Camptothecins and Topoisomerase I: A Foot in the Door.

Targeting the genome beyond Topoisomerase I with camptothecins and Novel Anticancer Drugs: importance of DNA Replication, Repair and Cell Cycle Checkpoints

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Abstract:

Camptothecins selectively target topoisomerase I (Top1) by trapping the catalytic intermediate of the Top1-DNA reaction, the cleavage complex. Hence, camptothecins represent a paradigm for targeting macromolecular interactions. Instead of preventing the binding of the two macromolecules they target (Top1 and DNA), camptothecins slow down the dissociation of these macromolecules. The activity of camptothecins underlines the usefulness of screening for drugs that inhibit the dissociation of macromolecules. Camptothecins and non-CPT Top1 inhibitors are being developed to improve the pharmacodynamics, pharmacokinetics and clinical pharmacology of camptothecins, and it is likely that drugs with improved anticancer activity will be discovered. Although Top1 is the only primary target of camptothecins, the mechanisms of camptothecins' anticancer activity rest beyond the formation of cleavage complexes. Indeed, Top1 cleavage complexes lead to replication- (and transcription-) mediated DNA damage. It is likely that DNA damage can be repaired more efficiently in normal than in cancer cells that are intrinsically deficient for DNA repair and cell cycle checkpoints. Evaluating such deficiencies in clinical samples is becoming possible. If specific deficiencies are associated with clinical responses, their detection should guide therapeutic decisions. Furthermore, targeting DNA repair (Tdp1) and checkpoints (Chk2) might increase the selectivity of Top1 inhibitors for tumors, thereby increasing the antitumor activity while reducing the side effects of Top1 inhibitors.

The aim of this brief review is a to give a rather subjective view of the current status and prospects regarding the targeting of Top1 by small molecules. We invite the reader to consult two recent and detailed reviews. One on novel Top1 inhibitors [1], and the other on DNA damage, repair and checkpoint pathways associated with Top1 cleavage complexes [2]. Both reviews contain a large number of additional references no included here for space limitation. We also invite the reader to our Website http://discover.nci.nih.gov/pommier/topo1.htm

Recent Development in the Pharmacology of Camptothecins

Camptothecins are among the most recently approved anticancer agents with two derivatives approved by the FDA: Camptosar® (Irinotecan hydrochloride; CPT-11) for advanced colo-rectal carcinomas and Hycamtin® (Topotecan) for ovarian cancers. Both drugs are water-soluble derivatives of the parent natural alkaloid camptothecin (CPT) [Fig. (**1**)], which was discovered and developed by the NCI starting in the early 70's, at approximately the same time and by the same groups as Taxol [3]. The anticancer activity of CPT was known well before CPT's mechanism of action, and CPT carboxylate had been tested clinically and discontinued at least in part because of side effects in spite of clear anticancer activity [3]. It is after discovering Top1 as the cellular target of CPT that Topotecan and Irinotecan (CPT-11) were both successfully developed. These successful drug developments demonstrate how target discovery can overcome hurdles in drug development. The discovery of Top1 as a new anticancer target took place under the auspices of the NCI approximately 20 years ago, via a US Federal (NCI) grant awarded to research groups from both academia and industry (the Johns Hopkins University, the University of Florida, and Smith Kline French at the time). In spite of the relative ease for their semi-synthesis, the commercial price of Hycamptin® (Topotecan) and Camptosar® (Irinotecan) was indexed on Taxol®, whose high cost was initially justified at least in part by its difficult synthesis and short supply.

