γH2 AX, 53BP1, pSer8-RPA32, pKap1 m and pNBS1 from patients with and without HR-deficiency genetic mutations.

We acknowledge that one trial (NCT05425862), where both doses of Talazoparib and CX-5461 were escalated to define the maximum tolerated dose (MTD) and recommended phase 2 dose (RP2D) for the combination, was temporarily suspended due to"enrolment suspension to further assess supplementary non-clinical study data". Notably, the study suspension reflects a precautionary measure to ensure patient safety based on evolving preclinical evidence, and the trial may resume pending additional data review. This cautious approach aligns with standard clinical development protocols for novel oncology therapeutics.

Effects of CX-5461 in non-neoplastic diseases

On one hand, Pol I-dependent rDNA transcription serves as the rate-limiting step in ribosome biogenesis and protein synthesis. On the other hand, beyond tumors, G4 structures also play significant roles in neurodegenerative diseases such as Alzheimer's disease and viral genomes. Therefore, although research on its role in non-tumor diseases remains relatively limited, preliminary studies indicate that CX-5461 may hold potential in the treatment of multiple non-neoplastic diseases.

Antiviral effect

Human cytomegalovirus (HCMV) is a β-herpesvirus that has infected over 40% of the adult population worldwide [92]. While HCMV is typically not regarded as pathogenic in healthy individuals with a fully functioning immune system, it can cause life-threatening conditions in immunocompromised patients, especially those undergoing congenital birth defects, organ, and stem cell transplants, as well as individuals with AIDS [93]. HCMV is capable of infecting and replicating within a diverse array of cell types, encompassing epithelial cells, macrophages, dendritic cells in glands and mucosal tissues, smooth muscle cells, fibroblasts, and vascular endothelial cells [94]. Prior reports have demonstrated that CX-5461 can impede viral DNA synthesis and virus production in human fibroblasts during both the early and late phases of human cytomegalovirus (HCMV) infection. Importantly, CX-5461 results in a more significant reduction in viral DNA synthesis after the initiation of this process compared to continuous treatment initiated early in the infection [95]. What's more, CX-5461's inhibition of rRNA synthesis leads to a marked decrease in both IE and pp65 mRNA and protein levels in human fibroblasts during the early stages post-infection. Moreover, CX-5461 reduces the transcripts of immediate-early protein O and glycoprotein B in herpes simplex virus-1 (HSV-1), indicating that RNA polymerase I (Pol I) is utilized by certain herpesviruses for their early transcription processes [96]. Another antiviral example of CX-5461 is its effect on the lytic reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV), a herpesvirus causally linked to cancer. It indicated that nucleolar stress induced by CX-5461 alone did not trigger the lytic cycle. However, the enhancement of the lytic cycle by CX-5461 was independent of p53 and was evident when combined with the expression of the viral protein K-Rta [97].

In recent years, G4 s have been identified in numerous disease-related viruses, including HIV [98], EBV [99], HBV [100], SARS-CoV-2 [93], and West Nile virus [101], with many of these G4 s exhibiting functional roles. For instance, bioinformatics predictions and in vitro experimental validations have identified 52 potential G4 s within the SARS-CoV-2 genome, which may regulate key genes involved in viral infection and genome replication, such as Nsp1, Nsp3, the S protein, and N protein [102]. Currently, there are limited cases where CX-5461 directly targets G4 structures in key viral genome genes to modulate viral replication and infection. However, research on other G4 ligands, such as Pyridostatin, which has been shown to inhibit the expression of the N protein of SARS-CoV-2 both in vivo and in vitro [103], suggests that it may be feasible for CX-5461 to inhibit viral infection by targeting G4 s in the viral genome. Nonetheless, this hypothesis requires further extensive in vivo and in vitro experimental validation.

Immunosuppression

At the same time, CX-5461 interferes with RNA Pol I activity, which can also impact the function of immune cells and inflammatory responses. Preliminary studies suggest that CX-5461 holds potential therapeutic effects for transplantation and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.

