Topoisomerase I inhibitors: camptothecins and beyond

Yves Pommier

Abstract | Nuclear DNA topoisomerase I (TOP1) is an essential human enzyme. It is the only known target of the alkaloid camptothecin, from which the potent anticancer agents irinotecan and topotecan are derived. As camptothecins bind at the interface of the TOP1–DNA complex, they represent a paradigm for interfacial inhibitors that reversibly trap macromolecular complexes. Several camptothecin and non-camptothecin derivatives are being developed to further increase anti-tumour activity and reduce side effects. The mechanisms and molecular determinants of tumour response to TOP1 inhibitors are reviewed, and rational combinations of TOP1 inhibitors with other drugs are considered based on current knowledge of repair and checkpoint pathways that are associated with TOP1-mediated DNA damage.

Topoisomerases
Topoisomerases are divided into type I and II. Type I enzymes cleave only one strand of duplex DNA whereas type II enzymes cleave both strands. Type I topoisomerases are further subdivided into type IA and IB. Type IA enzymes cleave the DNA by forming a 5′-phosphotyrosyl covalent bond and relax DNA supercoiling by a strand-passing mechanism, whereas type IB enzymes form a covalent bond with the 3′ end of the DNA, and relax DNA by controlled rotation.

Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, 20892-4255, USA.
e-mail: pommier@nih.gov
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DNA topoisomerase I (TOP1) is ubiquitous and essential in mammals; *Top1* knockout mice die early during embryogenesis[1]. Knocking out the orthologous gene in *Drosophila melanogaster* is also lethal[2]. However, yeast cells can survive without TOP1, although with genomic instability (in particular in their ribosomal DNA segments)[3]. Their survival is probably due to compensation by the other topoisomerases.

The mammalian genome encodes seven topoisomerase genes: four that encode type I topoisomerases and three that encode type II topoisomerases (TOP2α and TOP2β and SPO11). The 4 mammalian type I topoisomerase genes include nuclear topoisomerase I (generally abbreviated *TOP1*), the mitochondrial topoisomerase I (*TOP1MT*) gene[4,5] and two genes that encode TOP3α and TOP3β (reviewed in REFS 6,7). The type I topoisomerases have been subdivided into two groups, type IA and IB, on the basis of the side of the DNA break to which the enzyme becomes covalently bound as it forms its catalytic tyrosyl–DNA cleavage intermediate, referred to as the cleavage complex (reviewed in detail in REFS 6–8). TOP3 enzymes and bacterial TOP1 belong to the type IA group, as they form 5′-DNA tyrosyl adducts similar to the type II topoisomerases. TOP1 and TOP1mt belong to the type IB group, are the only known enzymes that form 3′-phosphotyrosyl bonds in eukaryotic cells and are the target of the topisomerase inhibitors that are described in this Review.

The biological function of TOP1

Relaxation of DNA supercoiling is a key function of Top1 enzymes. Nuclear DNA is a remarkably long polymer. A single mammalian genome corresponds to approximately 2 metres, which is squeezed into a cell nuclear volume of approximately 10^{-17} m^3. Cellular DNA must therefore be highly compacted, which creates many curved DNA domains (loops) and points of contact between these DNA domains. Moreover, DNA metabolism requires the two strands of the duplex to be separated for them to serve as templates for transcription, replication, recombination and repair. Because of the size and mass of the replication and transcription complexes it is plausible that such complexes do not rotate freely around the DNA helix. In addition, because of the limited free rotation of the DNA domain flanking a given replication or transcription complex, DNA supercoiling is generated by DNA metabolism (FIG. 1a). Therefore, DNA tends to be overwound (positively supercoiled) upstream of replication or transcription forks and underwound (negatively supercoiled) downstream of these forks. Furthermore, nucleosome formation constrains negative supercoiling by wrapping the DNA around the histone octamer, which tends to generate positive supercoiling in the flank of the nucleosome. Such supercoiling tightens the DNA duplex and needs to be relaxed by topoisomerases (FIG. 1b). Mammalian TOP1, TOP1mt TOP2α and TOP2β can relax both positive and negative supercoiling. Therefore, TOP1 enzymes tend to be concentrated in supercoiled chromatin regions (particularly in association with transcription or replication complexes)[7].

TOP1 relaxes DNA supercoiling in the absence of an energy cofactor by nicking the DNA and enabling the broken strand to rotate around the TOP1-bound DNA

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At a glance

- Topoisomerase I (TOP1) enzymes are essential in higher eukaryotes, as they are required to relax DNA supercoiling generated by transcription, replication and chromatin remodelling.
- Topoisomerases are particularly vulnerable to topoisomerase I inhibitors during their cleavage reaction, which is referred to as the 'cleavage complex'.
- TOP1 can be trapped by anticancer drugs as it cleaves DNA. Moreover, TOP1 can be trapped by endogenous alterations to DNA (mismatches, abasic sites, nicks and adducts) and apoptotic alterations to chromatin.
- Camptothecin is a natural product of which TOP1 is the only cellular target. Two camptothecin derivatives have recently been approved by the US Food and Drug Administration: topotecan for ovarian and lung cancers and irinotecan for colorectal cancer.
- Various non-camptothecin inhibitors of TOP1 are in development, including indolocarbazole, phenanthridine and indenoisoquinoline derivatives. Non-camptothecins are expected to be active in cancers that are currently resistant to camptothecins, and to have a greater therapeutic index.
- Co-crystal structures of TOP1 inhibitors illustrate the interfacial inhibition paradigm by which a small drug molecule can trap conformational intermediates of macromolecular complexes (in the case of TOP1 inhibitors, the TOP1 enzyme and its cleaved DNA substrate).
- The cytotoxic activity of TOP1 inhibitors is related to the interference of trapped TOP1 cleavage complexes with DNA replication and transcription.
- Deficiencies in both the checkpoint and DNA-repair pathways determine cellular sensitivity to TOP1 inhibitors. Therefore, the identification of such deficiencies in tumours should guide the rational use of TOP1 inhibitors. Targeting checkpoint and repair pathways should also increase the selectivity of TOP1 inhibitors in tumours that have pre-existing deficiencies in relevant redundant pathways.

DNA supercoiling
In relaxed normal B-DNA, each strand crosses the other once every 10.4 base pairs with a right-handed turn. DNA is negatively supercoiled ('underwound') when the strands cross each other at more than a 10.4 base-pair interval. DNA is positively supercoiled ('overwound') when the strands cross each other with a base-pair interval less than 10.4.

Nucleophilic attack
A nucleophile is a chemical compound or group that is attracted to nuclei (centres of positive charge) and tends to donate or share electrons. A nucleophilic chemical reaction consists of the donation of electrons from a nucleophile to another species known as an electrophile in order to form a chemical bond. Nucleophiles can take part in nucleophilic substitution, whereby a nucleophile becomes attracted to a full or partial positive charge on an element and displaces the group to which it is bonded.

strand (FIG. 1c; curved arrow). The crystal structures of TOP1 (REFS 9–11) show the enzyme encircling the DNA tightly like a clamp (Fig. 2a), which accounts for the fact that TOP1 controls the processive relaxation of supercoiled DNA[12]. Once the DNA is relaxed, TOP1 religates the breaks by reversing its covalent binding. Religation requires the 5′-hydroxyl-group at the DNA end to be aligned with the tyrosine-DNA phosphodiester bond (FIG. 1c). Under normal conditions, the cleavage intermediates are transient and religation is favoured over cleavage. The mechanistic similarities between TOP1 and other tyrosine recombinases indicate that TOP1 might also have a role in DNA recombination[13,14], as has been proposed for the replication of vaccinia virus[15] and hepadnaviruses[14].

Trapping of TOP1ccs by DNA modifications, drugs and during apoptosis. DNA topoisomerases are particularly vulnerable to topisomerase I inhibitors during their cleavage intermediate step (referred to as the cleavage complex). Despite their frequency throughout the genome, TOP1 cleavage complexes (TOP1ccs) are normally so transient that they are not detectable, but it is these complexes that are specifically and reversibly trapped by camptothecin and its pharmaceutical derivatives. It is also important to note that high levels of cellular TOP1ccs can accumulate owing to DNA modifications[8,16,17] or apoptosis[18–20] (FIG. 1d). Because the religation of TOP1ccs requires nucleophilic attack of the tyrosyl-DNA-phosphodiester bond by the free DNA end (the 5′-hydroxyl end; see FIG. 1c), it is crucial for the 5′-hydroxyl-DNA end to be perfectly aligned with the scissile tyrosyl-phosphodiester bond. Any misalignment prohibits religation and leads to an accumulation of TOP1cc. Therefore, TOP1ccs accumulate at sites of base mismatches, base oxidation, abasic sites, carcinogenic adducts and pre-existing DNA breaks (for reviews, see REFS 8,16) because of the misalignment of the 5′-hydroxyl end of the DNA caused by such lesions. The occurrence of apoptotic TOP1ccs has been documented in various conditions and in response to various apoptotic stimuli, including staurosporine (a ubiquitous apoptosis-inducer[21]), arsenic trioxide[18–20], tubulin and TOP2 inhibitors, and the activation of FAS or tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL) (O. Sordet and Y.P., unpublished observations). Apoptotic TOP1ccs are among the early biochemical changes that are observed during apoptosis. They result, at least in part, from oxidative DNA modifications generated by reactive oxygen species that are produced during apoptosis[19].

