

## Perspective

# Topoisomerase I (Top1): a major target of FL118 for its antitumor efficacy or mainly involved in its side effects of hematopoietic toxicity?

Fengzhi Li<sup>1</sup>, Xiang Ling<sup>1,2</sup>, Danni L Harris<sup>3</sup>, Jianqun Liao<sup>1,2</sup>, Yuping Wang<sup>2</sup>, David Westover<sup>4</sup>, Guohui Jiang<sup>5</sup>, Bo Xu<sup>6</sup>, Patrick M Boland<sup>7</sup>, Chunyang Jin<sup>3</sup>

<sup>1</sup>Department of Pharmacology & Therapeutics, Roswell Park Cancer Institute, Buffalo, New York, USA; <sup>2</sup>Canget BioTekpharma LLC, Buffalo, New York, USA; <sup>3</sup>Center for Drug Discovery, RTI International, Research Triangle Park, North Carolina, USA; <sup>4</sup>Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA; <sup>5</sup>Department of Pharmacology, School of Pharmacy, Qingdao University, Qingdao, China; <sup>6</sup>Departments of <sup>6</sup>Pathology, <sup>7</sup>Medicine, Roswell Park Cancer Institute, Buffalo, New York, USA

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**Abstract:** FL118 is a novel camptothecin (CPT) analogue that possesses exceptional antitumor efficacy in human tumor animal models. To date, two CPT analogues, irinotecan and topotecan, have been approved by the FDA for cancer treatment. FL118 exhibits superior antitumor activity over irinotecan and topotecan, and effectively overcomes the irinotecan- or topotecan-resistant human tumors in animal models. Accordingly, FL118 selectively inhibits the expression of multiple cancer-associated proteins (survivin, Mcl-1, XIAP, cIAP2, MdmX). However, FL118 has hematopoietic toxicity similar to irinotecan and topotecan, suggesting that FL118's hematopoietic toxicity may share a mechanism similar to irinotecan and topotecan. It is known that CPTs including irinotecan, SN-38 (active metabolite of irinotecan) and topotecan are topoisomerase I (Top1) inhibitors. However, the evidence from our studies failed to reveal that FL118 is a better Top1 inhibitor than SN-38. It was documented that Top1 expression level is positively associated with CPTs' sensitivity. Low Top1 expression links to CPTs' resistance. In contrast to these findings, we found that human colorectal tumor sensitivity to FL118 is irrelevant to the expression level of Top1 protein. FL118 can show high antitumor efficacy in Top1-negative tumors, while Top1 highly positive tumors can exhibit FL118 resistance. This suggests that the presence of Top1 target is not critical for FL118 antitumor activity. In other words, targeting Top1 by FL118 may not play a major role for its antitumor efficacy. However, studies indicate that FL118 can bind to, and inhibit Top1 activity. This raises the possibility that inhibition of Top1 by FL118 may predominantly be involved in hematopoietic toxicity, but not in FL118 antitumor activity. In this article, we will summarize existing observations and provide our up-to-date research results to support our opinion on this important topic.

**Keywords:** FL118, topoisomerase I, survivin, Mcl-1, XIAP, cIAP2, MdmX, camptothecin, irinotecan, topotecan

## Introduction

DNA topoisomerase I (Top1) is a ubiquitously expressed gene that is essential for mammalian cell proliferation during embryo development, as well as adult tissue and cell renewal over a lifetime. Top1 knockout mice die during early embryogenesis [1], and inhibition of Top1 activity interferes with normal tissue renewal and induces severe proliferative tissue toxicity (e.g. hematopoietic cell genesis from bone marrow). This is because the Top1 enzyme plays a critical role in cellular DNA replication and can

also facilitate many gene transcriptions [2]. Consistent with these facts, the major adverse events of the Top1 inhibitors from the camptothecin (CPT) analogues irinotecan (CPT-11) and topotecan (TPT) are the hematopoietic toxicity and diarrhea during cancer treatment in the clinic. The hematopoietic toxicity is likely due to the disruption of Top1-mediated DNA replication in normal bone marrow stem/progenitor cells. The diarrhea was proposed to be due to off-target effects related to the bis-piperidine that confers water-solubility in the case of irinotecan [2]. However, diarrhea induced by CPTs is

likely through a more general mechanism, since many anticancer drugs, including CPT analogues, without a bis-piperidine moiety have been known to induce diarrhea [3].

### Discovery of FL118, a CPT analogue with novel mechanism of actions (MOA)

Using the survivin gene as a target and biomarker in high-throughput screening of small molecule libraries, followed by hit-to-lead analogue characterization, we discovered a small-molecule (designated FL118) that possesses exceptional antitumor activity against colorectal and head-&-neck cancer in animal models of human tumors [4]. Structurally, FL118 is a CPT analogue with a methylenedioxy group linking to positions 10 and 11 of the A-ring. However, we found that mechanistically, FL118 selectively inhibits the expression of multiple antiapoptotic proteins (survivin, Mcl-1, XIAP, cIAP2) in the inhibitor of apoptosis (IAP) and Bcl-2 families. The inhibition of these proteins by FL118 is independent of the tumor suppressor p53 status either wild type (WT), mutant or null [4]. Importantly, individual genetic overexpression or silencing of these proteins revealed that each of these proteins plays a role in FL118-mediated cancer cell growth inhibition and apoptosis induction [4, 5]. Furthermore, in p53 WT colorectal cancer (CRC) cells, FL118 induces p53-dependent senescence by promoting MdmX/Mdm4 ubiquitination and degradation [6]. Intriguingly, in the absence of p53, FL118 exhibits an even stronger ability to inhibit CRC cell growth and induce apoptosis [6]. In our studies, we further demonstrated that forced expression of exogenous MdmX in HCT116 CRC cells further enhances FL118 ability to inhibit cell growth and induce apoptosis [6]. This suggests that MdmX is a unique biomarker and target for FL118: the higher the level of MdmX expression, the better it is for FL118 antitumor activity. Mechanistically, the inhibition of MdmX expression by FL118 is through FL118 switching Mdm2-mediated ubiquitination and degradation of the tumor suppressor p53 (oncogenic effects) to Mdm2-mediated ubiquitination and degradation of the oncogenic protein MdmX (tumor suppression effects) [6]. Intriguingly, the degradation of oncogenic protein MdmX by Mdm2 is independent of the DNA damage signaling regulator ATM and the status of p53 and p21 [6].