In rat aortic transplantation models, CX-5461 has been shown to mitigate the development of neointimal hyperplasia and vascular inflammation, partly by

inhibiting the differentiation, maturation, migration, and lipopolysaccharide-induced activation of macrophages derived from primary bone marrow cells [104]. Transcriptional sequencing indicated that CX-5461 can induce a molecular signature associated with cell cycle inhibition in primed macrophages, characterized by the downregulation of genes encoding cell cycle mediators and the concurrent upregulation of cell cycle inhibitors [105]. Additionally, in allogeneic skin transplantation models (BALB/c mice to C57BL/6 mice) and heterotopic heart transplantation models (F344 rats to Lewis rats), CX-5461 prevents acute allogeneic rejection by inhibiting the expansion of regulatory T cell populations (Tregs). In vitro, CX-5461 suppresses agonist-induced T cell activation, thereby continuously inhibiting the expression of key mediators of T cell-mediated allogeneic immunity, such as γ-interferon and interleukin-2. Mechanistically, CX-5461 partially relies on the p53-DUSP5 (dual-specificity phosphatase 5) axis and antagonism of the Erk1/2 mitogen-activated protein kinase pathway [106]. CX-5461 can promote the differentiation of Tregs through the P53 pathway while inhibiting conventional T cell-mediated allogeneic immunity and enhancing Treg-dependent immune tolerance [47]. Moreover, topical application of CX-5461 can prevent the development of imiquimod-induced psoriasis by reducing keratinocyte proliferation, T-cell infiltration, and pathological angiogenesis [107]. Therefore, CX-5461 represents a promising new immunosuppressant that may serve as an alternative to currently approved anti-rejection therapies.

Cardiovascular disease

The application of CX-5461 in cardiovascular diseases is also noteworthy. First, the anti-fibrotic effects of CX-5461 in primary cardiac fibroblasts were elucidated, showing that CX-5461 effectively inhibited spontaneous and mitogen-induced activation, proliferation, and differentiation into myofibroblasts. This inhibitory effect was predominantly mediated through the activation of the p53 signaling pathway, rather than by reducing the rate of ribosome biogenesis. Additionally, it revealed that CX-5461 triggered a non-canonical DNA damage response in cardiac fibroblasts, which served as the initiating upstream signal for p53 activation [48]. Throughout the advancement of pulmonary hypertension, CX-5461 is capable of inducing cell cycle arrest in human pulmonary arterial smooth muscle cells by augmenting the phosphorylation of p53. As a result, this halts the progression of pulmonary arterial remodeling, mitigates perivascular inflammation, and combats pulmonary hypertension. Furthermore, it enhances survival rates and can even partially reverse already established pulmonary hypertension in vivo [49]. Meanwhile, the same group found that CX-5461 can activate the ATR-p53 axis by enhancing the phosphorylation and acetylation modifications of p53, leading to G2/M cell cycle arrest in proliferating smooth muscle cells without causing significant apoptosis; As a result, it prevents balloon injury-induced neointima formation in rat carotid arteries in vivo [108] and decreases the incidence of vascular inflammation, thereby inhibiting arterial remodeling [104]. In contrast to the aforementioned study, another investigation reported that CX-5461 can accelerate the onset of aortic dissection by inhibiting the proliferation of aortic smooth muscle cells, generating reactive oxygen species (ROS), and inducing apoptosis [51]. The differing outcomes from these two studies may be attributed to the use of distinct animal models, and conducting more in-depth research on this topic is warranted.

Neurodegenerative disease

Plentiful studies suggest that G4 structures play an important role in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (AD), for instance, in AD patients, a large-scale GGGGCC repeat sequence is found at the C9ORF72 locus on chromosome 9, which is a potential site for extensive G4 structure formation [109–111], the specific role of these G4 structures in disease pathogenesis remains unknown. A recent study revealed that RNA G-quadruplex (rG4) formation in the hippocampus is markedly increased with aging and the severity of AD, and neurons exhibiting phosphorylated tau protein accumulation were observed to contain rG4 s, and rG4 structures were shown to directly drive tau aggregation [112]. Meanwhile, a stimulator of interferon genes (STING) G4-target system, named CRISPR-PDC [113], was designed with an identifier module to selectively target the sequence using clustered regularly interspersed short palindromic repeats (CRISPR), and a biotin-labeled pyridodicarboxamide (Bio-PDC) as the G4 regulator; the CRISPR-PDC system could effectively stabilize the G4 structure in the STING promoter, impede STING expression in living cells, attenuate cellular senescence, and restore the amyloid-β phagocytic capacity of microglia [113]. The above research indicated that targeting G4 may be a potential approach for treating neurodegenerative diseases.