Camptothecins and newer TOP1 inhibitors

Camptothecins. Camptothecin was first isolated from the bark of the Chinese tree, *Camptotheca acuminata*[22]. It was discovered and developed by the US National Cancer Institute (NCI) at about the same time and by the same groups that were also working on paclitaxel (Taxol)[22]. Camptothecin carboxylate was tested clinically in the mid 1970s and showed anticancer activity, but was discontinued because of its side effects[22]. It was not until after the discovery that TOP1 was the cellular target of camptothecin that the water-soluble derivatives of camptothecin — topotecan and irinotecan (also known as CPT-11) — were successfully developed[23].

The camptothecins are pharmacologically unique for several reasons. First, TOP1 is their only target, as has been shown using yeast cells, which become totally resistant to camptothecin when the *TOP1* gene is removed[24,25], and by the existence of single point mutations that render TOP1 immune to camptothecins in vertebrate cell lines selected for resistance to camptothecin [26]. Second, changing the stereochemistry of camptothecin by inverting its chiral centre at position 20 (synthetic 20-R-camptothecin; FIG. 2a) completely inactivates camptothecin. Therefore, it is remarkable that the natural alkaloid exists as the active 20-S-camptothecin enantiomer (referred to as camptothecin for simplicity) (FIG. 2a). Third, camptothecins penetrate vertebrate cells readily and target TOP1 within minutes of exposure. Camptothecin then binds reversibly to TOP1ccs. Because the cleavage complexes reverse within minutes of camptothecin removal, camptothecin is an incisive pharmacological tool, as drug exposure and the trapping of TOP1ccs can be precisely controlled. Finally, camptothecin and its derivatives have a relatively low affinity for TOP1ccs, as micromolar drug concentrations are required to detectably trap TOP1cc in biochemical assays, which indicates that camptothecin (which might function as an antibiotic in plants) was naturally selected for on the basis of its selectivity rather than its potency. For all the reasons listed above, camptothecin has become routinely used to explore replication-mediated

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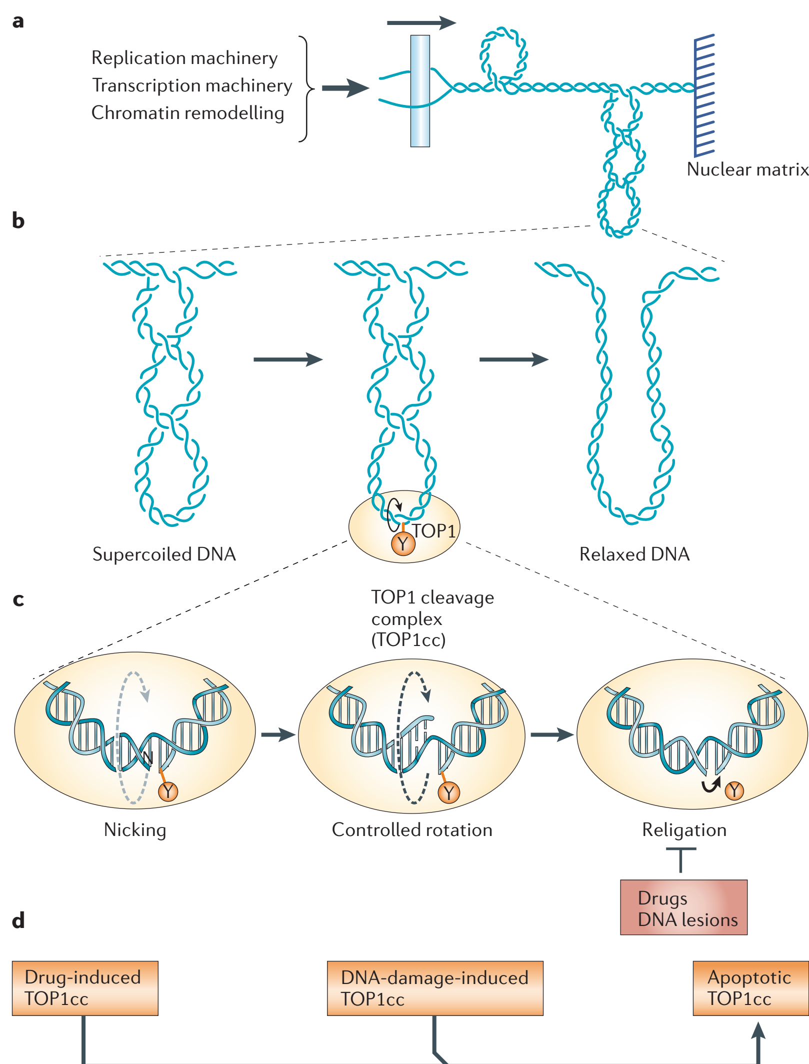

Figure 1 | **Relaxation of DNA supercoiling by TOP1-mediated DNA cleavage complexes, and the trapping of TOP1 cleavage complexes by drugs, DNA modifications and during apoptosis. a** | The generation of DNA supercoiling by DNA replication, transcription and chromatin remodelling. The unwinding of duplex DNA by macromolecular complexes tracking along the DNA (arrow) without rotating freely around the DNA double helix, which is also unable to rotate freely owing to its length or attachment to nuclear matrix regions, generates positive supercoiling ahead of the unwound segment and negative supercoiling behind (negative supercoiling not shown). **b** | The introduction of DNA single-strand breaks (nicks) by TOP1 provides swivel points that enable the rotation of the intact DNA strand around the break and facilitate DNA relaxation. The cleavage intermediate is referred to as a cleavage complex because TOP1 cleaves DNA by forming a covalent bond to the 3′ DNA terminus that it generates. The covalently linked catalytic tyrosine of TOP1 (Y723 for human TOP1) is shown as the yellow circle. **c** | An expanded view of DNA relaxation by a TOP1 cleavage complex (TOP1cc). The first step (left) is a transesterification reaction whereby the catalytic tyrosine (Y) becomes linked to the 3′ DNA end (nicking step). In the second step (middle), the torsional strain that results from DNA supercoiling drives the rotation of the 5′ end of the nicked DNA strand around the intact strand. TOP1 encircles the rotating nicked DNA and slows its rotation (FIG. 3a). This process is referred to as 'controlled rotation'. In the last step (right), the 5′ end of the nicked DNA is realigned with the corresponding 3′ end, which enables DNA religation (the closing step of the 'nicking–closing reaction'). TOP1ccs are normally transient because the closing step is much faster than the nicking step. Drugs and DNA lesions inhibit religation by misaligning the ends of the broken DNA. **d** | TOP1ccs can be stabilized under three conditions: by drugs such as camptothecin (left), by DNA lesions (damage) that misalign the 5′ end of the nicked DNA, and by DNA and TOP1 modifications that occur during programmed cell death (apoptosis).

(and transcription-mediated) DNA damage in various organisms, thereby providing a powerful way to study the genetic factors that are implicated in checkpoint regulation and DNA repair in response to TOP1-mediated DNA damage (reviewed in REFS 8,17).

Camptothecins have several limitations. First, TOP1ccs need to be maintained long enough to be converted into DNA damage (see next section and FIG. 4). However, camptothecins rapidly diffuse from TOP1ccs, which means that they must be given as a prolonged infusion to maintain persistent cleavage complexes. Second, camptothecins produce side effects (such as leucopaenia) that limit the dose that can be safely administered and, therefore, anti-tumour efficacy. The diarrhoea that is induced by irinotecan can be severe and is probably due to 'off-target' effects that are related to the bis-piperidine that confers water-solubility (FIG. 2). Third, the α-hydroxylactone E-ring of camptothecins is readily converted into a carboxylate (FIG. 2a), which is inactive against TOP1 and binds tightly to serum albumin. Two modifications of the camptothecin E-ring have been introduced to alleviate this. The addition of a methylene group, as in diflomotecan (BN80915), stabilizes the E-ring at the same time as retaining TOP1 inhibition. Diflomotecan is in early clinical trials (TABLE 1). Another way to stabilize the E-ring is to remove the lactone group, which completely blocks E-ring opening, as in the keto derivatives that retain high anti-TOP1 activity[27] (FIG. 2d). The cyclobutane methylenedioxy derivative, S39625 (FIG. 2), is expected to start clinical trials early next year.

Non-camptothecin TOP1 inhibitors. The search for non-camptothecin TOP1 inhibitors was initiated immediately after the discovery that TOP1 was the cellular target of camptothecins. The screening of chemical libraries and natural products with purified TOP1 and isolated DNA substrates led to the discovery of various TOP1 inhibitors (for review, see REF. 28), including the indolocarbazoles and phenanthroline derivatives. Simultaneously, computer analyses of drug-activity profiles in the NCI Developmental Therapeutics Program led to the discovery of the first indenoisoquinoline using comparative cytotoxicity-pattern profiles across the 60 cell lines of the NCI anticancer screen. This approach is commonly referred to as the COMPARE algorithm, developed by Kenneth Paull[29] (BOX 1).