Additionally, different from irinotecan, SN-38 and topotecan, which are substrates of the efflux pump proteins ABCG2/BCRP [7-11] and P-gp/MDR1 [12-16], FL118 is not a substrate of ABCG2 and P-gp, and can overcome treatment resistance resulting from the expression of ABCG2 [17] or P-gp [18]. Consistent with these observations, FL118 effectively overcomes irinotecan and topotecan resistance [18].

### Evidence for Top1-independent modulation of gene expression by topotecan

A critical question that we must ask ourselves is whether FL118 can inhibit the expression of multiple antiapoptotic and oncogenic proteins independent of Top1. In May 2016, Mabb, et al. published an interesting study in PLOS ONE [19]. In this study, the authors used multiple approaches to knock down or delete the Top1 gene (*TOP1*) in neurons to determine the role of Top1 in topotecan-mediated gene modulation. These authors found that in the presence of Top1, topotecan modulates much more gene expression than in the absence of Top1 through both Top1/DNA cleavage complex-dependent and -independent mechanisms [19]. These interesting findings triggered our enthusiasm to discuss the protein-encoding genes that were modulated by topotecan treatment in the neurons with *TOP1* knockdown. We analyzed the raw data provided in the study of Mabb, et al. for the 38 downregulated genes and 4 upregulated genes by topotecan in the neurons with conditional knockout (cKO) of *TOP1* (**Table 1**). We then asked ourselves whether the inhibition or induction of these genes by topotecan is Top1-independent or due to the incomplete cKO of Top1. We noticed approximate 75% of neurons with *TOP1* cKO (means: 25% is still WT Top1) in the studies. Based on this fact, if Top1-independent downregulation of these genes by topotecan is not involved, even though the 25% of WT Top1 neurons completely wipe out the 38 gene expression, there will be only a 25% downregulation of these genes overall. Thus, the 38 genes shown in **Table 1** (inhibition: from 1.97 to 5.17 fold) should be true Top1-independent, topotecan-regulated genes. Furthermore, 16 of the 38 genes were downregulated for  $\geq 2$ -fold after cKO of Top1 itself without topotecan treatment (not shown). Therefore, topotecan downregulation of these 16 genes is on a low base-

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**Table 1.** Gene modulation in mouse neurons by topotecan with topoisomerase I knockout

Genes inhibited	Top1 cKOveh value*	Top1 cKOtopot value	Fold inhibition
Cadm2	29.7	6.37	4.66
Nkain2	10.23	2.32	4.41
Syt1	180.8	70.4	2.57
Nrg3	9.91	2.8	3.54
Nbea	15.3	5.77	2.65
Kcnip4	11.68	3.18	3.67
Luzp2	23.6	7.5	3.15
Opcml	36.4	14.43	2.52
Negr1	60.1	26.2	2.3
Lphn3	9.41	3.29	2.86
Vps13b	2.64	0.85	3.11
Pcdh11x	5.13	1.67	3.07
Tenm1	3.51	1.03	3.41
Grm7	8.63	2.9	2.98
Grm5	18.6	7.24	2.57
Nrxn3	10.75	4.58	2.35
Galnt16	2.17	0.42	5.17
Robo2	8.32	3.24	2.57
Dgkb	12.49	4.98	2.51
Spock3	27.9	11.81	2.36
Erc2	23.7	11.18	2.12
Pcdh9	13.2	5.23	2.52
Mctp1	9.73	3.9	2.5
Dpp10	12.8	5.41	2.37
March1	8.34	3.44	2.42
Npas3	2.43	0.51	4.77
Htr2c	11.5	4.79	2.4
Gria4	17	7.21	2.36
Reln	10.07	2.46	4.09
Gucy1a2	9.35	4.17	2.24
Akt3	48.7	24.6	1.98
Ppfa2	11.98	4.93	2.43
Slc4a4	18.8	4.73	3.98
Gphn	13.4	5.7	2.35
Ptprd	10.4	4.76	2.19
Adarb2	1.84	0.39	4.72
Ctnna2	25	11.7	2.14
Epha5	20.7	10.5	1.97
Genes induced	Top1 cKOveh value	Top1 cKOtopot value	Folds induction
Cdkn1a	92.8	210.3	2.27
Phlda3	48.1	99.2	2.06
Slc5a3	7.16	15.8	2.21
Nes	8.7	20.9	2.4

\*Top1, topoisomerase I; cKO, conditional knockout; veh, vehicle treated; topot, topotecan treated.

