Clinical and cellular roles for TDP1 and TOP1 in modulating colorectal cancer response to irinotecan

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Abstract

Colorectal cancer (CRC) is the third most common cancer in the world. Despite surgery, up to 50% of patients relapse with incurable disease. First line chemotherapy utilises the topoisomerase 1 (TOP1) poison irinotecan, which triggers cell death by trapping TOP1 on DNA. The removal of TOP1 peptide from TOP1-DNA breaks is conducted by tyrosyl DNA phosphodiesterase 1 (TDP1). Despite putative roles for TDP1 and TOP1 in CRC, their role in cellular and clinical responses to TOP1 targeting therapies remains unclear. Here, we show varying expression levels of TOP1 and TDP1 polypeptides in multiple CRC cell lines and in clinical CRC samples. TDP1 overexpression or TOP1 depletion is protective. Conversely, TDP1 depletion increases DNA strand breakage and hypersensitivity to irinotecan in a TOP1 dependent manner, presenting a potential therapeutic opportunity in CRC. TDP1 protein levels correlate well with mRNA and with TDP1 catalytic activity. However, no correlation is observed between inherent TDP1 or TOP1 levels alone and irinotecan sensitivity, pointing at their limited utility as predictive biomarkers in CRC. These findings establish TDP1 as a potential therapeutic target for the treatment of CRC and question the validity of TOP1 or TDP1 on their own as predictive biomarkers for irinotecan response.
Introduction

Colorectal cancer remains one of the most significant malignancies to affect populations with 39,000 new cases per year and 16,000 deaths in the UK and 177,000 new cases per year and 58,000 deaths in the USA (1). Whilst surgical excision of localised tumours is the primary treatment modality, up to 50% of patients subsequently experience relapse and many more present with metastatic disease at the outset. Standard first line chemotherapy entails a combination of folinic acid, 5-fluorouracil and either irinotecan (FOLFIRI) or oxaliplatin (FOLFOX), for which there is broad equivalence in tumour response (2). Interestingly, irinotecan and oxaliplatin do not seem to generate common resistance mechanisms and the alternate combination is commonly used as second line therapy. Given the broad equivalence of efficacy in the first and second line settings between oxaliplatin and irinotecan, biomarkers predicting differential response would play a significant role in optimising treatment protocols.

Irinotecan (CPT-11) is a prodrug that is converted within the cell to its active metabolite SN38, a potent camptothecin-based topoisomerase 1 (TOP1) poison. Its efficacy in treating metastatic colorectal cancer was first demonstrated over a decade ago in clinical trials with response rates of 50% and improved overall survival approaching 24 months (3,4). Whilst forming a standard of care in the management of metastatic CRC, a role for irinotecan is also being investigated in the neoadjuvant treatment of locally advanced rectal cancer (5-7) where it is the subject of the ongoing ARISTOTLE trial (http://www.controlled-trials.com/ISRCTN09351447). The continuing inclusion of irinotecan in clinical trial protocols highlights the urgency and importance of identifying biomarkers for tumour response and novel means by which to improve irinotecan efficacy in the clinic. Both TOP1 and aprataxin levels have been shown to correlate with irinotecan sensitivity in CRC and PARP-1 inhibition may furthermore potentiate irinotecan sensitivity in the clinic (8-11).

TOP1 is an important cellular enzyme that allows for DNA relaxation, thus facilitating the processes of transcription and replication. In this role, TOP1 cleaves DNA to create a DNA single-strand break (SSB) to which it remains covalently bound to, thus allowing for rotation and relaxation of DNA (12). Once rotated, bound TOP1 ligates the nicked DNA and is released. TOP1 poisons prevent TOP1 ligase activity and subsequent release and therefore promote SSB persistence and DNA double-strand break (DSB) formation at replication forks, collectively
known as protein-linked DNA breaks (PDBs) (13-18). Unrepaired DSBs activate cell cycle arrest and trigger apoptosis-mediated cell death. The effectiveness of TOP1 poisons in killing cancer cells is therefore thought to be primarily dependent on levels of TOP1 and the rate of repair of TOP1-mediated DNA damage.