It is worth noting that two reports indicate that CX-5461 has a significant negative impact on learning and memory by inhibiting rDNA transcription [114, 115]. Therefore, it is necessary to investigate whether CX-5461 intervention exacerbates or alleviates the progression of neurodegenerative diseases through in vitro and in vivo studies.

CX-5461 and immunotherapy

Emerging evidence underscores the therapeutic potential of combining the CX-5461 with immune checkpoint inhibitors, driven by its dual role in inducing tumor-selective cytotoxicity and reprogramming immunosuppressive niches. Several mechanistic insights were involved in its reprogramming immunosuppressive, including the induction of immunogenic cell death (ICD), activation of the innate immune response via the cGAS-STING pathway, and modulation of the PD-L1/PD-1 axis.

Recently, a novel class of G4-binding organic-platinum hybrids, designated L^1-cispt and L^1-transpt, was discovered to induce nucleolar stress, activating the retinoic acid-induced gene I (RIG-I) pathway and triggering the cytosolic release of damage-associated molecular patterns (DAMPs) such as ATP and HMGB1, which in turn induce ICD [116]. These signals promote dendritic cell maturation and antigen cross-presentation, thereby priming cytotoxic T-cell responses. Preclinical studies show that L^1-cispt and L^1-transpt can increase tumor-infiltrating $CD8^+$ T cells and $CD4^+$ T cells in breast cancer models [116], highlighting the potential of G4 ligands in tumor immunotherapy.

Meanwhile, the recently clarified cGAS-STING signaling pathway is a vital downstream mechanism by which G4 ligands inhibit tumors. The cGAS-STING pathway, a fundamental component of the innate immune system, initiates an immune response by detecting abnormal DNA in the cytoplasm (such as micronuclei DNA or mitochondrial leakage DNA), playing an essential role in antitumor immune regulation [117]. In 2021, Pyridostatin and PhenDC3 were the first G4 ligands discovered to activate the cGAS-STING signaling pathway and innate immune gene expression by induce micronuclei formation and genome instability [118]. Following that, multiple G4 ligands and their combination therapies were identified to stimulate the cGAS-STING pathway and elicit a robust immune response in various tumor models, including TMPyP4 [119], the triphenylamine-based ligand A6 (targeting mitochondrial G4) [120], Pyridostatin combined with paclitaxel or DNA-PKcs inhibitor [121], Pyridostatin in conjunction with the adenovirus dl922-947 [122], and the pyridostatin Pyridostatin derivative PyPDS paired with cisplatin [123]. Regarding CX-5461, it has been reported that CX-5461 can induce cytosolic dsDNA accumulation and subsequent cGAS-STING activation, leading to IRF3 phosphorylation and the upregulation of CXCL10, IL-6, IFN-α/β, CCL5, and CXCL10 in colorectal cancer [37, 79].

Furthermore, CX-5461 was found to upregulate PD-L1 expression through STAT1 activation in CRC and breast cancer models [79]. This compensatory immune evasion mechanism creates a therapeutic vulnerability, as combining CX-5461 with PD-1/PD-L1 blockade demonstrates synergistic tumor growth suppression. Beyond immunotherapy combinations, CX-5461 exhibits radiosensitizing effects in ATRX-deficient gliomas and other malignancies by exacerbating replication stress [124, 125]. These findings collectively position CX-5461 and other G4 stabilizers as multifunctional agents for combinatorial immuno-oncology strategies.