Camptothecins are pharmacologically unique for at several reasons. First, Top1 is the only target of these drugs, as demonstrated in yeast cells, which become totally

immune to CPT when the Top1 gene is genetically removed [4], and as demonstrated by the existence of Top1 mutations rendering the enzyme immune to camptothecins in vertebrate cell lines selected for CPT resistance [5]. Secondly, changing the stereochemistry of CPT by inverting its chiral center at position 20 completely inactivates CPT (synthetic 20-R-CPT is inactive). Therefore, it is remarkable that the naturally selected drug is only 20-S-CPT (refer to as CPT for simplicity) [Fig. (**1**)]. CPT was first isolated from the bark of the Chinese tree, *Camptotheca acuminata*. It penetrates vertebrate cells readily and targets Top1 within minutes of exposure to low or even sub-micromolar drug concentrations. CPT then binds reversibly to the Top1 cleavage complexes. Because the cleavage complexes reverse within minutes following CPT removal, CPT is a sharp pharmacological tool (since the drug exposure can be precisely controlled) with high specificity (with only one target: Top1) in spite of a relatively low affinity as micromolar drug concentrations are required to readily detect the trapping of Top1 cleavage complexes. CPT has become a useful pharmacological tool to explore replication-mediated DNA damage in various organisms including yeast, thereby providing powerful ways to dissect genetic factors implicated in checkpoints and DNA repair in response to Top1 cleavage complexes (see below).

A second remarkable aspect of camptothecins is their specificity for binding to and inhibiting a precise step of the Top1 reaction. CPT binds neither to Top1 alone nor to DNA alone, but only to the complex formed by Top1 when it cleaves DNA (i.e. CPT selectively binds to the cleavage complex). We initially proposed [6] that the planar aromatic portion of CPT stacks between the base pairs flanking the cleavage site and prevents the religation of the DNA. Hence the title of the present review; CPT is like a foot in a door. CPT binds to the Top1-DNA complex, like a foot sneaking into an opened door and preventing the door from closing [Fig. (**2**)]. In this metaphor, the Top1-DNA complex would correspond to the open/cleaved DNA phosphodiester backbone. This stacking model was recently demonstrated by a co-crystal structure of Topotecan with the Top1-DNA complex [7], and extended more recently to CPT itself as well as to two non-CPT inhibitors, an indenoisoquinoline and an indolocarbazole [8]. CPT and the Top1 inhibitors stand as a pharmacological paradigm for searching inhibitors of macromolecular interactions. Indeed, instead of blocking the binding of the two

macromolecules (the DNA and Top1 in the present case), Top1 inhibitors prevent the dissociation of these 2 macromolecules. By generalizing this concept, one would propose to look for inhibitors of macromolecular interaction that, not only prevent the binding (which is presently most commonly the way targets are being pursued), but also for inhibitors that prevent the dissociation of target macromolecules.

Novel Non-Camptothecin Topoisomerase I Inhibitors

There are several reasons to develop new Top1 inhibitors. First, Top1 is a validated target since camptothecins exhibit anticancer activity. Second, it is likely that inhibitors with different chemical structures will exhibit differential (and hopefully greater) antitumor activity. For instance, among tubulin inhibitors, colchicine, vinca alkaloids and taxanes are very different clinically. Vincas and taxanes are potent anticancer agents whereas colchicines have no useful anticancer activity. Third, camptothecins have dose-limiting side effects including severe diarrheas and neutropenia. Fourth, camptothecins are rapidly inactivated in the bloodstream because of their labile lactone E-ring. This ring opens at physiological pH [Fig. (**1A**)], resulting in the carboxylate CPT derivative, which is inactive against Top1 and binds tightly to serum albumin. Although the E-ring opening is reversible at acidic pH [Fig. (**1A**)], only a small fraction of camptothecins is in the active lactone form.