Cellular removal of TOP1-DNA breaks involves proteasomal degradation of TOP1 to leave a small peptide linked to DNA via a 3’-phosphotyrosyl linkage that is removed by tyrosyl-DNA phosphodiesterase 1 (TDP1) prior to repair completion by the DNA single-strand break repair pathway (19-23). TDP1 is involved early on in the repair process and its activity is critical for repair (24-28). TDP1 is additionally able to process 3’-phosphoglycolate moieties that are induced by ionising radiation (IR) and therefore plays a role in the resolution of DNA damage associated with both TOP1 poisoning and IR (29-32). TDP1 inhibition may therefore be particularly well suited to improving the efficacy of radiotherapy delivered in combination with TOP1 targeting chemotherapies. Indeed, multiple drug screening efforts to identify TDP1 inhibitors are currently underway (33-35).

In this study, we examined the role of TDP1 in determining CRC responses to irinotecan. We observed a broad range of TDP1 and TOP1 protein levels in both CRC cell lines and clinical CRC samples. TDP1 and TOP1 protein correlate well with their respective mRNA levels whilst TDP1 protein expression correlates well with its catalytic activity in vitro. TDP1 depletion increased DNA strand breakage and hypersensitivity to irinotecan in a TOP1 dependent manner whilst TOP1 loss or TDP1 overexpression proved to be protective, presenting a potential therapeutic opportunity in CRC.
Materials and Methods

Cells

Human colorectal cancer cell lines were obtained from ATCC, LGC Standards, Middlesex UK in 2013. The cell lines were tested and authenticated by the supplier and further tested in our lab using morphological assessment, PCR-based methods and DNA staining (Hoechst 33258, sigma). Cells were confirmed to be mycoplasma free before embarking the study, stored in multiple early passages, and cultured for a period not exceeding 5 months. Cells were maintained in a 5% CO₂ incubator at 37°C. SW480 and SW48 were grown in Leibovitz’s L-15 media (20% FCS, 2 mM L-glutamine), DLD1 and LS174T were grown in DMEM (10% FCS, 2 mM L-glutamine) whilst Caco2 was cultured in DMEM (20% FCS, 2 mM L-glutamine) and RKO in RPMI (10% FCS, 2 mM L-glutamine). All media were Gibco products obtained from Life Technologies (Paisley, UK).

Whole cell extract preparation

Adhered cells were washed twice in ice cold phosphate buffered saline (PBS) and collected by scraping and centrifugation at 1,500 rpm for 5 min. Extraction was carried out using ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, 100 mM NaCl, 1% Triton X-100) supplemented with Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Burgess Hill, UK). Extraction was carried out on ice for 30 min, the lysate cleared by centrifugation at 13,000 rpm for 10 min and the supernatant collected as whole cell extract (WCE). Total protein concentration was determined by Bradford assay and the samples stored at -80°C.

Western blotting

WCE (40 µg) was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 125 V for 2 hr and transferred on to a Hybond-C Extra Nitrocellulose membrane (Fisher Scientific UK, Loughborough, UK) at 25 V for 90 min. The membranes were blocked in 5% PBS-milk for 1 hr prior to overnight incubation with antibodies against TDPI (ab4166; Abcam, Cambridge, UK), TOP1 (SC-32736; Santa Cruz Biotechnologies, California, USA) and actin (A4700; Sigma-Aldrich, Gillingham, UK) diluted in 5% PBST-milk to 1:2000, 1:1000 and 1:3000, respectively.
The membranes were thrice washed in PBST and incubated for 1 hr in 5% PBST-milk containing either HRP-labelled polyclonal rabbit anti-mouse or polyclonal goat anti-rabbit secondary antibodies (Dako, Ely, UK) at a 1:3000 dilution. The chemiluminescent detection reagent, SuperSignal West Pico Chemiluminescent Substrate (Fisher Scientific UK, Loughborough, UK) was used for blot development and bands quantified using Image J software.