Nano-delivery strategy of CX-5461

Despite the potential therapeutic strategies demonstrated by CX-5461 in various diseases, the contributing factor to its less-than-satisfactory treatment outcomes may be its inability to effectively accumulate at the site of action or subcellular targets. Meanwhile, CX-5461 is now administered intravenously or orally at a low pH (dissolved in 50 mM NaH_2PO_4, pH 4.5) [2], and relying on low pH to improve the solubility of CX-5461 can have adverse effects on pharmacokinetics, biodistribution, and therapeutic potential. This is also one of the important challenges faced by the clinical application of CX-5461. In recent years, nanomaterials have been utilized for the delivery of various small-molecule drugs due to their high loading efficiency, solubility promotion, surface modifiability, and specific targeting capabilities [126]. The application of nano-drug delivery for CX-5461 not only enhances therapeutic efficacy but also reduces side effects.

Right now, there are reports of multiple nano-delivery systems loaded with CX-5461. A prior study has utilized copper to bind CX-5461 through the nitrogen of the pyrazine ring (Cu-CX-5461) (Fig. 4A), synthesizing copper-complexed CX-5461 in liposomes, as well as the modified system prepared with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and cholesterol (Chol), named DMPC/Chol-Cu-CX-5461 (Fig. 4A), which can improve the apparent solubility of CX5461 by over 500-fold and has demonstrated enhanced anti-tumor effects in BRCA-deficient tumors [127, 128]. Recently, *Sophora Flavescens*-derived exosomes-like nanovesicles carrying CX5461 (SFELNVs@CX5461) for efficient CX5461 oral delivery was constructed (Fig. 4B), it uncovered that SFELNVs@CX5461 can inhibit pro-inflammatory factors (TNF-α, IL-1β, and IL-6) expression and promote the polarization of M2 macrophage to alleviate mice ulcerative colitis [129].

Furthermore, G4-based nanostructures are being investigated as drug delivery systems. A representative