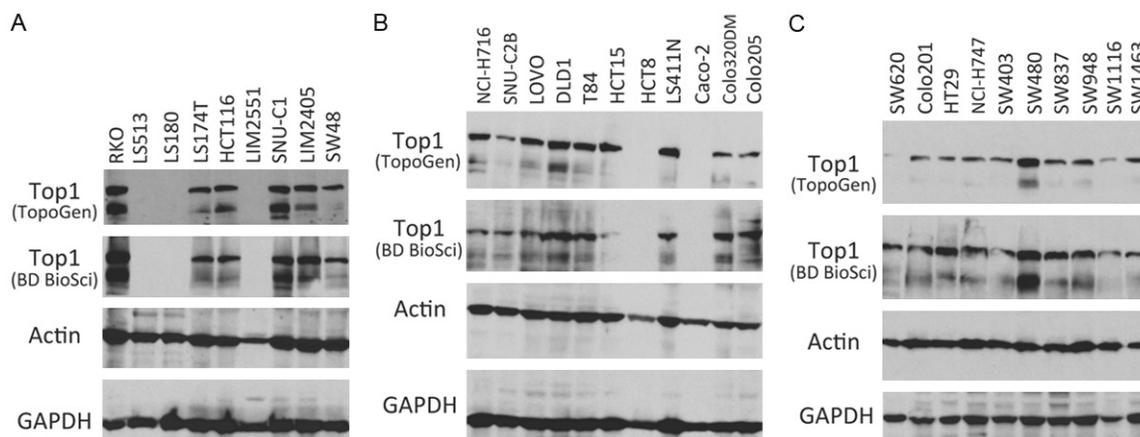
line expression level as a control (**Table 1**, blue). Furthermore, the *Htr2c* gene (*HTR2C*) was only downregulated in the topotecan-treated *TOP1* cKO neurons but not in WT neurons (**Table 1**, red). On the other hand, we recognized that if there is a  $\geq 10$  fold gene induction in 25% of neurons, the induction could override the overall gene expression level, even in the 75% of neurons that have no induction of such genes. If this were the case, we would be unable to distinguish whether the induction of the gene by topotecan is Top1-independent or Top1-dependent. However, in the case of the four upregulated genes shown in the bottom of **Table 1**, the first three genes were not induced in the WT neurons treated with topotecan, but were induced in the *TOP1* cKO neurons treated with topotecan (**Table 1**). The fourth *Nes* gene (*NES*) that was induced in both WT and *TOP1* cKO neurons that were treated with topotecan showed similar induction levels of less than 3 folds (2.45 versus 2.4 folds), which suggests that induction of *NES* is a Top1-independent event.

The key point that we want to emphasize is that the Supplemental Data provided in the study from Mabb, et al. [19] gives strong evidence that topotecan can inhibit or induce gene expression independent of Top1 expression/activity. This evidence provides a possibility that some novel CPT analogues (e.g. FL118) may mainly use Top1-independent mechanism to deliver their antitumor activity.

### Does FL118 need Top1 as a target for its superior antitumor efficacy?

In the initial FL118 discovery period, we largely used animal models of human tumors to screen the top candidates for antitumor activity. We found that FL118 is the top candidate that exhibits superior antitumor efficacy over other candidates, including irinotecan and topotecan, two FDA-approved Top1 inhibitors for cancer treatment. Since FL118 is structurally similar to irinotecan and topotecan, we thought that FL118 could be a much better Top1 inhibitor than irinotecan and topotecan. Thus, we tested them in the Top1-DNA complex biochemical cleavage assay [4]. Irinotecan is a pro-drug and showed very low activity in the *in vitro* experiment; we therefore used its active metabolite SN-38 in parallel with FL118 to compare their relative ability to inhibit Top1 enzyme activity.

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**Figure 1.** Topoisomerase I (Top1) expression in 30 colorectal cancer (CRC) cell lines: Individual CRC cells grown to 80% confluence were lysed and subjected to western blot analysis to determine the expression of Top1 protein. Two Top1 antibodies were used in this analysis. One is purchased from TopoGen (TG2012-4, Lot 12FB04) and the other is purchased from BD Biosciences (X-21, RUO). Both actin and GAPDH are internal controls for total protein loading evaluation.