Early structure-activity relationship studies suggested that the E-ring alpha-hydroxylactone was required for activity. However, two chemical families derived from the CPT structure have questioned this assumption [Fig. (**1B**)]. In the homocamptothecins, the E-ring has been converted from a 6- into a 7-membered ring. This methylene addition increases the E-ring stability at physiological pH, and thereby the fraction of active drug [for review see [9]]. The clinically chosen derivative, diflomotecan bears fluoro substitutions at both positions 9 and 10 [see Fig. (**1A**)]. Homocamptothecins have two advantages over camptothecins: 1) they are more potent Top1 inhibitors than camptothecins in biochemical assays; 2) they are less susceptible to cellular drug efflux by the ABCG2 transporter [10]. Homocamptothecins have in

common with camptothecins: 1) the trapping Top1 at sites very similar to those trapped by camptothecins, and 2) their cellular cross-resistance with CPT in cells with Top1 mutations [11], which suggests that homocamptothecin binds to the same binding site as camptothecins in the Top1-DNA complex. One potential limitation of homocamptothecins is their irreversible inactivation by opening of the lactone E-ring. Binding of homocamptothecin to Top1 has recently been shown to stabilize the E-ring lactone form [12]. The second novel family of lactone derivative of CPT are ketonic derivatives, which are active Top1 poisons, and are completely stable because their E-ring cannot open [13] [Fig. (**1B**)].

Non-camptothecin inhibitors structurally different from camptothecins include the indolocarbazoles, the phenanthridines, and the indenoisoquinolines [reviewed in [1]]. The indolocarbazoles are the most advanced with two derivatives in clinical trials [Fig. (**1C**), left panel]. More than 150 indenoisoquinolines [Fig. (**1C**), right panel] have been synthesized with the goals of obtaining: 1) more potent Top1 poisons; 2) drugs with original structures; 3) cytotoxic agents at low concentrations in various cell types; 4) drugs that selectively target Top1, as determined by the drug resistance of cells devoid of Top1; 5) drugs that target Top1 differentially from camptothecins and remain active against CPT-resistant Top1's [14]. Novel indenoisoquinolines fulfilling these criteria are in preclinical evaluation. A recent development has been the generation of three Top1-DNA co-crystal structures: one with an indenoisoquinoline, one with an indolocarbazole, and the other with the natural CPT bound to Top1-DNA complexes. In all three structures, a single drug molecule is bound at the interface of the base pairs flanking the cleavage site, and forms hydrogen bonds with key residues of the enzyme [8]. The drugs'polycyclic ring systems overlap with those of Topotecan bound to the Top1-DNA complex. The differential trapping of Top1 cleavage complexes by each drug family at different Top1 sites results primarily from the optimum stacking of the each drug's polycyclic ring systems with the base pairs flanking the cleavage site. For instance, indenoisoquinolines tend to trap the Top1 sites with C(-1) whereas camptothecins trap sites with T(-1) and G(+1) [14].

Novel Development in Targeting the Repair/Response Pathways to Camptothecins

One of the key questions regarding the use of camptothecins and Top1 inhibitors as anticancer agents is what determines their anticancer activity. Indeed, Top1 is present both in cancer and normal tissues (it is essential for multicellular organisms), and there is no evidence that Top1 is more sensitive to camptothecins in tumors than normal tissues. Top1 overexpression may, however contribute to the selectivity of camptothecins against some colon carcinomas with chromosome 20 amplification because the Top1 gene is localized to chromosome 20q [15]. However, to our knowledge, there is no clinical report addressing the possible relationship between clinical response to camptothecins and chromosome 20q amplification in tumors, and so far no obvious relationship has been established between Top1 levels and cellular response *in vitro* [16] or in tumors.

The most likely explanation for the selectivity of camptothecins for cancer cells is the existence of preexisting DNA repair and cell cycle checkpoint deficiencies in tumors. Indeed, Top1 trapping is required but is not sufficient for the production of DNA damage. In cancer cells, it appears that DNA damage results primarily from replication-mediated DNA double-strand breaks as replication forks collide with Top1 cleavage complexes on the leading strand for DNA synthesis [#1, Fig. (**3**)]. Top1 cannot religate these cleavage complexes as the 5'-hydroxyl generated by Top1 is bound with the newly replicated daughter strand. Although transcription-mediated DNA damage can, at least in principle arise from a comparable mechanism, it appears that, if such lesions exist, they are not a prevalent cause of death, at least in tissue culture model systems. Hence, one could consider that the "secondary" targets of camptothecins are DNA repair and cell cycle checkpoint deficiencies.