**Quantitative PCR**

Total mRNA was purified from cells using the RNeasy kit (Qiagen, Crawley, UK) and mRNA concentration measured using the NanoDrop 1000 spectrophotometer (Nanodrop, Willmington, USA) at OD 230 nm. The Superscript First Strand RT-PCR system (Invitrogen, Paisley, UK) was used to generate cDNA from 1 µg total mRNA. Real time PCR analysis was carried out on the MX3005p qPCR system (Agilent Technologies, Santa Clara, USA) using Blue qPCR Sybr low ROX mix (Fisher Scientific, Loughborough, UK) and primers targeting exon crossing regions for TDP1 (Forward 5'-CCCCTTCAGTTTTACCTCAC-3', Reverse 5'-'AGTCCACGTCAAAGCAGTAG-3'), TOP1 (Forward 5'-'ACGAATCAAGGGTGAGAAGG-3', Reverse 5'-CGATACTGGTTCCGGATCTT-3') and GAPDH (Forward 5'-'ACATCGCTCAGACACCATT-3', Reverse 5'-'TGTAGTGGAGGTGAATGAGG-3') ordered from Eurogentec (Southampton, UK). Relative quantitation (RQ) of mRNA was calculated as RQ =2^ΔΔCT, where CT is the average cycle threshold for three readings.

**TDP1 activity assay**

*In vitro* 3'-tyrosyl-DNA phosphodiesterase activity was measured using a gel-based 3'-tyrosyl-DNA phosphodiesterase activity assay. Reactions (10 µL total) were carried out in assay buffer (25 mM HEPES, pH 8.0, 130 mM KCl, 1 mM DTT) containing 50 nM 5'-Cy5.5 labelled substrate, 5'-(Cy5.5)GATCTAAAAGACT(pY)-3' (The Midland Certified Reagent Company, Texas, USA), and indicated amounts of WCE (ng) or recombinant human TDP1 protein described in (25). The reactions progressed at 37°C for 1 hr and were quenched with 10 µL loading buffer (44% deionized formamide, 2.25 mM Tris-borate, 0.05 mM EDTA, 0.01% xylene cyanol, 1% bromophenol blue) prior to heating at 90°C for 10 min and separation on a 20% Urea SequaGel (Fisher Scientific, Loughborough, UK) run at 190 V for 2 hr in 1X TBE. Gels were
imaged using the FujiFilm Fluor Imager FLA-5100 at 635 nm and bands quantified using Image J software.

**Clonogenic survival assay**

CRC sensitivity to irinotecan (CPT-11) and irradiation (IR) was measured by clonogenic survival assay. Briefly, adhered cells seeded at dose-dependent densities (300 to 2400 per 10 cm dish) were treated with gamma radiation (1-4 Gy using a $^{137}$Cs γ-ray source at a rate of 1 Gy/9s exposure) or with media containing CPT-11 (Sigma-Aldrich, Gillingham, UK) or DMSO and incubated in a 5% CO$_2$ incubator at 37°C for 7 to 12 days to allow for colony formation (>50 cells). The colonies were fixed and stained using 0.4% methylene blue in 50% methanol prior to counting. The surviving fraction was calculated as the surviving colony fraction (colonies counted/total cells seeded) of the treatment plates divided by that of the untreated plates.

**Plasmid transfection**

Cells were transfected with the pCI-neo-Myc vector or that encoding human TDP1 using Lipofectamine LTX plus reagent (Invitrogen, Paisley, UK). Briefly, plasmid amounts (µg) were suspended in 200 µL FCS-free media and mixed with 200 µL FCS-free media containing 7.5 µL LTX reagent and 3 µL Plus reagent prior to a 5 min incubation at room temperature. The mixture was added to 6 cm dishes containing 6 x 10$^5$ adhered cells in 3 ml FCS-media and the cells incubated at 37°C for 24 hr prior to harvesting for further use.