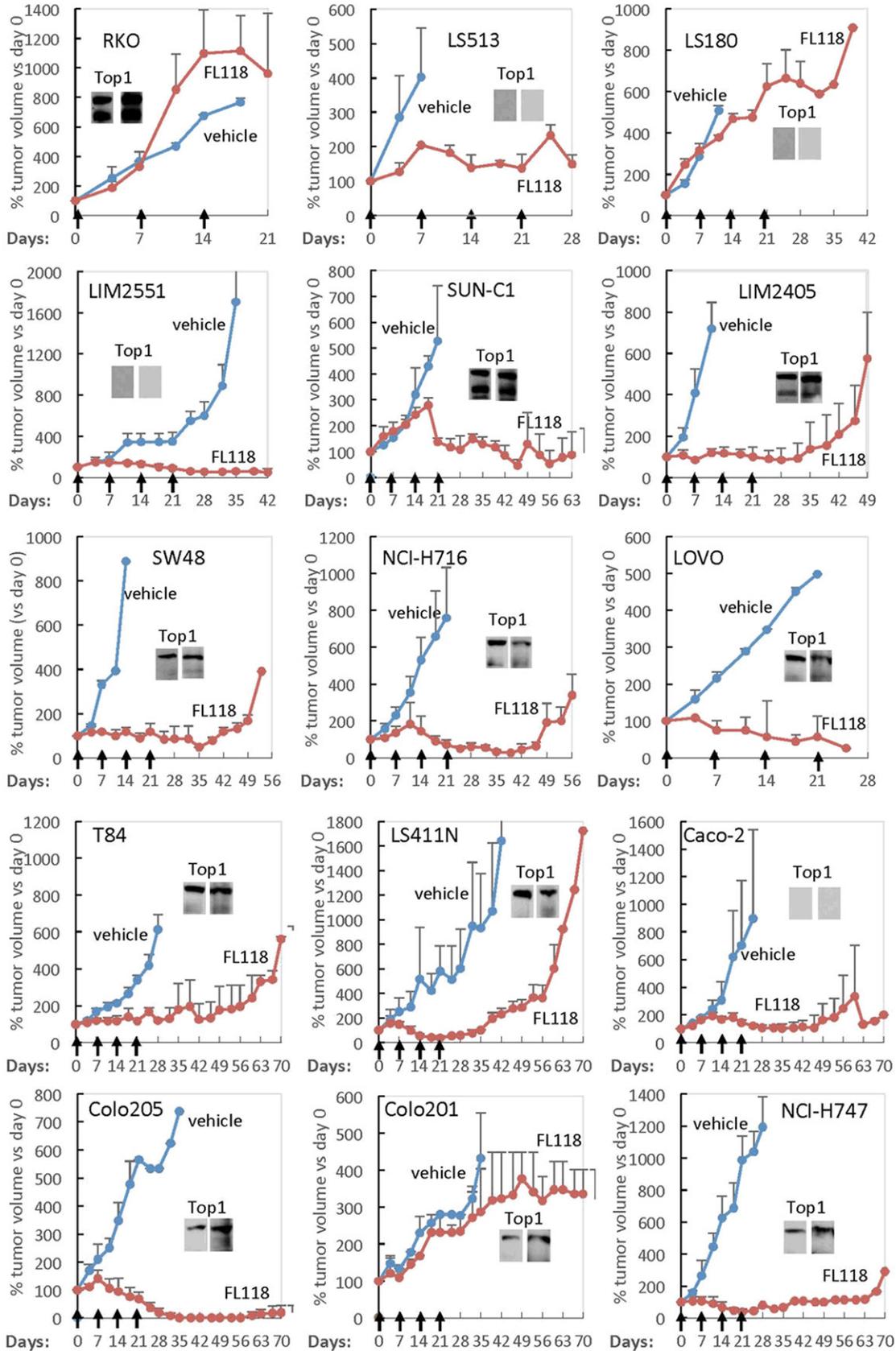
The experiment revealed that even at a 1  $\mu\text{M}$  concentration, the highest SN-38 dose that can be reached by irinotecan *in vivo*, FL118 did not exhibit a better ability to inhibit Top1 activity of converting supercoiled DNA into relaxed DNA by nicking the DNA (in fact, FL118 at 1  $\mu\text{M}$  only exhibited approximately one-half of inhibition that SN-38 showed) [4]. In contrast, FL118 can effectively inhibit cancer cell growth at far below a nM level [4].

Alternatively, it was reported that mutations of *TOP1* in CRC cells result in high CPT-resistance [20]. Actually, plants use mutations in *TOP1* as a self-defensive strategy to avoid harming themselves with their own production of CPT [21]. We compared the relative resistance of cancer cells to CPT, SN-38, topotecan and FL118 before and after Top1 mutations, respectively, using the Du145 parental prostate cancer cells (Top1 gene WT) in parallel with the Du145-derived two sub-cell lines (RC0.1, RC1) with Top1 R364H mutations [22]. The data showed that in the parental DU145 cells (Top1 WT), the relative potency (RP) of CPT, SN-38, topotecan and FL118 calculated from their IC50 (set topotecan RP as 1) is in turn 3.17, 4.75, 1.0 and 41.7. In contrast, in the Top1-mutated RC0.1 cells, the RP of CPT, SN-38, topotecan and FL118 (still set topotecan RP as 1) is in turn 0.97, 5.24, 1.0 and 778; and in the Top1-mutated RC1 cells, the RP of CPT, SN-38, topotecan and FL118 is in turn 2.69, 13.2, 1.0, and 572 [23]. Thus, the RP of

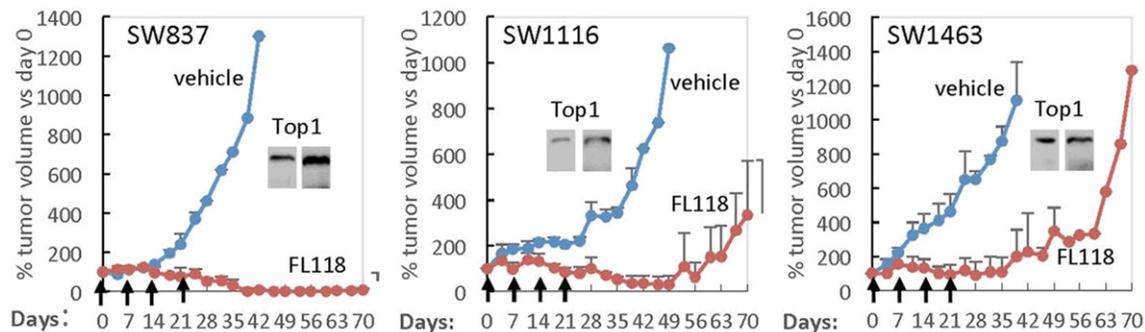
FL118 in comparison with other CPTs (CPT, SN-38, topotecan) is significantly enhanced after Top1 mutation.