Progress has been remarkable in recent years regarding the elucidation of the repair and checkpoint pathways involved in the removal of Top1 cleavage complexes [for review see [2] and http://discover.nci.nih.gov/pommier/topo1.htm] The use of yeast strains with known genetic deficiencies has been a powerful approach, and basic researchers in DNA repair and checkpoints are now using CPT as an inducer of replication-mediated DNA damage in yeast. Some of the Top1 repair pathways are illustrated in Figure **3** [for further details see [2] and

http://discover.nci.nih.gov/pommier/topo1.htm]. First, Top1 cleavage complexes associated with replication-mediated DNA damage can be reversed by replication fork reversal with formation of a "chicken foot" structure [#2, Fig. (**3**)]. Consequently, the 5'-hydroxyl can be readily religated with the broken DNA. The stalled replication fork would then restart on an intact DNA substrate in coordination with the activity of the Bloom (and possibly Werner/RecQ) helicase. In the absence of replication fork reversal, schematically, two types of lesions need to be repaired: 1) replication-mediated DNA double-strand breaks [#3, Fig. (**3**)], and 2) irreversible Top1 cleavage complexes (commonly referred to as suicide complexes) [#4-7, Fig. (**3**)]. After which, replication fork restart would have to occur. The most specific Top1 repair enzyme appears to be tyrosyl DNA phosphodiesterase (Tdp1) (#5). Tdp 1 hydrolyzes the 3'-phosphodiester covalent bond between the Top1 catalytic tyrosine and the 3'-DNA end. Tdp1 is associated with the XRCC1 repair complex in mammalian cells [17]. However, Tdp1's activity is limited unless Top1 is first proteolyzed. Consistently, Top1 ubiquitination and proteolysis (#6) [18, 19] are rate limiting for the repair and survival of CPT-treated cells. More recently, experiments in genetically altered yeast strains demonstrated alternative pathways for excising Top1 covalent complexes [#8, Fig. (**3**)] [for further details see [2]]. Instead of cleaving the peptide-nucleic acid bond (as for Tdp1), two endonuclease pathways can remove the segment of DNA bound to Top1. Mus81/Eme1 appears associated with recombination intermediates, whereas Rad1/Rad10 could function on simpler 5'-flap substrates. The phylogenic conservation of the Top1 repair pathways between yeast and humans is consistent with the occurrence of Top1-mediated DNA damage under physiological conditions (in the absence of exposure to camptothecins), as a large spectrum of endogenous and carcinogenic DNA lesions (oxidative and alkylation base damages, abasic sites, carcinogenic adducts and DNA nicks) are known to trap Top1 cleavage complexes [20]. From a cancer therapeutic viewpoint, the remarkable progress in understanding the repair of Top1-mediated DNA lesions provides us with a defined set of molecular markers that are known to be altered in and involved with the carcinogenic process, and that can be evaluated and used as molecular markers for therapeutic response.