**mRNA silencing**

For mRNA silencing, 10 µL Lipofectamine 2000 RNAiMAX reagent (Invitrogen, Paisley, UK) added to 250 µL FCS-free media was incubated at room temperature for 5 min and added to 250 µL FCS-free media containing the indicated siRNA sequences. The mixture was incubated at room temperature for 20 min before adding to 6 cm dishes containing 3 x 10$^5$ cells in 3 ml FCS-media. For TDP1 siRNA, cells were treated first in suspension and again 6 hr later. For combined TDP1/TOP1 siRNA treatment, TOP1 siRNA was carried out 16 hr after the second TDP1 siRNA treatment. The plates were incubated at 37°C for a total of 72 hr for TDP1 siRNA and 48 hr for TOP1 siRNA prior to harvesting for further use. Both TDP1 and TOP1 siRNA sequence pools were bought as Dharmacon ON-TARGETplus smartpools (Fisher Scientific,
Loughborough, UK) whilst the BLAST validated scrambled siRNA sequence (5’-UUCUUCGAACGUGUCACGU-3’) and individual TDP1 siRNA sequences (TDP1si-05: 5’-GGAGUUAAGCCAAGUAUA-3’, TDP1si-06: 5’-UCAGUUACUUGAUGCUUA-3’, TDP1si-07: 5’-GACCAUAUCUAGUAGU-3’, TDP1si-08: 5’-CUAGACAGUUUCAAAGUGA-3’) were obtained from Eurogentec (Southampton, UK).

Alkaline comet assay

DNA single strand breaks were measured by alkaline comet assay (ACA) (36). Cells in suspension were treated with media containing DMSO or 50 µM CPT-11 for 1 hr. The cells washed once in ice-cold PBS and mixed 1:1 with 1.2% low-melt agarose prior to layering onto pre-chilled frosted glass slides pre-coated with 0.6% agarose. The slides were subsequently immersed in ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA pH 8.0, 10 mM Tris-HCl, 1% Triton X-100, 1% DMSO; pH 10) for 1 hr at 4°C followed by DNA unwinding in electrophoresis buffer (50 mM NaOH, 1 mM EDTA, 1% DMSO) for 45 min prior to electrophoresis at 12 V for 25 min. The slides were neutralised overnight in 0.4 M Tris-HCl (pH 7.0) and stained with Sybr-Green (1:10,000 in PBS) for 10 min. The tail moments for 100 cells per sample were scored using the Comet Assay IV software (Perceptive Instruments, UK).

Immunostaining

Cell monolayers grown to sub-confluent densities on coverslips were treated with media containing DMSO or 1 µM CPT-11 for 1.5 hr. The cells were washed twice in warmed PBS and left to recover in fresh media for 2 hr. After washing twice in ice-cold PBS, the cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature and permeabilised in 0.2% Triton-X in PBS for 3 min on ice. The coverslips were washed thrice in PBS, blocked in 2% BSA-Fraction V for 30 min prior to incubation with γ-H2AX primary antibodies (JBW301, Millipore, Watford, UK) diluted to 1:800 in 2% BSA-Fraction V for 45 min. The coverslips were thrice washed in PBS and incubated with FITC labelled secondary antibodies (Sigma-Aldrich, Gillingham, UK) diluted 1:300 in 2% BSA-Fraction V for 45 min. The coverslips were rinsed in PBS three times and mounted on to slides using Vectashield containing DAPI (Vector Laboratories, Peterborough, UK) prior to visualising and scoring on a Nikon Eclipse e-400 microscope.
Immunohistochemistry

Tissue microarray slides consisting of 59 formalin fixed, paraffin embedded colorectal cancer samples with documented tumour demographic and correlated overall survival data were obtained from Imgenex (IMH-306, CA, USA). These were deparaffinised and stained with antibodies for TDP1 (NB100-81642, Novus Biologicals, CO, USA) and TOP1 (NCL-TOPO1, Leica Microsystems, Milton Keynes, UK), both at 1:200 dilution using a Bond Max Autostainer. Cores were classified as none, low, moderate or high for TDP1 and TOP1 staining, with samples recorded if >5% cells stained accordingly. Rectal adenocarcinoma samples with known chemoradiotherapy response outcomes were obtained with appropriate consent from a cohort of patients treated on the RICE study (5). Specifically these were from patients with locally advanced rectal adenocarcinomas treated with a neoadjuvant regimen of pelvic radiotherapy (45Gy in 25 fractions over 5 weeks) with concurrent oral capecitabine (650mg/m² twice daily, continuously days 1-35) and irinotecan (60mg/m² intravenously once weekly, weeks 1-4) prior to surgical resection. These were similarly stained for TDP1 expression and classified as above.