If Top1 inhibition by FL118 does make a small contribution to FL118 antitumor activity, the observation from Top1 mutation may imply important consequences. That is, although all 4 of the compounds (CPT, SN-38, topotecan and FL118) showed increased IC50 values after Top1 mutation, FL118 was much less affected. This resulted in a substantial increase in FL118's relative potential (RP). This difference could cause CPT, SN-38 and topotecan to be ineffective, while FL118 can still be effective to kill cancer cells after Top1 mutation. On the other hand, Top1 expression level/enzyme catalytic activity has been reported to affect CPT compound sensitivity in killing tumor cells. For example, it was shown that reduced Top1 expression and/or Top1 catalytic activity is associated with increased resistance to CPT compounds [24-26], while increased Top1 in cancer cells sensitizes CPTs (irinotecan and SN-38) [27-29]. Intriguingly, in contrast to these documented findings from the literature, we found that the sensitivity of CRC cell line-established xenograft tumors to FL118 is irrelevant to Top1 expression levels. CRC xenograft tumors with high Top1 expression can be highly resistant to FL118 treatment. For example, RKO tumors highly express Top1 and are also highly resistant to FL118 (Figures 1A and 2 first row left). In contrast, CRC xenograft tumors

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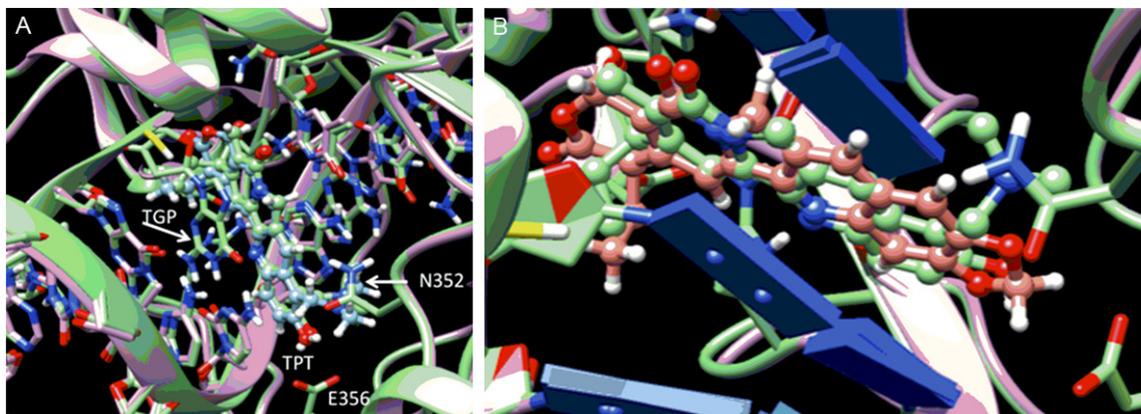
**Figure 2.** The sensitivity of CRC xenograft tumors to FL118 treatment is not associated with the expression of the CPTs target Top1: Individual xenograft tumors were first established from the corresponding CRC cell lines (RKO, LS513, LS180, LIM2551, SUN-C1, LIM2405, SW48, NCI-H716, LOVO, T84, LS411N, Caco-2, Colo205, Colo201, NCI-H747, SW837, SW1116, SW1463) by subcutaneous injection of 2 million cells at the flank area of SCID mice, respectively. Then the established tumors were inoculated into SCID mice at the flank area for testing FL118 sensitivity. FL118 treatment was initiated at the time when the inoculated individual xenograft tumors reached 100-200 mm<sup>3</sup> (designated day 0). FL118 was administered with the schedule of weekly x 4 (arrowed) via p.o. (per oral) routes at a dose of 10 mg/kg (MTD: maximum tolerated dose). Individual tumor curves were derived from the mean tumor sizes  $\pm$  SD from up to five mice. The small image insert within each xenograft tumor histogram is the cut-and-past Top1 expression from **Figure 1**.

with low/negative Top1 expression can be sensitive to FL118 treatment. For example, LIM2551 tumors do not express Top1 and are also highly sensitive to FL118 treatment (**Figures 1A** and **2** second row left). In this regard, we determined the Top1 expression in all of our available 30 CRC cell lines (**Figure 1**). In parallel, we determined the CRC cell-established xenograft tumor sensitivity to FL118 treatment in SCID mice. While the *in vivo* experiments haven't been completely finished, available tumor sensitivity to FL118 from 18 CRC xenograft tumors is presented in **Figure 2**. The *in vivo* data indicated that CRC tumors with either high Top1 expression or low/negative Top1 expression can be resistant or sensitive to FL118 treatment (**Figure 2**). This finding is contrast to the finding in the literature for the relationship of Top1 expression and CPTs' sensitivity [24-27, 29].

### What is the role of FL118 interaction with Top1/DNA complex?

The data presented in **Figures 1** and **2**, showing FL118 antitumor efficacy is not associated with Top1 expression, suggest that FL118 may not use Top1 as a target for its antitumor activity. However, several studies using the *in vitro* Top1 biochemical cleavage assay found that most CPTs, including FL118 [10,11-MD-20(S)-CPT] and closely related compounds (RS racemic), exhibit an association of antitumor activity with

inhibition of Top1-mediated DNA cleavage and re-ligation activity in *in vitro* Top1 biochemical cleavage assays [30, 31]. In this regard, we have analyzed 29 CPT analogues including CPT, SN-38, topotecan, FL118 and many FL118 analogues using molecular docking and the molecular mechanics generalized Born model with solvent accessible surface area (MMGBSA) [32] rescoring to compare poses in the Top1/DNA complex. Our *in-house* studies and published work reveal that MMGBSA rescoring of top-ranked (10-20) docking poses (protein/DNA-inhibitor complex configurations) allows discrimination of the physiologically relevant pose/configuration ~83% of the time in large test assessments (e.g. PDB-BIND) [33] with improved affinity correlation for the lowest MMGBSA rescored pose. We conducted this study using two different docking approaches, Autodock VINA and Schrodinger GLIDE SP/XP. In both cases, we started with the crystal structure of the topotecan-Top1-DNA complex [34] (PDB 1K4T). Protein preparation for GLIDE docking followed typical work flows and the use of OPLS3 refinement (sidechain completion/hydrogen addition followed by steepest descents and conjugate gradient energy minimization) of the crystal structure components prior to docking grid computation. To prepare for Autodock VINA docking, we initially prepared the protein/DNA complex using AMBER14 [34] libraries and using GAMEES-UK [35] computation of potential derived charges for 5'-thio-



**Figure 3.** A. Overlap of lowest MMGBSA docked pose of topotecan (TPT, green, ball and stick) with crystallographic TPT (cyan, ball and stick) in Top1/DNA complex. B. Lowest MMGBSA docked pose of TPT (green carbons) and FL118 (brown carbons) show analogous amino acid and base pair interactions. Heteroatoms are colored by normal conventions (red = oxygen, blue = nitrogen).