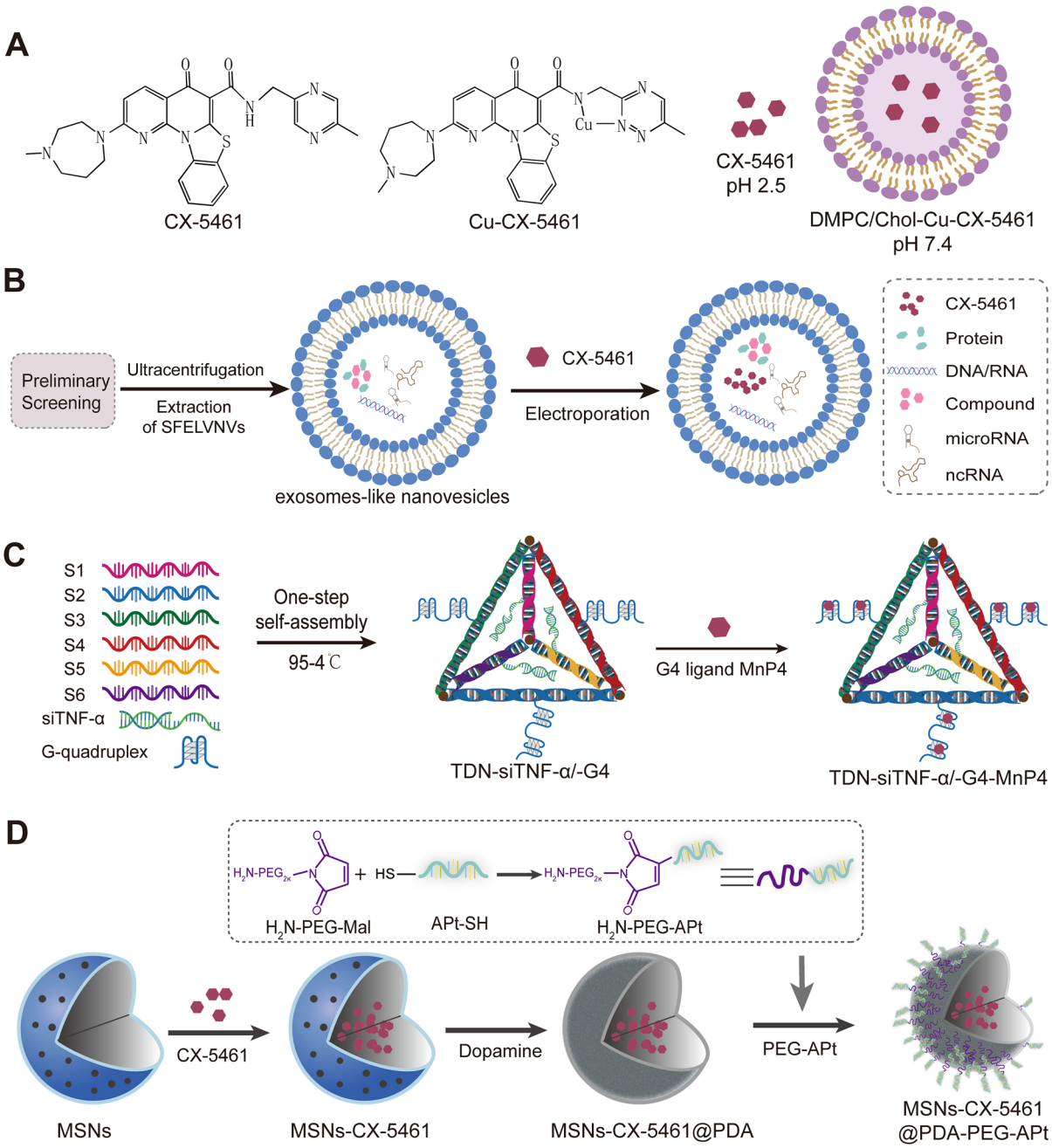

Fig. 4 The potential nano-delivery strategies of CX-5461. **A** Schematic representation of CX-5461, Cu-CX-5461, and DMPC/Chol-Cu-CX-5461; DMPC/Chol-Cu-CX-5461 at neutral pH can enhance the apparent solubility of CX5461 by over 500-fold and improve anti-tumor effects. **B** Illustration of the *Sophora Flavescens*-derived exosome-like nanovesicles (SFELNVs@CX5461) carrying CX5461 with other functional components. **C** Schematic diagram of the multifunctional nano-delivery system based on tetrahedral DNA (TDN) for transporting the G4 ligand. The G4 ligand achieves nano-delivery by binding to the G4 structures formed on the TDN (particularly the G4 s formed by the DNA aptamer AS1411), and this delivery system recognizes the highly expressed nucleolin on the surface of tumor cell membranes via the G4 structure, facilitating active targeting of tumor cells. **D** Structural diagram of the nano-delivery system based on mesoporous silica nanoparticles (MSNs) for delivering CX-5461, named MSNs-CX-5461@PDA-PEG-APt, which utilizes AS1411, polyethylene glycol (PEG), and polydopamine (PDA), achieving high stability, a high loading capacity of CX-5461, and active targeting of tumor cells

example is the TDN-siTNF-α/-G4-MnP4 nano-delivery system (Fig. 4C), which employs a tetrahedral DNA nanostructure (TDN) as a universal functional unit [130]. This system introduces tumor necrosis factor-α small interfering RNA (siTNF-α) by DNA hybridization while incorporating the metallo-antioxidant manganese porphyrin (MnP4) as a G4 ligand via π-π stacking interactions. It was found that TDN-siTNF-α/-G4-MnP4 can silence TNF-α in macrophages through siTNF-α, polarizing them into an anti-inflammatory M2 phenotype, and scavenge intracellular reactive oxygen species (ROS) via MnP4, therefore effectively inhibiting oxidative stress and cell death, demonstrating rapid and remarkable therapeutic efficacy in acute liver failure treatment [130].