Indolocarbazoles[30] are the most advanced non-camptothecin TOP1 inhibitors in clinical development. The indolocarbazole derivative developed by Bristol Myers is now in clinical trials (BOX 1). Several other indolocarbazoles are also undergoing clinical trials in Japan[31]. Nitidine and phenanthridine derivatives were developed in the mid 1990s. Topovale (ARC-111) is the lead phenanthridine currently in clinical development[32] (BOX 1). Among more than 400 indenoisoquinolines evaluated as TOP1 inhibitors and antiproliferative agents[33], several derivatives have recently been selected for clinical development (BOX 1).

The indenoisoquinolines have several different characteristics (and advantages) compared with the camptothecins. First, they are chemically stable, as

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they do not contain the labile hydroxylactone E-ring of camptothecins. Second, they trap TOP1ccs at different DNA sequences compared with the camptothecins, and therefore target the genome at different sites, which might translate to different cellular effects and clinical-activity profiles. Third, the TOP1ccs that are induced by indenoisoquinolines in biochemical systems and in cells are markedly less reversible than those that are induced by camptothecins[34,35]. Therefore, the pharmacokinetics of indenoisoquinolines should enable the formation of persistent TOP1ccs, which could potentially give rise to shorter infusion times.

Camptothecins and non-camptothecins as paradigms for interfacial inhibitors. Initial studies showed that camptothecin traps TOP1ccs by a unique mechanism. Camptothecin does not bind detectably to TOP1 alone, and binds only weakly to DNA alone[36]. DNA sequencing of TOP1 cleavage sites led us to propose that camptothecin might trap TOP1ccs by forming a ternary complex with a drug molecule stacked between the base pairs that flank the TOP1-associated DNA cleavage site (BOX 2)[37]. Indeed, TOP1ccs that are trapped by camptothecin are those at which the base pairs that flank the broken DNA are a thymine at the -1 position and a guanine at the +1 position (FIG. 3). Moreover, only the natural 20-S camptothecin enantiomer is active, which indicated the existence of a stereo-specific binding site for the drug at the interface of the TOP1–DNA cleavage complex[38]. The stacking hypothesis was confirmed approximately 10 years later by crystallographic analyses with the clinical derivative topotecan[11]. More recently, co-crystal structures of ternary complexes have been obtained for the parent camptothecin alkaloid, two indenoisoquinolines and one indolocarbazole[39,40] (FIG. 3). In each case, the drug binds simultaneously both to the DNA by hydrophobic stacking interactions and to TOP1 by a network of hydrogen bonds (FIG. 3). Strikingly, the TOP1 residues that are involved in these H-bonds are those that were previously identified as point mutations in camptothecin-resistant cells[26]. In all five ternary-complex

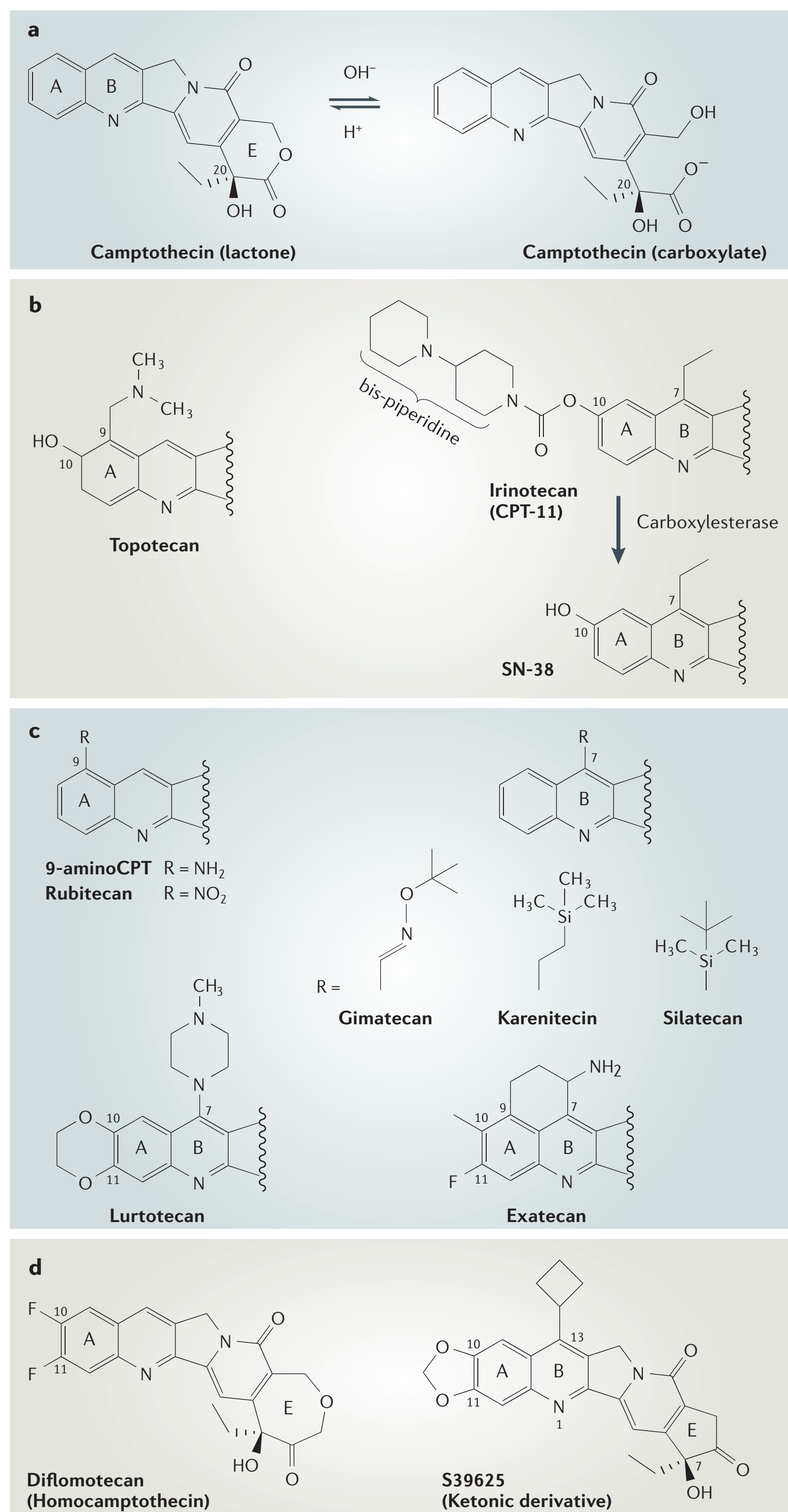

Figure 2 | **The chemical structure of camptothecin and its derivatives. a** | Camptothecin is a 5-ring heterocyclic alkaloid that contains an α-hydroxylactone within its E-ring that is unstable at physiological pH. For all camptothecin derivatives, the carboxylate form is inactive as a TOP1 inhibitor and is sequested by tight binding to serum albumin. As a result, shortly after administration only a small fraction of camptothecin or its derivatives are in the active lactone form. **b** | The two US Food and Drug Administration-approved water-soluble camptothecin derivatives, topotecan and irinotecan. Irinotecan is a weak TOP1 inhibitor. Its carboxybispiperidine group has to be hydrolysed by carboxyesterases to yield its active metabolite SN-38. **c** | Camptothecin derivatives in clinical trials (TABLE 1) with substitutions on the A and B rings. **d** | These two derivatives differ from camptothecins by chemical modifications that stabilize their E-ring. The 7-membered E-ring derivatives (left) are referred to as ‘homocamptothecins’. Their E-ring opens less readily than the E-ring of camptothecins but cannot re-close after opening. Diflomotecan is in clinical trials (TABLE 1). The 5-membered E-ring of the ketonic derivatives (right) does not open and is therefore stable. The 10,11-methylenedioxy and 7-cyclobutane derivative S39625 has been selected for clinical development.