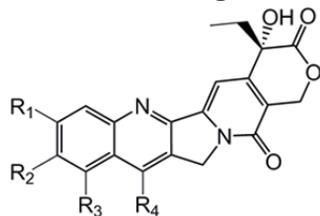
2'-deoxy-guanosine phosphonic acid (TGP) which were incorporated into an XLEAP prepared representation of the modified base. Docking with standard GLIDE SP [36] protocol (5000 poses for initial docking phase/keeping 400 initial poses for energy minimization and best scoring assessment wherein only poses within 100 of the top pose are considered) or Autodock VINA [37] with exhaustiveness = 80 and collection of 20 poses for each ligand was conducted. In both cases we performed MMGBSA rescoring of the docked poses. In the case of GLIDE poses, the Pime-MMGBSA (OPLS3/VSQP) approach was used and in the case of VINA, AMBER14-MMGBSA rescoring was performed allowing for full protein/DNA/docked ligand minimization. The lowest MMGBSA poses for each ligand were then collected to examine the probable 'bound' configuration for the putative ligands of the protein-DNA complex.

**Figure 3A** shows the lowest MMGBSA energy minimized VINA/MMGBSA pose for topotecan (TPT, green, ball and stick) with a stacking interaction between TGP on one face of the ligand and an adenine/thymine base pair on the opposite side of the ligand. As seen in **Figure 3**, the root-mean-square deviation (RMSD) fit of the docked/free-energy rescored minimized pose (green) compared to the crystal structure solution (cyan) is less than an angstrom with similar interactions with surrounding residues. The TPT ligand in this docked pose interacts with Asp533 of Top1 (not shown in **Figure 3A**)

through a hydrogen bonding interaction and has  $\pi$ - $\pi$  stacking interactions with the TGP on one side and adenine on the other. The ligand in this intimate intercalated fashion is bordered by Glu356 and Asn352 of Top1 at the bottom of this panel. As the lowest MMGBSA docked pose of TPT resembles the crystal structure configuration of TPT, we next examined the predicted pose for FL118 in **Figure 2B** where it shows the overlap with the docked pose of TPT. The ligands are shown to have a similar disposition with respect to the interacting residues. **Table 2** reports the VINA/MMGBSA scores for 29 CPT derivatives. While FL118 has a slightly less negative MMGBSA score than topotecan (-49.5 vs -50.5), several FL118 derivatives tested are seen to have more negative MMGBSA scores (i.e., predicted to be better Top1 inhibitors) than topotecan (**Table 2**). In consonance with the MMGBSA scores, we found that compared to FL118, most FL118 analogues with more negative MMGBSA scores (i.e. predicted to be better Top1 inhibitors) exhibit greater toxicity (body weight loss) than FL118. This is likely due to their stronger inhibition of Top1 than FL118 in animal models (**Figure 4**).

While MMGBSA rescoring of docking poses improves relative Top1/DNA complex binding affinity prediction, correlations rarely give Pearson  $r > 0.8$ . The method is semi-quantitative due to theoretical approximations (e.g. neglect of explicit water, lack of inclusion of conformational entropies), as well as the challenges of predicting flexible ensemble poses that are

**Table 2.** CPT analogues structure and VINA/MMGBSA results



Compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	MMGBSA (kcal/mol)
CPT	H	H	H	H	-47.5
Topotecan (TPT)	H	OH	CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub>	H	-50.5
SN-38	H	OH	H	Et	-48.5
7-Methyl-CPT	H	H	H	CH <sub>3</sub>	-48.9
7-Ethyl-CPT	H	H	H	Et	-49.4
7-Hydroxymethyl-CPT	H	H	H	CH <sub>2</sub> OH	-49.4
7-Chloromethyl-CPT	H	H	H	CH <sub>2</sub> Cl	-49.9
FL118	10,11-MD		H	H	-49.5
7-Methyl-FL118	10,11-MD		H	CH <sub>3</sub>	-52.2
7-Ethyl-FL118	10,11-MD		H	Et	-53.2
7-Hydroxymethyl-FL118	10,11-MD		H	CH <sub>2</sub> OH	-53.6
7-Chloromethyl-FL118	10,11-MD		H	CH <sub>2</sub> Cl	-54.4
7-Phenyl-FL118	10,11-MD		H	Ph	-57.8
7-(p-Tolyl)-FL118	10,11-MD		H	(4-CH <sub>3</sub> )Ph	-55.7
7-(4-CF <sub>3</sub> )phenyl-FL118	10,11-MD		H	(4-CF <sub>3</sub> )Ph	-54.8
7-(4-F)phenyl-FL118	10,11-MD		H	(4-F)Ph	-54.0
7-(4-Cl)phenyl-FL118	10,11-MD		H	(4-Cl)Ph	-54.8
7-Benzyl-FL118	10,11-MD		H	Bn	-63.4
7-(4-F)benzyl-FL118	10,11-MD		H	(4-F)Bn	-59.8
DFMD-CPT	10,11-DFMD		H	H	-49.5
7-Methyl-DFMD-CPT	10,11-DFMD		H	CH <sub>3</sub>	-49.4
7-Ethyl-DFMD-CPT	10,11-DFMD		H	Et	-50.7
7-Hydroxymethyl-DFMD-CPT	10,11-DFMD		H	CH <sub>2</sub> OH	-53.5
7-Chloromethyl-DFMD-CPT	10,11-DFMD		H	CH <sub>2</sub> Cl	-49.5
ED-CPT	10,11-ED		H	H	-51.1
7-Methyl-ED-CPT	10,11-ED		H	CH <sub>3</sub>	-50.7
7-Ethyl-ED-CPT	10,11-ED		H	Et	-54.3
7-Hydroxymethyl-ED-CPT	10,11-ED		H	CH <sub>2</sub> OH	-53.2
7-Chloromethyl-ED-CPT	10,11-ED		H	CH <sub>2</sub> Cl	-53.9