Results

To examine the role of TDP1 in colorectal cancer (CRC), we first characterised the cellular variation of TDP1 and TOP1 protein levels in a panel of six CRC cell lines (RKO, SW480, SW48, Caco2, DLD1 and LS147T). Whole cell extracts prepared from each of the six cell lines were analysed by Western blotting using antibodies against TDP1, TOP1 and actin (Figures 1A-1C). Since actin is normally used as a loading control for the comparison of isogenic samples in a Western blot and our panel consists of non-isogenic cell lines, we instead loaded equal amounts of extract (40 µg) and normalised TDP1 and TOP1 levels directly to that obtained for the highest expressor, the RKO cell line. Our results show varying levels of TDP1 and TOP1 expression within the CRC panel. For diagnostic and drug design purposes, it is important to understand the nature of such variation. We thus carried out quantitative PCR to measure the levels of TDP1 and TOP1 mRNA. Purified total mRNA was reverse transcribed into cDNA that was further amplified using primers against exon-crossing regions for TDP1 and TOP1 cDNA. The cycle threshold obtained was used to quantify relative TDP1 and TOP1 mRNA levels normalised to that obtained for the RKO cell line (Figures 1D and 1E). Pearson’s correlation coefficients for
TDP1 protein and mRNA levels \((r=0.884, \ p=0.019)\) and TOP1 protein and mRNA levels \((r=0.870, \ p=0.024)\) were significantly positive (Figures 1F and 1G). We conclude from these experiments that TDP1 and TOP1 display remarkable variation in CRC cell lines and that protein expression correlates well with corresponding levels of mRNA.

To gain further insight into the extent of TDP1 and TOP1 variation in CRC, we used a commercially available tissue microarray (TMA) containing 59 CRC samples to assess TDP1 and TOP1 protein levels. Within normal colonic epithelium, TDP1 staining was seen to be primarily nuclear, in both epithelial and crypt cells (Figure 2A). The 59 CRC samples demonstrated a range of protein expression for both TDP1 and TOP1 (Figures 2B and 2C) with the majority showing moderate to strong staining for both TDP1 (69\%) and TOP1 (77\%). Though there was no significant overall correlation between TDP1 and TOP1 protein levels \((R=0.24, \ p=0.075)\), it was noted that of the 42 samples that scored moderate/high for TOP1, 72\% showed moderate to high TDP1 staining. Interestingly, there appears to be no difference in staining frequencies between tumours selected from different anatomical sites (Table 1). We also carried out TDP1 immunohistochemistry (IHC) on 125 rectal cancer specimens acquired from the RICE trial of neoadjuvant irinotecan, capecitabine and radiotherapy treatment (5) (Figure 2D and 2E). Whilst a variation in TDP1 was again identified, overall these specimens exhibited lower expression of TDP1 than those in the TMA samples. We conclude from these observations that TDP1 and TOP1 expression also varies considerably in clinical CRC samples.