Finally, DNA repair is coupled with cell cycle checkpoints, which explains that checkpoint deficiencies (which are known to be carcinogenic: Rb, ATM, Mre11, Nbs1, BRCA1, Chk2, p53) sensitize cancer cells to DNA damaging agents, especially when they are associated with DNA repair defects. For instance, yeast cells rendered defective for Tdp1 are not resistant to camptothecins unless a key checkpoint gene, Rad9 (homolog of human BRCA1) is simultaneously deficient. Hence, checkpoint proteins drive (channel) the repair of DNA lesions toward specific repair pathways. For instance, in the absence of Sgs1, the yeast homolog for Bloom's and Werner's helicases, DNA damage has to be repaired by homologous recombination rather than by replication fork reversal [#2 in Fig. (**3**) becomes deficient]. Hence, several concepts with therapeutic implications emerge: 1) cancer cells might be selectively sensitive to camptothecins and other DNA damaging agents because of their intrinsic deficiencies in checkpoints and DNA repair; 2) targeting checkpoint and/or repair might enhance the selectivity of DNA damaging for cancer cells that are already partially deficient for such pathways. Accordingly, we found that the cell cycle checkpoint abrogator, UCN-01 (7-hydroxystaurosporine), which is in clinical trials, selectively enhances the antiproliferative activity of CPT in p53-deficient cells [21, 22]. We recently discussed the rationale for developing, and using Chk2 inhibitors [23] in association with camptothecins and the novel non-CPT Top1 inhibitors to increase the lethality of Top1-mediated DNA damage. The rationale would be to use Chk2 inhibitors to abrogate cell cycle checkpoints in the tumor while decreasing the toxicity of DNA damaging agents by decreasing apoptosis in normal intestinal and hematopoietic cells. Hence, the paradigm is that the same checkpoint/repair deficiencies that confer growth advantage in the carcinogenic process also render the cancer cells more sensitive to DNA damaging agents. Thus, DNA repair and checkpoint deficiencies are a double-edge sword for the tumor, allowing growth but conferring sensitivity to DNA damaging agents including Top1 inhibitors. Better understanding of the repair and checkpoint pathways will provide valuable insights for rationale drug associations with Top1 inhibitors.

Figure Legends:

Figure 1: Structure of some Top1 inhibitors. **A**. Camptothecins: the lactone E-ring opening is pH-dependent. It is induced within minutes at physiological pH but is reversible at acidic pH. The two FDA-approved derivatives are Topotecan (Hycamtin®; SmithKlineBeecham; approved by the FDA in 1998 for ovarian cancer and more recently for small cell lung cancer) and CPT-11 (Irinotecan = Camptosar®; Yakult licenced to Pharmacia-Upjohn; approved by the FDA in 2000 for relapsing colorectal carcinomas). CPT-11 is a prodrug, and needs to be converted to SN-38 by carboxylesterases. **B**. The two E-ring modified camptothecin derivatives: homocamptothecin derivatives (Beaufour-Ipsen Laboratories) are in early clinical trials; and the ketonic derivative (Servier Laboratories) is in preclinical development. **C**. Two indolocarbazoles in clinical trials are shown at left. One of the indenoisoquinoline (MJ-III-65) in preclinical development is shown at right.

Figure 2: The Top1 cleavage/religation reactions proceed by two enzyme catalyzed transesterification reactions. In the forward reaction, the Top1 catalytic tyrosine (Y; tyrosine 723 in humans) attacks a DNA backbone phosphodiester, thereby generating a single-strand break with a covalent tyrosyl-DNA bond at the 3'-end and a free 5'-hydroxyl ("open door"). In the reverse reaction, which is favored under normal conditions, the 5'-hydroxyl attacks the phosphodiester-tyrosyl bond, thereby releasing the enzyme and religating the DNA ("closed door"). Camptothecins (the natural alkaloid and its derivatives) as well as the non-CPT derivatives [see Fig. (**1**)] trap the Top1 cleavage complex by binding at the enzyme-DNA interface and preventing Top1-mediated DNA religation ("foot in the door"; see text for further description of the metaphor). Note that CPT binding is non-covalent and rapidly reversible upon drug dilution/removal.

Figure 3: Some of the pathways involved in the repair of replication-associated Top1-mediated DNA damage. **1**. By stabilizing ("trapping; increasing the half-life of") a large fraction of the cellular cleavage complexes, CPT uncouples DNA relaxation by Top1 ahead of replication forks with DNA polymerization at the replication forks. Once a