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Table 1 | **Camptothecin derivatives in clinical use**

Camptothecin derivative	Status	Remarks	Indications
Topotecan hydrochloride (Hycamtin)	FDA approved (GlaxoSmithKline)	Intravenous infusion (water-soluble)	Metastatic ovarian cancer (second line); SCLC (second line)
Irinotecan hydrochloride (Camptosar)	FDA approved (Pfizer*)	Intravenous infusion (water-soluble)	Metastatic colorectal cancer (first line with 5FU/leucovorin)
9-NC; Rubitecan (Orathecin)	Phase II/III (SuperGen)	Oral administration; converted to 9-AC	Pancreatic cancer
9-AC; IDEC-132	Phase II	Intravenous infusion; oral; intraparenteral	Ovarian cancer (intraparenteral)
Exatecan mesylate (DX-8591f); DE-310[¶]	Phase II/III (Daiichi)	Intravenous infusion	Various carcinomas (sarcomas?)
Lurtotecan GI-147211 NX 211	Phase II (Gilead[‡])	Intravenous infusion; liposomal (NX 211)	Ovarian and other carcinomas
Gimatecan (ST-1481)	Phase I/II (Novartis[§])	Oral administration; lipophilic	Glioblastoma, SCLC and solid tumours
PEG–camptothecin; Prothecan	Phase II (Enzon Inc.)	PEGylated derivative; intravenous infusion (water-soluble)	NSCLC and other solid tumours
Karenitecin; BNP-1350	Phase II (Bionumerik Pharmaceuticals)	Oral administration; lipophilic	Glioblastomas, melanomas and NSCLC
Silatecan; DB-67	Preclinical	Lipophilic	Glioblastomas
Diflomotecan; BN 80915	Phase II (Ipsen)	Intravenous infusion	Advanced metastatic cancers: colon, breast and prostate

*Licensed from Yakult Honsha Co. Ltd, Japan, and Daiichi Pharmaceutical Co. Ltd, Japan. [‡]Licensed from Glaxo Wellcome. [§]Licensed from Sigma-Tau. [¶]Carboxymethyldextran polyalcohol carrier covalently linked through a peptidyl spacer. 5FU, 5-fluorouracil; FDA, US Food and Drugs Administration; NSCLC, non-small-cell lung cancer; PEG, poly(ethyleneglycol); SCLC, small-cell lung cancer.

structures, each polycyclic aromatic ring of the drug intercalates between the -1 and +1 base pairs that flank the TOP1 cleavage site[40] (FIG. 3). The crystal structures of camptothecin-resistant mutant enzymes[41] raise to two further points. First, single point mutations do not prevent the co-crystallization of camptothecin in its normal orientation despite the marked resistance conferred by these mutations. This illustrates the dynamic nature of the camptothecin–TOP1–DNA interaction, and indicates that the loss of a single hydrogen bond does not preclude drug binding, but most probably weakens drug retention in the TOP1–DNA complex, thereby resulting in resistance. Second, the crystal structures show that point mutations distal from the drug-binding site affect drug binding by allosteric effects on the amino-acid residues that form the direct hydrogen bonds with camptothecin[40,41].

Determinants of response to TOP1 inhibitors

Because TOP1 inhibitors bind reversibly to TOP1ccs, and TOP1 readily religates the cleaved DNA after drug removal, TOP1 inhibitors do not directly damage DNA. It is TOP1 itself that damages DNA in connection with DNA-helix-tracking processes — primarily replication and transcription (FIG. 1a). As TOP1 religation activity is slowed down by the drugs, replication and transcription complexes 'catch up' and 'collide' with the TOP1–DNA cleavage complexes, thereby generating irreversible TOP1 covalent complexes as the 5′ end of the nicked DNA template becomes misaligned with its substrate (FIG. 4). Therefore, it is DNA replication and/or transcription that convert the TOP1ccs that are reversibly trapped by drugs into irreversible TOP1 covalent complexes and DNA damage. For this reason, TOP1 inhibitors that trap TOP1ccs are commonly referred to as 'TOP1 poisons'. A number of genes have crucial roles in the cellular responses downstream of replication- and transcription-mediated DNA damage (TABLE 2). These genes are commonly altered in tumours, which might explain, at least in part, the increased sensitivity of tumour cells to TOP1 inhibitors compared with normal cells.

Replication-mediated DNA double-strand breaks and downstream cellular responses. Replication-fork collision is the primary cytotoxic mechanism of TOP1 inhibitors in dividing cells (FIG. 4a). Indeed, cancer cells in culture tend to be resistant to camptothecin when they are outside of S-phase[42,43] or when replication is arrested at the time of camptothecin treatment[44,45]. Moreover, like other cell-cycle-specific agents, camptothecin is increasingly cytotoxic with increasing time of drug exposure. Camptothecin kills fewer than 50% of cells when they are exposed to the drug for less than 1 hour[44]. This is different from TOP2 inhibitors, which can be highly cytotoxic (over 90%) even in the absence of active replication[44]. The nature of the replication-mediated DNA damage has been characterized in replicating SV40 (REFS 45,46) and in the ribosomal RNA gene cluster in mammalian cells[47]. TOP1ccs are converted to double-strand breaks by 'replication run-off'[47] as the leading strand is replicated up to the last nucleotide at the 5′ end of the TOP1 cleavage complex (FIG. 4a).

Replication double-strand breaks produce several well-characterized molecular responses. At the chromatin level, the H2AX histone variant, which represents

Chiral centre
From the Greek word cheir (which translates as 'hand' in English) to mean objects that have non-superimposable mirror images. It is synonymous with stereocentre (for instance an asymmetric carbon atom) whose spatial structure determines whether the enantiomer is in the R configuration (from the Latin '*rectus*': right) or the S configuration (from the Latin '*sinister*': left).

Leucopaenia
Low white-blood-cell count.

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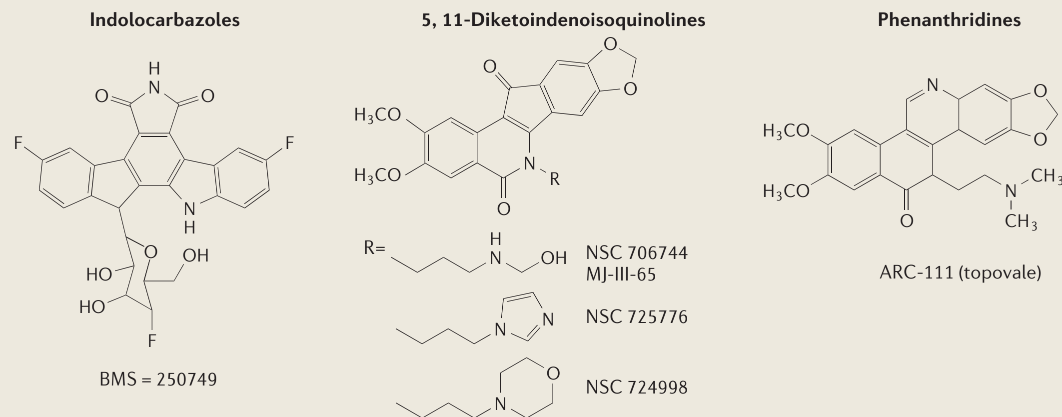
Box 1 | **New non-camptothecin TOP1 inhibitors in preclinical development**



Rationale for developing non-camptothecin TOP1 inhibitors:

- Camptothecins are among the most effective anticancer drugs recently introduced in cancer chemotherapy. Therefore, TOP1 is a validated target for cancer treatment.
- Drugs with common targets are known to have different pharmacological and anticancer activity (for example, TOP2 poisons or tubulin inhibitors).
- Camptothecins have limitations.

10–20% of histone H2A[48], becomes phosphorylated minutes after the formation of camptothecin-induced replication double-strand breaks[49]. The phosphorylation of H2AX is a ubiquitous response to DNA double-strand breaks that occur in non-replicating[48] or replicating DNA[49]. The phosphorylated form of H2AX, termed γ-H2AX, can be detected by immunofluorescence[48] or immunostaining[50], as it accumulates and forms a nuclear focus around each double-strand break. γ-H2AX is an extremely sensitive marker for double-strand breaks that are produced by DNA-damaging agents, but also by genomic instability[50] and apoptosis[51]. Consequently, γ-H2AX is currently being evaluated as a biomarker to monitor tumour response to camptothecin and non-camptothecin TOP1 inhibitors. H2AX is also functionally important, as genetic inactivation of H2AX sensitizes both yeast and mammalian cells to camptothecin[49,52] (TABLE 2).

Box 2 | **Definition and characteristics of interfacial inhibitors**

Interfacial inhibitors bind to a stereospecific site at the interface of molecular complexes as they undergo conformation changes during a reaction. Paradigms are:

- Camptothecin for protein–DNA complexes (for example, TOP1–DNA–camptothecin ternary complex)[11,37,40].
- Brefeldin A for protein–protein complexes (for example, ARF–GTP–EF–brefeldin A)[125].

Drug binding to the interfacial 'hotspot' reversibly traps the macromolecular complex in a normally transient conformation, which initiates the cytotoxic response.
There are many examples of interfacial inhibitors among natural products[126,127] such as:

- TOP1 and TOP2 inhibitors, taxol, colchicine, α-amanitin, cyclosporine, rapamycin, ribosome-targeted antibiotics and, recently, vinblastine[128].
- The interfacial inhibitor concept has immediate implications for drug discovery. Interfacial inhibitors belong to the class of 'uncompetitive inhibitors'.
- To discover new interfacial inhibitors, assays should be based on stabilization rather than the dissociation of macromolecular complexes.