TDP1 is a key ‘bottleneck’ repair factor for damage caused by TOP1 targeting therapies and is therefore regarded as a promising therapeutic target for inhibition (32, 37). Our findings that CRC possess varying levels of TDP1 prompted us to test if TDP1 plays a role in CRC responses to irinotecan. We initially depleted TDP1, TOP1 or both from RKO and SW480 cell lines using siRNA-targeting sequence pools (4 sequences per pool). Cells were subsequently subjected to clonogenic survival assay to measure cell sensitivity to CPT-11 (Figures 3A and 3D) or irradiation (Figures 3B and 3E), and for Western blotting analysis using antibodies against TDP1, TOP1 and actin (Figures 3C and 3F). We established conditions that led to >80\% depletion of both endogenous TDP1 and TOP1 on their own and in combination. Using a gel-based TDP1 activity assay, we furthermore confirmed that TDP1 siRNA treated RKO cells possess only 10\% of their 3’-tyrosyl-DNA phosphodiesterase activity compared to scramble.
siRNA treated RKO cells (Figures 3G and 3H). The survival assay data show that for both cell lines, TDP1 siRNA treated cells were significantly more sensitive to CPT-11 compared to scrambled siRNA treated cells whilst the opposite was true for TOP1 depleted cells. Notably, these data further demonstrate that TDP1 depletion no longer sensitises cells to CPT-11 if TOP1 is additionally depleted. TDP1 depleted RKO cells also demonstrated a slight increase in sensitivity to irradiation whilst this difference was more pronounced in SW480 cells. To rule out the possibility of off-target effects, we additionally show that depletion of TDP1 using four separate TDP1-targeting siRNA sequences also results in increased sensitivity of RKO cells to CPT-11 (Figures 3I and 3J). We further show that TDP1 depleted RKO cells accumulate more DNA single-strand breaks in the presence of CPT-11 as measured by alkaline comet assays (Figure 3K) and possess elevated levels of cytotoxic DNA double-strand breaks as shown by immunostaining with γ-H2AX (Figure 3L), compared to mock treated counterparts. Together, we conclude that TDP1 depletion sensitises CRC cells to irinotecan treatment in a TOP1 dependent manner and that TDP1 inhibition may further sensitise CRC to radiation therapy.

We next set out to test if TDP1 overexpression would protect CRC cells from CPT-11 mediated cell death. Three CRC cell lines, RKO, SW480 and DLD1, were transfected with equimolar amounts of pCI-neo-myc plasmid that was either empty or encoding human TDP1. A clonogenic survival assay was carried out during which cells were treated with CPT-11 for the duration of colony formation (Figures 4A, 4C and 4E). The results showed that whilst TDP1 overexpression is protective in RKO and SW480 cell lines, the level of protection did not reach statistical significance in the less sensitive DLD1 cell line. Western blotting analysis confirmed TDP1 overexpression in all cell lines (Figures 4B, 4D and 4F), which was further illustrated by a greater TDP1 catalytic activity in TDP1 transfected cells compared to control cells, for all three cell lines tested (Figures 4G and 4H). Next, we examined if the difference in CPT-11 survival is due to the ability of TDP1 to tolerate CPT-11 induced DNA strand breakage. Our data demonstrate that RKO cells overexpressing TDP1 accumulate less DNA single-strand breaks (Figure 4I) and less DNA double-strand breaks (Figure 4J) than controls, as measured by alkaline comet assays and γ-H2AX foci formation, respectively. We conclude that TDP1 overexpression protects irinotecan-responsive CRC cells from irinotecan-mediated cytotoxicity.
The experiments outlined above suggest the potential of assessing TDP1 status as a useful clinical diagnostic tool to characterise patient samples. It is, however, essential to establish if TDP1 protein levels correlate with its cellular activity. To test this, we measured TDP1 activity in CRC whole cell extract using a 5’-Cy5.5 labelled oligonucleotide substrate containing a 3’-phosphotyrosine modification that is processed by TDP1 into an oligonucleotide product containing a 3’-phosphate group (Figure 5A). WCE prepared from each of the six CRC cell lines were subjected to this assay and reaction products separated by 20% denaturing PAGE prior to gel imaging (Figure 5B) and band quantification (Figure 5C). We observed a variation in TDP1 activity that correlates significantly with TDP1 protein level (R= 0.937, p= 0.006 for 30 ng WCE and R= 0.895, p= 0.016 for 60 ng WCE) (Figures 5D and 5E). These observations suggest that measuring TDP1 protein level as a readout of its catalytic activity is suitable for the design of future clinical diagnostic tools.