Meanwhile, AS1411, also known as AGRO100, is undergoing or completed multiple Phase II clinical trials. AS1411 is a 26nt ssDNA aptamer d(GGTGGTGGTGGTTGTGGTGGTGGTGG), composed of two classic G4 structure sequences with two layers of G-quartets [131], and capable of recognizing nucleolin (NCL) [132]. NCL is a protein that is overexpressed on the surface of various cancer cells, including gastric cancer, B-cell chronic lymphocytic leukemia, cervical cancer, and breast cancer. Therefore, by modifying nanostructures with AS1411, the enrichment of the nano-system at tumor sites can be enhanced by targeting the highly expressed NCL on the tumor cell membrane [133, 134]. As a drug carrier, AS1411 can carry chemotherapy drugs (such as triptolide), gene editing tools (such as CRISPR/Cas9), or nanoparticles to target tumor cells [132, 135]. It demonstrated that AS1411 can serve as a supramolecular carrier to deliver C_8, an acridine-based G-quadruplex ligand to HeLa cancer cells, exhibit strong binding affinity, suppress *c-MYC* transcription, and reduce ligand cytotoxicity in non-malignant cells [136]. Additionally, a novel type of mesoporous silica nanoparticles (MSNs) loaded with CX-5461, named MSNs-CX-5461@PDA-PEG-APt, has been constructed using AS1411, polyethylene glycol (PEG), and polydopamine (PDA) (Fig. 4D). This nano-delivery platform has a high CX-5461 loading capacity due to the high surface area of the MSNs, PDA acts as a gatekeeper to prevent the leakage of CX-5461 from the MSNs, PEG grafted onto the surface of PDA increases stability and biocompatibility under physiological conditions, and the aptamer AS1411 promotes the nucleolar aggregation of CX-5461. Therefore, this nucleolar-targeting nano platform loaded with CX-5461 can treat cancer by inducing pro-death autophagy and has no significant toxicity to major organs [137]. Notably, this nanoparticle-based delivery of CX-5461 orchestrated by AS1411 operates independently of direct CX-5461/AS1411 interactions.

Consequently, nano-delivery strategies exhibit substantial potential for achieving CX-5461-targeted tissue delivery, particularly to the tumor. These approaches not only enhance intratumoral drug accumulation with spatiotemporally controlled release profiles but also significantly mitigate photosensitivity-related adverse effects, positioning this field as a frontier for innovative therapeutic exploration. Nevertheless, current research on CX-5461 nanoformulations remains scarce. Developing diversified CX-5461-targeted nanoplatforms, especially those exploiting direct AS1411/CX-5461 molecular interplay, holds critical clinical relevance, given AS1411's established safety profile from multiple Phase II clinical trials.

Individualized G4-targeting strategy

Although the nucleic acid sequences of G4 s are inherently distinct, the core structure of G4 s, formed by the stack of multiple G-tracts, and the flat, hydrophobic surface it presents for G4 ligand binding by π-π stacking are fundamentally similar. This characteristic leads to most G4 ligands exhibiting limited selectivity or specificity for diverse G4 structures. Indeed, to date, numerous ligands capable of binding to and stabilizing G4 s have been discovered or modified; however, ligands with individualized G4-targeting ability remain scarce, and CX-5461 is no exception. Meanwhile, bioinformatics analyses have predicted that the human genome contains at least 376,000 potential G4 sequences [138], while high-throughput sequencing has identified 716,310 distinct G4 s intracellularly, with 451,646 not been predicted by bioinformatics methods [59]. Due to their widespread and diverse distribution, the formation of G4 s may regulate the expression of genes that promote disease progression, as well as those that inhibit it. Therefore, the modification of CX-5461 specific target to individual G4 is particularly indispensable and important for targeted therapies in conditions such as tumors, as well as for reducing extensive, nonselective, collateral mutagenesis in cells reported recently [139].

The current dual-targeting approach, which integrates the G4 ligands with nucleic acid sequence readers to simultaneously target both the G4 structure and its neighboring nucleic acid sequence, exemplifies an optimal strategy for precise G4 targeting. By relying on base complementary pairing, the nucleic acid reader coupled with the G4 ligand can specifically recognize and bind to the ssDNA adjacent to G4, or the dsDNA nearby (Fig. 5A, B). Reported nucleic acid readers to date include peptide nucleic acids (PNAs), locked nucleic acids (LNAs), etc.