replication fork catches up with a Top1 cleavage complex, a replication-mediated double-strand break is generated on the leading strand [24]. **2**. Replication fork reversal by formation of a "chicken foot"-like structure can allow the dissociation of the newly replicated DNA strand from its template. The broken template can then be religated by Top1. Resolution of the "chicken foot" structures probably requires RecQ helicase (such a Bloom'syndrome helicase). **3**. Repair of a replication-mediated double-strand break can involve either or both homologous recombination (HR) or/and non-homologous end joining (NHEJ) [for details see [2] and http://discover.nci.nih.gov/pommier/topo1.htm]. **4**. Invasion by a DNA strand bearing a 5'-hydroxyl can free Top1. However, this type of religation, which Top1 can perform effectively *in vitro* would generate a recombination. **5**. The most specific Top1 excision repair pathway involves Tdp1, which can hydrolyze the Tyrosyl-DNA covalent bond thereby regenerating the tyrosyl (Y) residue on Top1, and leaving a 3'-phosphate (P) that need to be hydrolyzed [probably by PNKP – for details see [2] and http://discover.nci.nih.gov/pommier/topo1.htm]. **6**. Tdp1 is markedly more efficient after proteolysis of Top1, which exposes the tyrosyl-DNA bond to be hydrolyzed. **7**. Two 5'-endonuclease complexes have recently been implicated in the excision of the DNA strand covalently bound to Top1.

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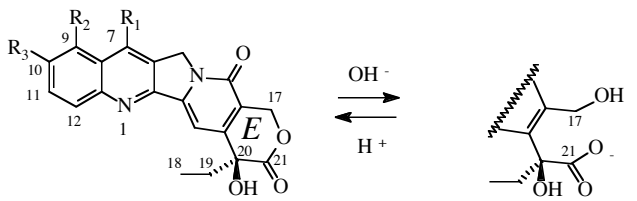
A



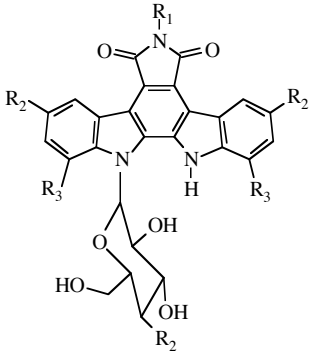
lactone form *Carboxylate form*

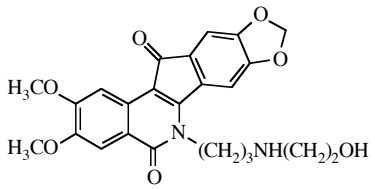
	R1	R2	R3
Camptothecin	H	H	H
Topotecan	H	$CH_2 \cdot N(CH_3)_2$	OH
SN-38	$CH_2 \cdot CH_3$	H	OH
CPT-11	$CH_2 \cdot CH_3$	H	O–CO–N N