Several other phosphorylation responses are induced by TOP1-associated replication double-strand breaks. They include the phosphorylation of replication protein A2 (RPA2), ataxia telangiectasia mutated (ATM), CHK2, CHK1, the Bloom syndrome helicase (BLM) and p53. RPA2 forms heterotrimers with RPA1 and RPA3, and is required to stabilize single-stranded DNA during replication and repair. RPA2 phosphorylation occurs within 1 hour of camptothecin treatment and persists throughout DNA synthesis inhibition, indicating that it is functionally linked to the replication checkpoint[53]. The kinase primarily responsible for camptothecin-induced RPA2 and H2AX phosphorylation is DNA-dependent protein kinase (DNAPK) in combination with ATM[49,53]. Camptothecin-induced replication double-strand breaks also activate the ATM–CHK2–p53 axis[54,55] and induce the phosphorylation of BLM[56]. A defect in ATM–CHK2–p53 activation tends to increase the cytotoxic effect of camptothecin (TABLE 2). On the other hand, CHK1 is primarily activated by the ataxia telangiectasia and RAD3-related (ATR) kinase (FIG. 5). Therefore, replication-associated DNA double-strand breaks that are produced by TOP1ccs cause a pleiotropic response that involves many of the sensors and effectors of the DNA double-strand break network[54,57–59] (FIG. 5).

Transcription-associated DNA lesions and cellular responses. TOP1 has several key functions for transcription. First, it is required for transcription elongation, as TOP1 removes the supercoiling that is caused by transcription complexes[7] (FIG. 1). Second, TOP1 represses basal transcription initiation by binding to TFIID, which promotes the assembly of TFIID–TFIIA complexes[60,61]. This function is independent of the DNA-relaxing activity of TOP1[60]. Third, TOP1 has been

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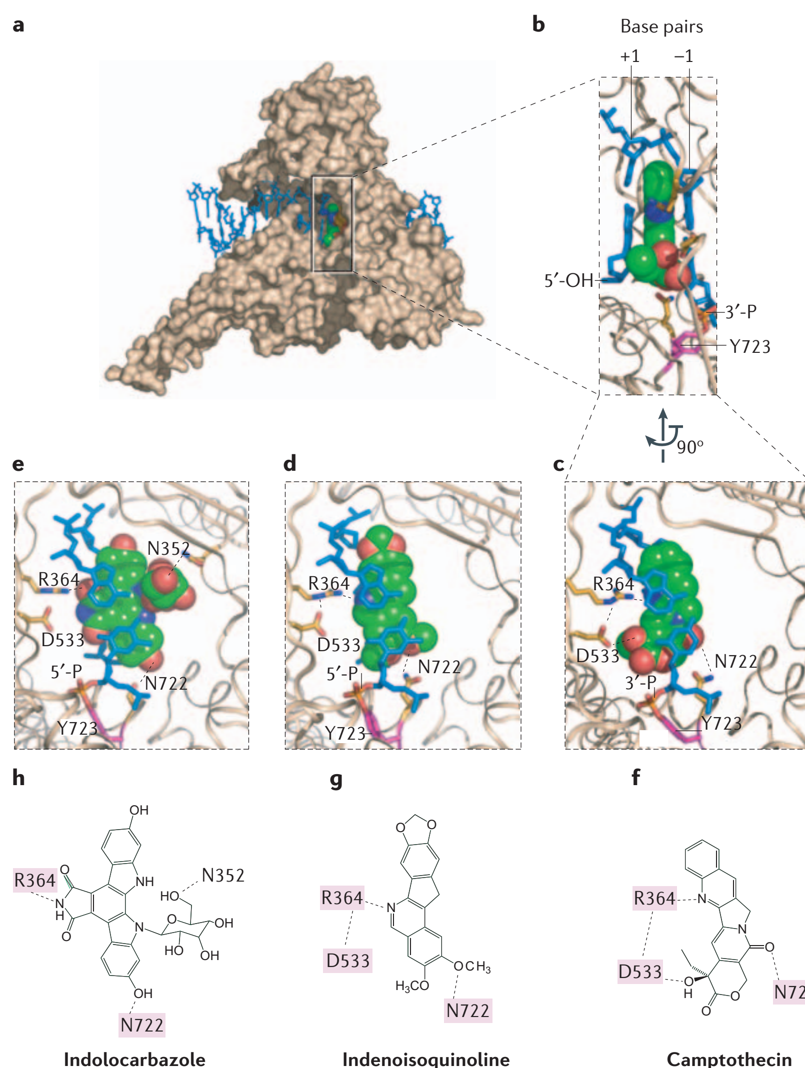

Figure 3 | **The atomic structures of camptothecin and non-camptothecin TOP1 inhibitors as they trap a TOP1 cleavage complex and function as interfacial inhibitors. a** | Overview of a ternary TOP1 cleavage complex (TOP1cc) trapped by camptothecin. Camptothecin is shown in green in the centre of the boxed area. The TOP1 polypeptide is shown in surface representation (in brown). The N-terminal domain of TOP1 (about 200 residues) is not shown, as its atomic structure has not been determined. The DNA double helix (in blue) appears in lateral view going through TOP1. **b** | Enlarged view of the cleavage site induced by TOP1 and trapped by camptothecin. The drug (shown in spherical representation with carbon atoms in green, oxygen in red and nitrogen in blue) is stacked between the base pairs that flank the TOP1 cleavage site. By convention, the base covalently linked to the TOP1 catalytic tyrosine (Y723; shown in magenta) is referred to as the –1 position. The base at the 5′ end of the nicked DNA is referred to as +1. The base pairs that flank the TOP1 cleavage site are shown in blue in stick representation. The nick is on the lower strand, and the religation of the 5′ end of the nicked DNA is precluded by the presence of the drug intercalated inside the cleavage site. The TOP1 polypeptide is shown in ribbon representation. **c** | Hydrogen-bond network between camptothecin and TOP1 residues. The view is a 90° vertical rotation of panel **b**. Hydrogen bonds are shown as dashed lines between the TOP1 residues: asparagine 722, aspartate 533 and arginine 364. The stacking of the drug between the base pairs that flank the cleavage site can be seen for the +1 base pair (blue sticks), which cover the drug molecule. The –1 base pair is stacked under the drug and is not visible in this orientation. **d**–**e** | Hydrogen bonds for the indenoisoquinoline and the indolocarbazole, respectively. The orientation is the same as in panel **c**. Again, note the -1 and +1 base-pair stacking. **f**–**h** | Hydrogen bonds between the drugs and the indicated TOP1 residues. TOP1 residues in the pink boxes correspond to mutations that confer camptothecin resistance.

associated with phosphorylation of proteins from the SR (serine-arginine-rich) family of splicing factors[62], and has been proposed to function as a cofactor for splicing independently of its DNA nicking–closing activity[63]. Therefore, camptothecin and its derivatives are potent blockers of transcription elongation[64–67].

Camptothecins also block ribosome formation[68], a function that might have contributed to the recently reported inhibition of hypoxia-induced factor 1α (HIF1α) translation by low-dose camptothecins[69]. Although the overall level of transcripts decreases rapidly following TOP1 inhibition, specific genes are differentially affected, as shown by microarray analyses[70], and camptothecins induce the expression of many genes including *JUN*[71]. The effects of camptothecins on transcription initiation are also gene-specific. Although initiation is stimulated in some genes, such as dihydrofolate reductase (*DHFR*)[64], it is inhibited in others, such as epidermal growth factor receptor (*EGFR*)[72]. Inactivation of the *EGFR* promoter has been attributed to the specific binding of TOP1 to *JUN* following JUN phosphorylation by Jun N-terminal kinase (JNK)[72].

Transcription complexes, similar to replication complexes, have been proposed to convert reversible TOP1ccs to irreversible TOP1 covalent complexes[65,73] (FIGS 4b,5). However, in highly proliferative cancer cells, transcription seems to contribute much less than replication to the anticancer activity of camptothecins — it is only at high (>1 μM) concentrations that the cytotoxicity of camptothecins becomes independent of replication[43,44]. Nevertheless, transcription-dependent DNA lesions associated with TOP1ccs can induce apoptosis in post-mitotic cells such as neurons[74] and lymphocytes (O. Sordet and Y. P., unpublished observations).

The nature of transcription-mediated DNA damage and downstream cellular responses is less well characterized than that of replication-induced damage. At present, there is no evidence for the existence of a 'transcription checkpoint'. The hypersensitivity of Cockayne syndrome cells, which are deficient in transcription-coupled repair, indicates a possible link with transcription-associated DNA repair (TABLE 2). Besides forming irreversible TOP1 covalent complexes, transcription collisions can induce the proteasomal degradation of both TOP1 and RNA polymerase II (REFS 67,75). However, such proteolytic degradation does not occur in all cell lines, and requires exposure to high camptothecin concentrations over several hours. Because degradation tends to be defective in transformed rather than normal cells, Liu and colleagues have proposed that TOP1 degradation in normal tissue might function as a protective mechanism by which normal cells adapt to camptothecin by depleting TOP1; whereas tumour cells do not[76]. Another consequence of trapped TOP1ccs during transcription might be the accumulation of negative supercoiling downstream of the transcription block, which could favour the formation of R-loop structures (DNA:RNA hybrids; FIG. 4b) that are known to be cytotoxic and mutagenic. TOP1 inhibition by camptothecin has also been reported to block the SR-kinase activity of TOP1. In which case, splicing might be inhibited as pre-mRNA processing and

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splicing becomes functionally defective (FIG. 4b)[63]. The inactivation of the SR-protein ASF could also favour the formation of R-loops[77]. More studies are needed to better understand the transcription effects of TOP1ccs.