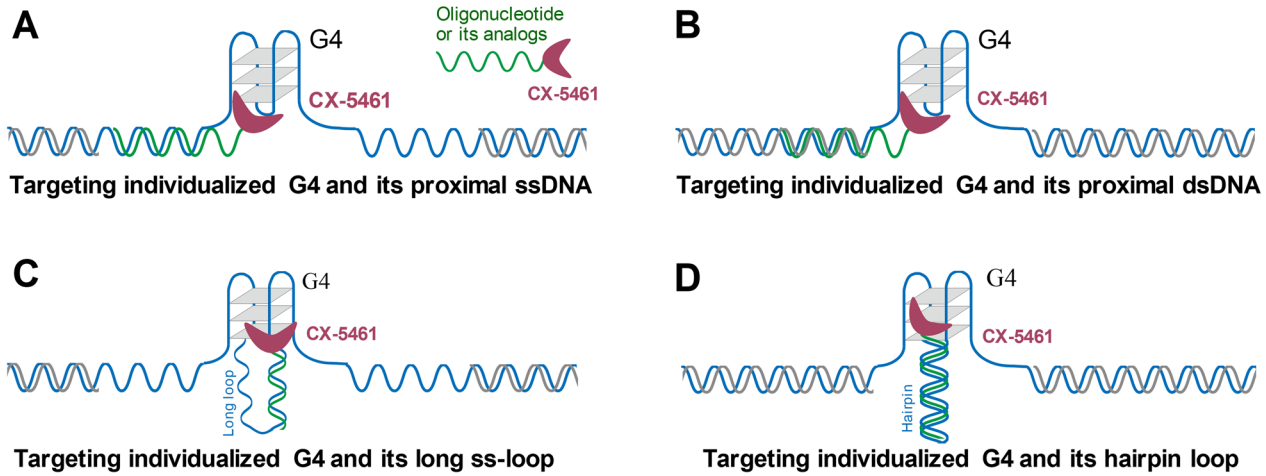

Fig. 5 Preconceived individualized G4-targeting strategies of CX-5461 conjugated with oligonucleotide or its analogs (peptide nucleic acids, locked nucleic acids, etc.)

PNAs are synthetic analogs of natural nucleic acids that can engage in complementary base pairing with natural DNA or RNA through Watson −Crick base pairing with high resistance to nuclease degradation [140]. Miscellaneous PNAs without G4 ligands have been excogitated to recognize G4 sequences to form hetero-duplexes or hetero-quadruplexes [141–143]. PNA itself has also been reported to be able to invade the *BCL2* promoter sequence and bind specifically to the complementary single strand of the G4 sequence, namely the i-motif sequence [144] and the adjacent single strand of the i-motif, thereby specifically inducing the formation of the *BCL2* promoter G4 [145, 146]. Recently, a PNA probe equipped with a pro-reactive ligand sequence specifically alkylates the targeted G4 or i-motif through light irradiation. In this process, the ligand, upon recognizing and binding to the desired G4 or i-motif, positions the pro-reactive warhead in a more controlled and reproducible manner, thereby inducing the necessary proximity for the alkylation reaction to proceed selectively [147]. Meanwhile, the G4 ligand naphthalene diimide (NDI) conjugated to PNA, was able to specifically recognize the G4 of choice within the unique long terminal repeat (LTR) promoter of the HIV-1, consisting of overlapping and therefore mutually exclusive G4 and can induce and stabilize the least populated G4 at the expenses of the more stable ones in the HIV-1 LTR region [148]. Furthermore, a G4 ligand, cyclic imidazole/lysine polyamide (cIKP), was combined with a hairpin pyrrole/imidazole polyamide (hPIP), differing from PNA, to enable the targeted recognition of G4 and its proximal dsDNA (Fig. 5B) [149]; the concept of simultaneous recognition of G4 and its adjacent dsDNA offers a novel strategy for ligand design.