CPT = Camptothecin, MD = Methyleneedioxy, DFMD = Difluoromethyleneedioxy, ED = Ethyleneedioxy.

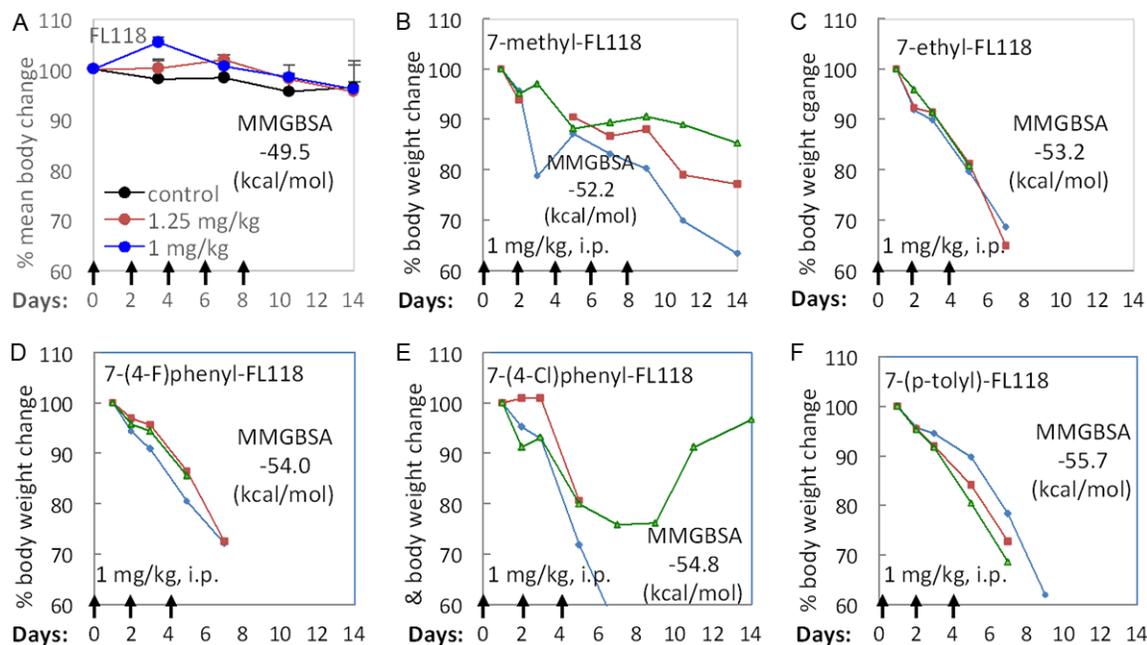
free-energy rescored. Nevertheless, the results from **Table 2** suggest that favorable interactions with the Top1/DNA complex (more negative MMGBSA score) link to a greater toxicity potential of FL118 analogues tested *in vivo* (**Figure 4**).

Based on the literature-documented Top1 biochemical cleavage data and our molecular modeling results with relevant animal model studies, further discussion of some critical

points may benefit future research in the pertinent field.

In the Top1 biochemical cleavage assay, the concentration of the drug used in these studies was usually in the range of 1-100  $\mu$ M and drug concentrations below 1  $\mu$ M showed no significant Top1 inhibition activity [30, 31]. A key concern here is whether the *in vitro* result obtained from any concentration higher than 0.5-1  $\mu$ M from the Top1 biochemical cleavage assay

## Topoisomerase I: a target for FL118 toxicity versus efficacy



**Figure 4.** Body weight changes (a marker of toxicity) of mice after treatment with FL118 or a FL118 analogue: A. SCID mice were treated with FL118 (q2dx5). Individual body weight change curves were derived from the mean  $\pm$  SE from five mice. B. Individual SCID mice were treated with 7-methyl-FL118 (q2dx5). Two mice were euthanized on day 14 and one on day 16. C. Individual SCID mice were treated with 7-ethyl-FL118 (q2dx3). One mouse was euthanized on day 5 and the other two on day 7. D. Individual SCID mice were treated with 7-(4-F)phenyl-FL118 (q2dx3). One mouse was euthanized on day 5 and the other two on day 7. E. Individual SCID mice were treated with 7-(4-Cl)phenyl-FL118 (q2dx3). One mouse was euthanized on day 5 and one on day 7. F. Individual SCID mice were treated with 7-(p-tolyl)-FL118 (q2dx3). Two mice were euthanized on day 7 and one on day 9. The reason for euthanization of mice is because body weight loss for  $\geq 20\%$  and/or mice in a moribund state.

experiment could mimic the *in vivo* activity of FL118 to inhibit Top1. Our view on this issue is negative in terms of our findings from the experimental studies and molecular docking of FL118 and FL118 core structure-based analogues. This view is based on two facts: (a) FL118 effectively inhibits cancer cell growth in the concentration range of 0.01 to 10 nM, depending on cancer cell types and/or genetic backgrounds [4]; and (b) our pharmacokinetics (PK) studies with animal models of human tumors indicated that the peak of FL118's concentration in blood is under 100 nM with a short half-life ( $< 1.8$  hours), while the peak of FL118's concentration in tumors can be around 300 nM (rapidly accumulated in tumor) with a much longer half-life (6.8-12.8 hours) [18]. This means that FL118 may not even be able to reach a sustained 1  $\mu$ M concentration in the *in vivo* situation. Furthermore, molecular docking results also indicated that FL118 is not a better Top1 inhibitor than topotecan (Table 2). However, FL118 has much better antitumor activity

than topotecan [4] and can overcome topotecan-resistant tumors [18].