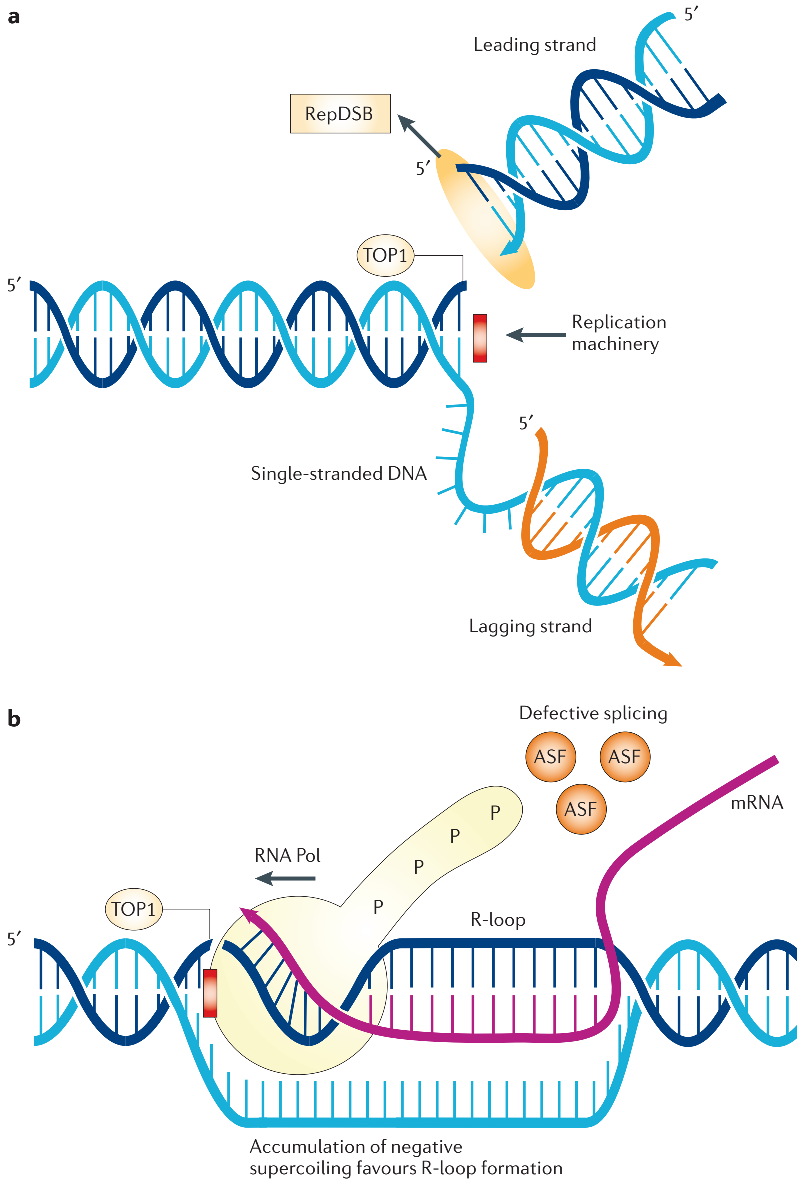

Figure 4 | **Conversion of TOP1 cleavage complexes into DNA damage by replication-fork collision and transcription. a** | When a TOP1 cleavage complex (TOP1cc) is on the leading strand (dark blue) for DNA synthesis, DNA polymerase elongates the nascent leading strand (light blue) up to the last base flanking the TOP1 cleavage site, thereby generating a replication double-strand break (RepDSB). Religation of the TOP1cc is blocked by the pairing of the template and leading strand immediately downstream of the TOP1cc. Because of the discontinuous replication on the lagging strand, a single-stranded DNA segment (light blue) probably exists immediately downstream of the TOP1 covalent complex. **b** | When a trapped TOP1cc is on the transcribed strand (dark blue), the RNA polymerase can reach the cleavage complex and arrest RNA elongation (dark purple). The RNA–DNA duplex prevents the religation of the TOP1cc, and TOP1 inhibition leads to an accumulation of negative supercoiling that could promote the formation of an R-loop. Inhibition of TOP1 SR-kinase activity would also inactivate splicing because of ASF hypophosphorylation. In cancer cells treated with camptothecins, replication-mediated double-strand breaks (**a**) are probably the main mechanism that generates DNA damage.

Repair of irreversible TOP1 covalent complexes. Yeast studies have shown that several biochemical pathways repair TOP1 covalent complexes. The human orthologues of many of the yeast genes involved in TOP1 repair are mutated in hereditary diseases that predispose to cancer. These mutations also sensitize cells to camptothecins (TABLE 2). We refer the reader to recent reviews for a full description of the repair of TOP1-mediated DNA damage (see REFS 8,17). Briefly, the TOP1 repair genes can be grouped into three main pathways: the tyrosyl-DNA-phosphodiesterase (TDP1) excision pathway that involves the XRCC1 and base excision repair (BER) complex; the endonuclease pathways centred around MRE11, MUS81 and RAD1 and homologous recombination (in which RAD51 and RAD52 orthologues have an important role); and the fork-regression pathway centred around the BLM helicase complex with TOP3α (REF. 8).

TDP1 was identified as an enzyme that hydrolyses a tyrosyl residue from the 3′ end of DNA in yeast[78]. In humans, inactivating mutations of *TDP1* are responsible for the disease autosomal recessive spino-cerebellar ataxia with axonal neuropathy (SCAN1)[79] (TABLE 2). The hypersensitivity of SCAN1 cells to camptothecin is independent of replication, which indicates that TDP1 is primarily involved in the repair of transcription-associated DNA damage[80,81]. In addition, the expression of recombinant TDP1 in cells with the SCAN1 mutation (H493R) results in the formation of TDP1–DNA intermediates[82]. TDP1 forms macromolecular complexes with the BER complex including XRCC1 and poly(ADP-ribose) polymerase (PARP), which are also both implicated in the repair of TOP1ccs[8,83,84] (TABLE 2). Indeed, PARP1-deficient cells are hypersensitive to camptothecins[8,85,86] (TABLE 2), and PARP inhibitors potentiate the effects of camptothecins[86,87]. Both the BLM and MUS81 pathways are probably primarily associated with the repair of replication-mediated double-strand breaks. Homologous recombination (RAD51 and RAD52) also has a crucial role in repairing replication double-strand breaks (TABLE 2). The apparent redundancy of the TOP1 repair pathways is consistent with the existence and importance of TOP1ccs under physiological conditions[8,16,19]. Further investigations are needed to clarify whether specific pathways repair specific lesions in particular cells. For example, a working model is the preferential repair of transcription-associated DNA lesions by the TDP1–BER pathway[81] (FIG. 5) in post-mitotic cells, such as neurons or lymphocytes, and the preferential repair of replication-associated DNA lesions by homologous recombination in actively proliferating cells.

Therapeutic combinations with TOP1 inhibitors

As for most other anticancer agents, the prescription of TOP1 inhibitors is currently based on the cancer histology and tissue of origin. Irinotecan is approved for colon carcinomas, whereas topotecan is approved for ovarian cancers, although both drugs target TOP1 in a similar way. The different indications have been determined empirically over the course of clinical trials, rather than by the molecular characteristics of the tumours.

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Despite early reports that suggested the potential value of determining TOP1 levels in tumours[88,89], current therapeutic protocols do not take tumour expression levels of TOP1 protein or mRNA into account nor do they evaluate chromosome 20q amplification status (the TOP1 genomic locus is at 20q12–q13.1) before giving camptothecin-based therapies. The most sensitive biomarkers activated by the trapping of TOP1ccs by camptothecins and non-camptothecin TOP1 inhibitors in experimental systems are γ-H2AX[49], CHK2 phosphorylation at threonine 68 by ATM and DNA-PK[90], and ATM activation by autophosphorylation at serine 1981 (REFS 54,90). Studies are ongoing (see the National Cancer Institute Developmental Therapeutics Program (NCI-DTP)) to evaluate these markers in clinical material. Key challenges are to further understand the molecular determinants of response to camptothecins (FIG 5; TABLE 2), to select the patients whose tumours should be selectively sensitive to TOP1 inhibitors and to combine therapies accordingly. All of these challenges require validated biomarkers.