LNA is a uniquely modified antisense nucleic acid molecule that, when paired with DNA/RNA, induces a conformational shift to the A-form of the double helix structure. The conformational shift hinders nucleases from recognizing the phosphodiester bonds, thereby improving the stability of the hybrid double-stranded nucleic acids [150]. Currently, locked nucleic acids (LNAs) themselves have been reported to affect the formation of G4 structures [151–154]. Recently, a G4-ligand-conjugated oligo named GL-O was synthesized and demonstrated selective targeting of an individual G4 DNA. In this construct, the azido-functionalized quinazoline-pyrimidine G4 ligand targets the terminal G-tetrad of the G4, and a DNA oligonucleotide complementary to the single-stranded DNA (ssDNA) flanking the target G4. When three nucleotides at both the 3' and 5' ends of the DNA oligonucleotide in the GL-O were replaced with LNAs, the binding affinity and G4 stabilization were further enhanced [155].

Although there are currently no reports on the dual-targeting of G4 and its adjacent sequences using CX-5461 coupled with oligonucleotides and their analogs (ssDNA, PNA, and LNA), various dual-targeting strategies related to other G4 ligands provide alternative approaches for the individualized G4-targeting of CX-5461. Additionally, considering that non-canonical G4 structures featuring long loops or hairpin loops have been reported to be abundantly present in the genome [156–158] and that the presence of hairpin loops within G4 s can further enhance their structural stability [159, 160], we can hypothesize that oligonucleotides and their analogs conjugated with CX-5461, could also be designed to target to the long single-stranded loop (ss-loop) or hairpin loop within the

G4 structure (Fig. 5C, D). This approach is anticipated to improve the targeting specificity of CX-5461 and further promote the stability of G4 s; however, it requires extensive in vitro and in vivo experimental validation.

Prospect

As a First-in-Class anticancer drug that has received 'Fast Track Designation' from the FDA, CX-5461 has been synthesized for over a decade, and during this period, its research in various diseases, particularly in cancer, has been extensively conducted. Initially known for inhibiting rDNA transcription, CX-5461 has evolved to be recognized as a G4 stabilizer and a topoisomerase poison, with its mechanism of action gradually refined. Although the initial Phase I clinical trial results were somewhat underwhelming, it did highlight its excellent biosafety and relatively few side effects. Subsequent animal experiments and clinical study results suggest that CX-5461 exhibits promising therapeutic effects on tumors with DNA-repair deficiencies, such as those related to PALB2 and BRCA2 mutations, which sparked enthusiasm for further research, and provides a crucial basis for patient selection in subsequent clinical studies.

Currently, several issues regarding CX-5461 warrant further attention. Firstly, its mechanism of action, especially the molecular basis of its toxicity to topoisomerase, remains to be fully elucidated and is distinct from other reported topoisomerase poisons. Secondly, beyond rRNA synthesis, G4 structures, and topoisomerases, additional potential targets of CX-5461 need to be explored, including i-motif structures. Furthermore, the effects of CX-5461 on tumors with DNA repair deficiencies other than BRCA2 mutations require investigation. It is worth inspecting whether gene defects in other DNA repair-related signaling pathways, such as mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and double-strand break repair (DSBR), also play pivotal roles in CX-5461 treatment, similar to BRCA2 defects, which will provide a theoretical foundation for selecting patients for future clinical trials. Additionally, while there are already several reports on the combination of CX-5461 with other treatment modalities, further exploration of combination therapies is needed. Moreover, recent reports have indicated that CX-5461 may act as a potent mutagen, underscoring the urgency for research on targeted drug delivery for CX-5461 and the development of individualized G4-targeting strategies.

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Author contributions
Hong-Xia Li: conceptualization, data curation, investigation, funding acquisition, writing-original draft, writing-review & editing; Yi-Meng He: investigation, methodology; Jing Fei: investigation, methodology, software; Man Guo: software; Chen Zeng: investigation; Pi-jun Yan: software; Yong Xu: resources, validation; Gang Qin: conceptualization, resources, software, supervision; Fang-Yuan Teng: funding acquisition, resources, writing-original draft, writing-review & editing.

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Competing interests
The authors declare they have no competing interests.

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