B

Homocamptothecins Ketonic derivative

C

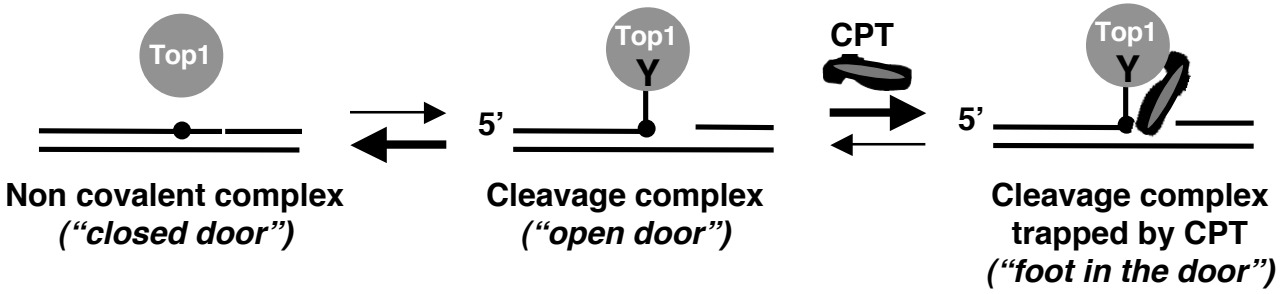




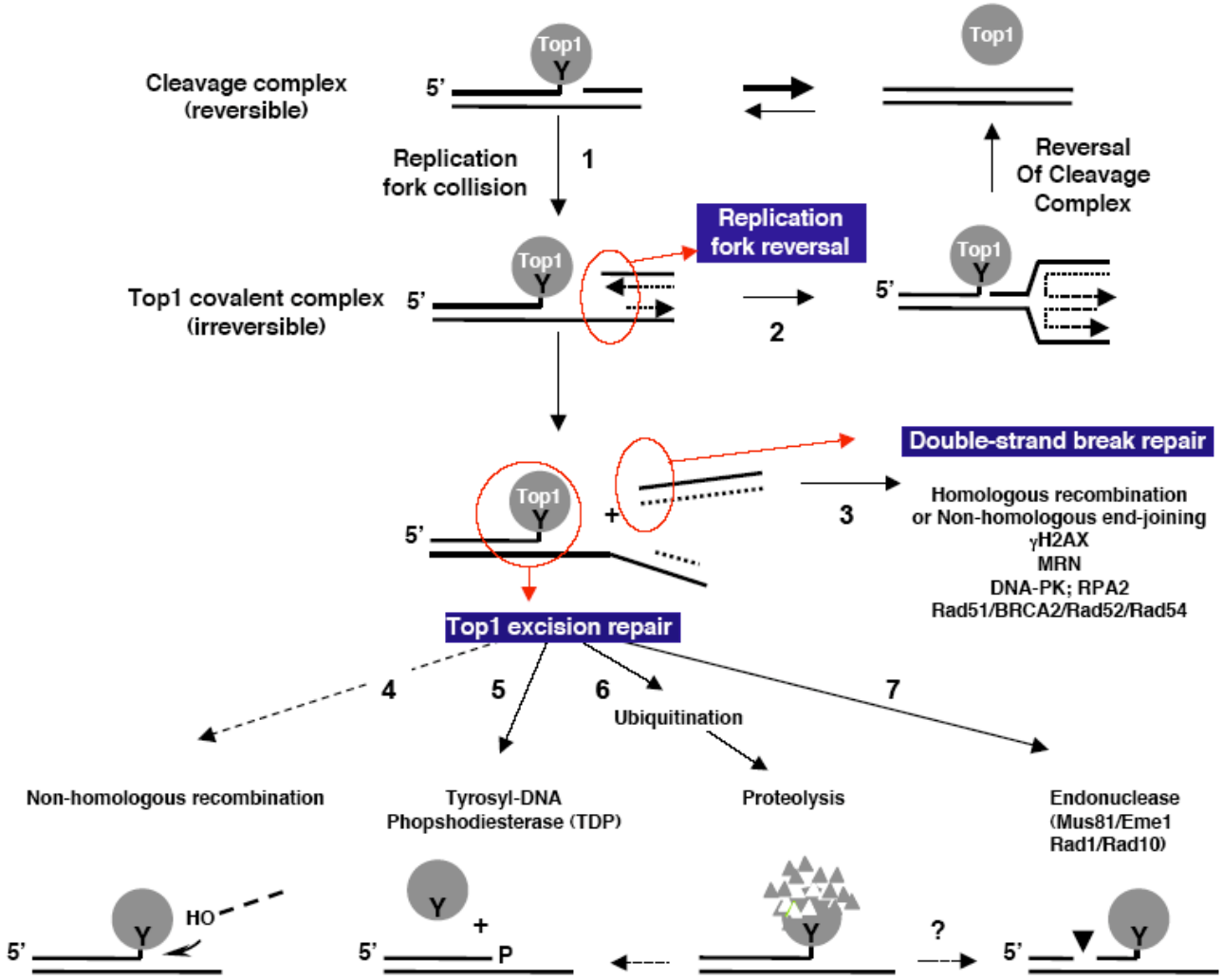
MJ-III-65

	R1	R2	R3
NB-506	NHCHO	H	OH
BMS-250749	H	F	H

Pommier, Fig. 1



Pommier, Fig. 2



Pommier, Fig. 3