Toward a rationale for the use of TOP1 inhibitors. Improving the use of camptothecins and new TOP1 inhibitors will probably require molecular correlates. Finding such correlates could come from several

Table 2 | **Genetic alterations that sensitize mammalian cells to TOP1 poisons**

Genes	Functions	Refs
APTX	Mutated in AOA1; encodes aprataxin, which associates with XRCC1	129
ATM	Mutated in AT; a protein kinase from the PI3K family that is implicated in the DSB response and CHK2 activation	54,130,131
ATR	Mutated in Seckel syndrome (SCKL); a protein kinase from the PI3K family implicated in replication stress and CHK1 activation	132
BCL2	Mutated in B-cell lymphoma; suppresses apoptosis	133
BLM	Mutated in BS; helicase from the RecQ family involved in genomic stability	56
BRCA1	Mutated in familial breast cancers; DNA damage response; TC-NER	134
BRCA2	Mutated in FANC and familial breast cancers; BRCA2 = FANCD1; promotes RAD51 loading and homologous recombination (HR)	135
CSA and CSB	Mutated in Cockayne syndrome (CS); involved in TCR/BER	136
CHK1	Checkpoint kinase phosphorylated and activated by PI3K (ATR)	137,138
CHK2	Mutated in Li-Fraumeni syndrome with normal p53; checkpoint kinase phosphorylated and activated by PI3K (for example, ATM)	55,137,139
DNA-PK	Protein kinase from the PI3K family; implicated in DSB response	53,140
FEN1	Flap and gap endonuclease; processing of stalled replication forks	141
H2AX	Core histone; phosphorylated in response to DSB (γ-H2AX foci)	49
Lamin A	Mutated in Hutchinson-Gilford progeria syndrome (HGPS)	142
NBS1	Mutated in Nijmegen breakage syndrome (NBS); Scaffolding protein forming a complex with MRE11 and RAD50 (MRN complex); DSB repair and recombination pathways	143
PARP1 and PARP2	Involved in BER with XRCC1 and TDP1	8,84–86
PNKP and hPNK	Processing of DNA ends: 3′-DNA-phosphatase and 5′-DNA-kinase	144
RAD51C	One of the five RAD51 paralogues; implicated in DNA strand exchange and homologous recombination	145
TDP1	Mutated in SCAN1; hydrolysis of 3′-phosphodiesters (phosphotyrosyl and phosphoglycolate) and phosphamides (TDP1 cleavage complex)	79–82
TP53	Mutated in Li-Fraumeni syndrome; encodes p53; involved in checkpoint and apoptosis control	146,147
WRN	Mutated in Werner syndrome; RecQ helicase involved in genomic stability	148,149
XRCC1	BER; binds to TDP1, PARP, β-polymerase, ligase III and aprataxin	83,150
XRCC2	One of the five RAD51 paralogues: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3; implicated in DNA strand exchange and homologous recombination	150–152
XRCC3	One of the five RAD51 paralogs; implicated in DNA strand exchange and homologous recombination	152

AOA1, Ataxia-oculomotor apraxia 1; AT, ataxia telangiectasia; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; BER, base excision repair; BLM, Bloom syndrome (BS); CSA and CSB, Cockayne syndrome (CS) complementation groups A and B; DNA-PK, DNA-dependent protein kinase; DSB, DNA double-strand breaks; FANC, Fanconi anaemia; NBS, Nijmegen breakage syndrome; NER, nucleotide excision repair; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidyl inositol 3-kinase; PNKP, polynucleotide kinase phosphatase; SCAN1, spino cerebellar ataxia axonal neuropathy; TCR, transcription-coupled repair; WRN, Werner syndrome.

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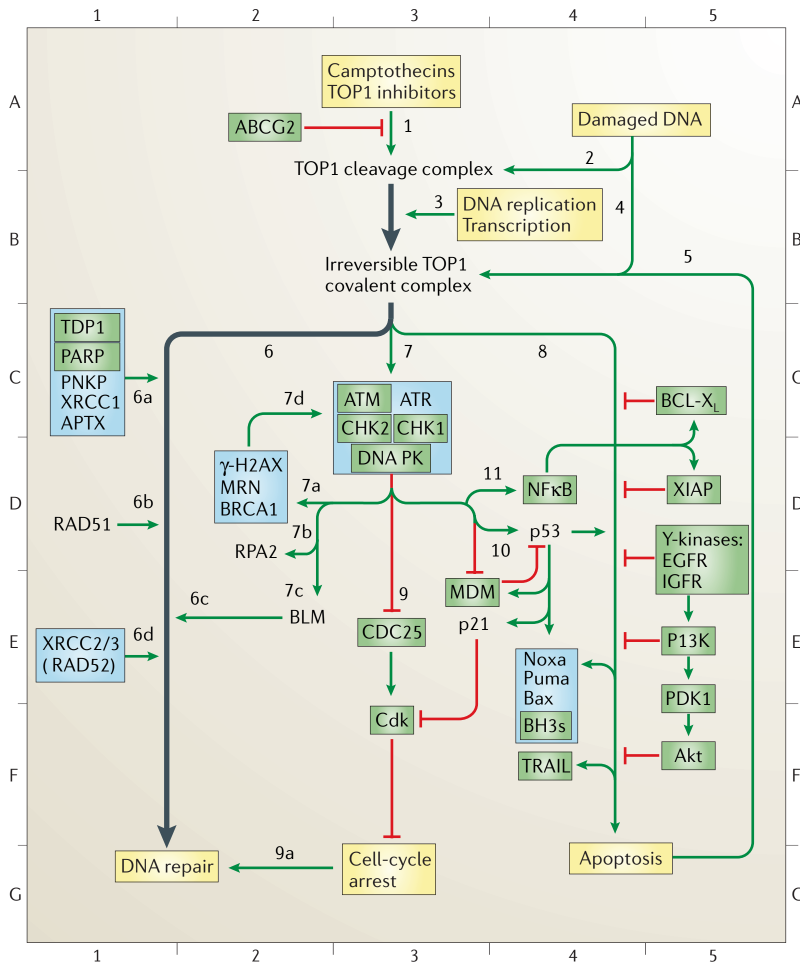

Figure 5 | **Molecular pathways involved in cellular responses to TOP1 cleavage complexes.** Annotation conventions are derived from molecular interaction map notation. Green lines refer to activation, red lines to inhibition and black lines to conversion. Inputs and outputs are indicated in yellow boxes. Camptothecins are actively pumped out of cells by the ATP-binding cassette sub-family G (ABCG2) transporter (A2). Drugs (1, A3) and DNA lesions (2, A4) induce reversible TOP1 cleavage complexes, which are converted into irreversible TOP1 covalent complexes by replication and transcription (3, B3). Irreversible TOP1 covalent complexes can also be produced when TOP1 cleaves damaged DNA (4, B4) and during apoptosis (5, B5). Irreversible TOP1 covalent complexes can be repaired (6, C2), induce cell-cycle arrest (7, C3 and 9, D3) or apoptosis (8, C4). DNA repair involves at least three pathways: the TDP1, PARP and XRCC1 base excision repair (BER) complexes (6a, C1); homologous recombination with RAD51 (6b, D1) and the RAD52 complex (6d, E1); and the Bloom syndrome helicases (BLM)–TOP3α pathway that suppresses homologous recombination (6c, E2) (reviewed in REFS 8,17). Cell-cycle arrest involves checkpoint kinases including ataxia telangiectasia mutated (ATM), ataxia telangiectasia and RAD3 related (ATR) and DNA-dependent protein kinase (DNA-PK), and their relays CHK2 and CHK1 (7, C3). Some of the regulators and targets of the checkpoint kinases are histone H2AX, MRE11–RAD50–NBS1 (MRN complex) and BRCA1 (7a, D2), replication protein A (RPA2) (7b, D2), BLM (7c, E2), and CDC25 (9, E3). γ-H2AX, MRN and BRCA1 further activate the ATM–CHK2 axis[54] (7d, C2). Several pathways involved in checkpoint activation are shown: inhibition of CDC25 phosphatases (9, E3), the inactivation of MDM2 (E3) and the activation of p53 (10, D4). *CDKNIA*, which encodes p21, is an important p53-responsive gene, which in turn inhibits cyclin-dependent kinases (F3). Cell-cycle arrest facilitates DNA repair (9a, G2). p53 activates apoptosis both directly and by transactivating pro-apoptotic genes (E4). The apoptotic pathway is commonly attenuated in cancer cells because of p53 mutations, and because of the constitutive activation of receptor tyrosine kinases (Y-kinases) (D5), phosphatidylinositol-3-kinase (PI3K) (E5) and Akt (F5). TOP1 inhibitors tend to suppress apoptosis by activating nuclear factor κB (NFκB) (11, D4), which increases the expression of anti-apoptotic genes such as *XIAP* (chromosome X-linked inhibitor of apoptosis) (D5) and BCL-X_L (C5). The sites of action of new targeted therapies are indicated in the green boxes.

approaches that include: the molecular dissection of cellular responses in experimental models (including yeast and non-metazoan models) to provide insights into the downstream molecular pathways that determine cellular sensitivity or resistance to TOP1ccs[8,17] (TABLE 2); the molecular characterization of tumours to determine the presence of molecular determinants of response before therapy; and, as mentioned above, the identification of tumour biomarkers to monitor response in individual tumours. FIG. 5 uses the graphical conventions developed by Kohn *et al.*[91] (see NCI-discover site) to summarize some of the cellular pathways that determine the cellular response to TOP1 inhibitors. In the section below, we will focus on four determinants of response to TOP1ccs: one upstream of TOP1ccs (cellular drug accumulation, primarily under the control of the ATP-binding cassette transporter ABCG2) and three downstream from TOP1ccs (DNA repair, growth arrest linked to cell-cycle checkpoints and apoptosis). Each of the downstream determinants is under the control of several key regulatory elements that usually function in parallel. Defects in the downstream determinants are crucial to the oncogenic process, and it is probable that rational therapeutic combinations could be guided by pre-existing defects that are tumour specific. Selectivity could therefore be achieved by targeting a pathway that the cell has become reliant on for the cellular response to TOP1 covalent complexes, as parallel pathways have become mutated in a particular tumour; a concept known as synthetic lethality.