The critical question is whether the inhibition of Top1 activity by FL118 plays a major role in FL118's exceptional antitumor activity or is mainly involved in its side effects of hematopoietic toxicity. FL118 possesses exceptional antitumor activity in animal models of human tumors, and inhibits multiple antiapoptotic proteins (survivin, Mcl-1, XIAP, cIAP2), oncogenic protein MdmX [4-6, 18], and overcomes both ABCG2 and P-gp resistance in cancer cells [17, 18, 38]. However, we failed to find that FL118 is a better Top1 inhibitor than SN-38/irinotecan and topotecan, in either the Top1 biochemical cleavage assay [4] or after Top1 mutation in cancer cells [23]. Furthermore, the data from Figures 1 and 2 demonstrate that FL118 antitumor efficacy is not associated with Top1 expression. In other words, CRC xenograft tumors with either high or low/negative Top1 expression can be sensitive or resistant to

FL118 treatment, suggesting Top1 expression is irrelevant to FL118 antitumor efficacy.

Although use of a range of 1-100  $\mu\text{M}$  for drug concentrations in Top1 biochemical cleavage assay may not well mimic the FL118 *in vivo* situation (1-100  $\mu\text{M}$  *in vitro* versus  $\leq 1 \mu\text{M}$  *in vivo*), it would be safe to propose that FL118 at its *in vivo* therapeutic doses may still have the ability to negatively affect Top1 enzyme activity. However, this may not make a major contribution to FL118's antitumor efficacy, especially when considering the data shown in **Figures 1** and **2**. The fact is that FL118 possesses much-better antitumor activity than irinotecan and topotecan [4], and could overcome irinotecan- or topotecan-resistant human tumors in animal models [18]. However, FL118 exhibits hematopoietic toxicity (as well as diarrhea) similar to irinotecan and topotecan. Practically, due to the much higher *in vivo* potency of FL118 in comparison with irinotecan and topotecan as we previously found [4], the side effects (hematopoietic toxicity and diarrhea) for FL118 appear less severe. Nevertheless, previous studies have shown that CPTs-induced Top1-mediated lesions and DNA damage signaling in primitive hematopoietic cells confer significant oncogenic potential [39]. Therefore, in order to decrease irinotecan or topotecan-induced hematopoietic toxicity, clinical chemotherapy including irinotecan or topotecan has been used in parallel with peripheral-blood stem cell or bone marrow transplantation [40-42]. Thus, the hematopoietic toxicity resulting from FL118 treatment is likely due to the inhibition of Top1-mediated DNA replication by FL118 during hematopoietic cell renewal from bone marrow stem/progenitor cells.

### Concluding remarks

There is no doubt that Top1 is not an ideal target for cancer treatment since normal tissue/cell renewal needs Top1 for DNA replication and cell proliferation. Blocking Top1 function would induce serious toxicity in renewing tissues (e.g. hematopoietic toxicity). Intriguingly, examples of potent CPT compounds that are not dependent on Top1 expression level were reported previously. Jaxel et al. showed that while CPT analogues 10-NH<sub>2</sub>-(RS)-CPT and 11-CN-(RS)-CPT showed very poor Top1 inhibition, these two CPT compounds extended sur-

vival time much longer than other CPTs with strong Top1 inhibition in the L1210 leukemia metastatic mouse model [30]. The disagreement between antitumor activity and the potential inhibition of Top1 enzyme activity argues 10-NH<sub>2</sub>-(RS)-CPT and 11-CN-(RS)-CPT may use alternative targets instead of Top1 for their anti-leukemia activity. Based on this observation and in consideration of the recent finding in neurons (**Table 1**), as well as our data shown in **Figures 1** and **2**, we propose that inhibition of Top1 activity by FL118 may not play a major role in FL118's exceptional antitumor activity; instead, inhibition of Top1 by FL118 may mainly be involved in the side effects of hematopoietic toxicity and possibly in diarrhea as well. Therefore, we believe that the next generation of low toxicity and high efficacy anticancer agents that are structurally relevant to FL118 should focus on the development of compounds having weaker affinity to the Top1-DNA complex, while keeping their strong ability to inhibit the expression of survivin, Mcl-1, XIAP, cIAP2 and MdmX.

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### Disclosure of conflict of interest

FL118 and FL118 core structure-based analogues will be further developed in Canget BioTekpharma LLC ([www.canget-biotek.com](http://www.canget-biotek.com)), a Roswell Park Cancer Institute-spinoff company. FL, XL and YW are initial investors of Canget for development of FL118 and FL118 core structure-relevant anticancer agents.

**Address correspondence to:** Dr. Fengzhi Li, Department of Pharmacology & Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263, USA. Tel: 716-845-4398; Fax: 716-845-8857; E-mail: fengzhi.li@roswellpark.org; Chunyang Jin, Center for Drug Discovery, RTI International, Research Triangle Park, North Carolina, USA. E-mail: cjin@rti.org

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