Role of ABC transporters in resistance to camptothecins. The human genome contains 48 genes that encode ABC transporters[92], and the overexpression of these drug efflux transporters confers a high degree of resistance to various anticancer drugs[93]. Camptothecin and irinotecan are relatively poor substrates for ABCB1 (also known as MDR1)[94], but are affected by ABCG2 (also known as MXR or BCRP). In addition to camptothecin derivatives, the spectrum of resistance conferred by ABCG2 in human cells includes mitoxantrone, anthracyclines, methotrexate and tyrosine kinase inhibitors, with no resistance to vinca alkaloids or taxanes[92]. Initial evidence came from the observation that a mitoxantrone-resistant human carcinoma cell line (MCF7/MX) had high-level cross-resistance to CPT-11, SN-38 (the active form of irinotecan) and 9-aminocamptothecin, but had resistance to neither camptothecin nor 10,11-methylenedioxy-camptothecin[95,96]. ABCG2 has been implicated in topotecan resistance in human ovarian cell lines[97] and in lung cancer resistance to irinotecan[98]. Some camptothecin-resistant yeast strains also have alterations in the ABC transporters (SNQ2 and PDR5) and their upstream regulator PDR1 (REF. 99). The SNQ2 transporter, similar to ABCG2, shows limited sequence similarity to MDR1 and MRP, and is most similar to the *D. melanogaster white* gene[100].

Efforts to overcome multidrug resistance with ABC transporter inhibitors have been disappointing. Nevertheless, analyses of the mechanism by which imatinib mesylate can reverse resistance to irinotecan showed an unexpected mechanism — imatinib is a

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substrate for ABCG2 and can function as a competitive inhibitor, leading to the cellular accumulation of irinotecan[101]. This empirical result indicates that combining different anticancer drugs that are substrates of common ABC transporters might saturate the transporter and increase anticancer activity[102]. It also exemplifies the pharmacokinetic and pharmacodynamic challenges that can unexpectedly occur when chemotherapeutic drugs are combined.

Taking advantage of the defective checkpoint pathways that characterize tumours. Efficient DNA repair requires cells to stop replicating and dividing in response to checkpoint activation (FIG. 5). A clear example of this type of synthetic lethality is the case of DNA-repair-deficient yeast cells with *TDP1* deficiency, which become markedly sensitive to camptothecins after the inactivation of their cell-cycle checkpoint gene *RAD9* (which encodes the orthologue of proteins that contain the BRCA1 C-terminal domain (BRCT) such as BRCA1, 53BP1 and NBS1 in humans)[103]. Therefore, defects in cell-cycle checkpoints might adversely affect DNA repair and increase the antiproliferative activity of camptothecins.

The abrogation of cell-cycle checkpoints can be achieved using small-molecule inhibitors of the protein kinases that activate the cell-cycle checkpoint(s), such as CHK1, CHK2, ATM, ATR and DNA-PK (FIG. 5). A proof of principle for this approach is the remarkable synergism produced by the addition of 7-hydroxystaurosporine (UCN-01) immediately after camptothecin treatment[53,104]. UCN-01 is a relatively broad serine-threonine protein kinase inhibitor that inhibits CHK1 (REF. 105), CHK2 (REF. 106) and 3-phosphoinositide-dependent protein kinase 1 (PDPK1)[107] (and thereby Akt; FIG. 5). Unfortunately, the synergism observed in cell culture[53,104] has been difficult to evaluate in clinical trials because of the poor pharmacokinetics of UCN-01 in humans[108]. CHK1 and CHK2 inhibitors are in preclinical development at present, and it will be interesting to see whether these prove to be synergistic with TOP1 inhibitors. Moreover, CHK2 has a dual function, as it activates both apoptosis and cell-cycle checkpoints[54]. Therefore, in tumour tissues where apoptosis is defective, as in the case of tumours with mutated or inactivated p53, CHK2 inhibitors would be expected to function primarily by preventing DNA repair because of its main function as a checkpoint inhibitor that can induce cellular arrest in a p53-independent manner. On the other hand, in normal tissues the CHK2 inhibitor would be expected to limit apoptosis and therefore dose-limiting toxicity. The resulting net effect would then be an increased therapeutic index of the TOP1 inhibitors[54,109]. Recently, inhibitors of ATM kinase[110] and DNAPK[111] have been reported. Clinical trials of such derivatives in combination with camptothecins is awaited, especially in patients whose tumours show genomic instability and constitutive activation of the ATM–CHK2 pathway[50].

Taking advantage of defective repair pathways in tumours. A significant number of tumours have defective DNA-repair pathways, including mismatch repair, nucleotide excision repair or double-strand break repair. The apparent redundancy of the repair pathways involved with the excision of TOP1 covalent complexes (see previous section and FIG. 5) indicates that several pathways must operate together for optimum cell survival. Therefore, therapeutic selectivity could, in principle, be achieved by first identifying pre-existing defective pathways in the tumour and then by targeting the remaining pathways. A proof of principle for this approach is the case of camptothecin-treated yeast cells that are not particularly sensitive to TDP1 inactivation unless the RAD1 or MRE11 pathways are also inactivated[8,112,113].

A number of DNA-repair inhibitors are being developed. Among the most advanced are PARP inhibitors[86,114,115] (FIG. 5). Their synergistic effects with camptothecins and their inhibition of TOP1cc repair has been known for a long time[87], and was confirmed recently with a new PARP inhibitor[86]. Accordingly, PARP-deficient and knockout cells[8,17] are hypersensitive to camptothecins[8,85,86] (TABLE 2), and the PARP inhibitor A14361 increases the anti-tumour activity of irinotecan in mouse models[86]. Several PARP inhibitors are in clinical development[115], and clinical trials that combine PARP and TOP1 inhibitors are warranted. PARP functions in the XRCC1–BER complex with TDP1, but the development of TDP1 inhibitors (FIG. 5) is in the initial stages only[116]. As for yeast[103,112], the rationale is the selective sensitization of tumour cells to TDP1 inactivation when they are checkpoint-deficient. Accordingly, a recent report showed the selective cytotoxicity of PARP inhibitors in BRCA-deficient tumours[114].

Taking advantage of tumour apoptotic defects. A significant fraction of tumours require the inactivation of apoptosis to counteract the activation of oncogenes such as *MYC* and the presence of endogenous DNA damage related to genomic instability[117]. Therefore, reactivating apoptotic pathways can attack tumours in the absence of a DNA-damaging agent. This might be exacerbated when cells are treated with TOP1 inhibitors. Moreover, TOP1 inhibitors are potent activators of effector caspases, both by activating BAX[118] and the extrinsic pathway that involves FAS and caspase 6 at the membrane level[119]. On the other hand, TOP1 inhibitors also activate nuclear factor κB (NFκB) and tend to suppress apoptosis[120,121]. Therefore, the relative balance between activation of the pro- and anti-apoptotic pathways governs the cellular response to TOP1 inhibitors. FIG. 5 outlines the complexity of the pathways involved, the site of action of currently available targeted therapies[122] are shown as green boxes.

Several therapeutic approaches can be envisaged to activate apoptosis in tumour cells, such as the direct activation of the extrinsic pathway with recombinant TRAIL, activation of the mitochondrial pathway with BH3-mimetics and reactivation of p53 with MDM2 inhibitors (FIG. 5). Several therapeutic approaches are also available to inactivate anti-apoptotic pathways, many of which are involved in carcinogenesis. Targeted anti-apoptotic therapies include inhibitors of the growth factor tyrosine kinases (EGFR, insulin-like growth factor receptor, platelet-derived growth factor receptor and

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vascular endothelial growth factor receptor), phosphatidylinositol-3-kinase (PI3K), PDP1, Akt and NFκB. As a first approximation, the proteasome inhibitor bortezomib can be viewed as an inhibitor of NFκB, as it inhibits the degradation of the inhibitor of κB (IκB)[123]. Bortezomib functions synergistically with SN-38 (REF. 124), possibly because camptothecins are potent activators of NFκB[120] downstream of ATM[121] (FIG. 5). Therefore, the combination of TOP1 inhibitors with NFκB inhibitors might prove to be efficacious.

Conclusions

Although camptothecins are therapeutically effective, they are not curative as single agents. Several approaches need to be considered to improve the effectiveness of TOP1 inhibitors. First, the development of new inhibitors with activity against different cancers, improved pharmacokinetics and lower toxicity are needed. Second, further investigation of the molecular determinants of drug activity in model systems should lead to the development of molecular tools to classify tumours on the basis of whether they have a molecular network matching drug-specific pathways. Third, a rationale for the combination of TOP1 inhibitors with other drugs or biological treatments on the basis of the molecular network of individual tumours is needed. And fourth, reliable, sensitive and non-invasive biomarkers are required to follow the early response or lack of response to TOP1 inhibitors in combination with other treatments so that therapies can be rapidly and effectively adapted.

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Competing interests statement
The authors declare no competing financial interests.

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