A key clinical challenge facing the use of irinotecan is the lack of response in some patients. Therefore, we set out to test if TDP1 levels could be used as a diagnostic marker for irinotecan response. To relate TDP1 and TOP1 protein levels to irinotecan response, we measured the inherent CPT-11 sensitivity of the CRC cell lines using a clonogenic survival assay. Cells were treated continuously with CPT-11 for the duration of colony formation prior to fixing, staining and counting. The results showed a significant variation in CPT-11 sensitivity between the six CRC cell lines, the least sensitive of which was DLD1 and the most sensitive was LS417T (Figure 6A). However, subsequent analyses showed weak or lack of correlation between CPT-11 surviving fraction at 0.5 µM CPT-11 (Figure 6B) or 1.0 µM CPT-11 (Figure 6C) and TDP1 protein levels (R= -0.377, p= 0.461 and R= -0.504, p= 0.308, respectively) or TOP1 protein levels (R= -0.169, p= 0.749 and R= -0.284, p= 0.585, respectively). Since TDP1 repairs TOP1-mediated DNA damage, we normalised TDP1 levels to that of TOP1 but yet again found no significant correlation with sensitivity at 0.5 µM CPT-11 (n=6, 2-tailed: R= -0.270, p=0.605) or 1.0 µM CPT-11 (R= -0.278, p=0.594) (Figures 6B and 6C, right). For this latter analyses, removal of an apparent outlier cell line (SW48) highlighted a positive correlation, as would be expected if TDP1 and TOP1 were dominant determinants for irinotecan sensitivity (Figures 6B and 6C, dotted line; n=5, 2-tailed: R= 0.688, p= 0.199 and R= 0.773, p= 0.125). These observations suggest that TDP1 and TOP1 protein levels alone do not correlate with irinotecan
sensitivity in CRC cell lines, and that TDP1/TOP1 ratio may serve as a promising biomarker for irinotecan response.

To test if this is also true in a clinical setting, we examined 125 rectal cancer samples acquired from the RICE trial for irinotecan/radiotherapy response. Criteria for irinotecan/radiotherapy responsiveness were pathological complete remission (pCR) or microfoci of residual tumour (micR) only in the post-treatment (i.e. definitive surgical resection) specimen. Chemo/radiotherapy response was comparable between tumours with none/low TDP1 levels (35% pCR/micR positive) and those with moderate/high levels of TDP1 (39% pCR/micR positive) (Figure 6D), highlighting a lack of correlation between TDP1 level on its own and rectal cancer response to irinotecan/capecitabine/radiotherapy treatment. Of the 59 colorectal cancer cases on the TMA, with a mean follow up of 122 months, 23/59 (39%) patients had died, 11 of confirmed cancer. Neither TDP1 nor TOP1 expression was prognostic for overall survival (log rank test p=0.451 for TDP1 and p=0.241 for TOP1) or cancer-specific survival in these 59 patients (noting that numbers of events were small), although specific treatment details were unavailable. Together these data challenge the utility of TDP1 or TOP1 alone as biomarkers for predicting the response of CRC to irinotecan but establish TDP1 as a potential therapeutic target in CRC.

Discussion

The topoisomerase 1 poison irinotecan has a major role in the chemotherapeutic management of advanced colorectal cancer. However its use in the clinic is limited since some patients do not respond whilst others develop resistance. This study is aimed to gain an insight into the role of the key DNA repair enzyme TDP1 and irinotecan sensitivity in CRC. Our findings demonstrate that both TDP1 and TOP1 expression levels vary in a panel of CRC cell lines and in patient CRC samples as measured by IHC. We show that the majority of CRC samples assessed for TOP1 possessed either moderate or high levels. This is consistent with published data that suggest that CRC, especially rectal cancers, possess high levels of TOP1 (9,38). In contrast to TOP1, no data is currently available on the expression and distribution of TDP1 in colorectal cancer. Here we report that most CRC samples in the commercially available TMA possessed moderate to high levels of TDP1 whereas a larger proportion of the RICE trial samples of locally advanced rectal
cancers expressed little or no TDP1. Whether this variation results from differences in sample preparation (e.g. whole section as opposed to TMA) or reflects true biological differences between tumour sites and stage is not clear. Future work characterizing additional clinical sample sets will aim to address this question and also how TDP1 expression might vary within individual tumours (intra-tumoral heterogeneity) and how this might change with time as the tumors are exposed to different treatments.

Unlike TOP1, the role for TDP1 in CRC cellular response to irinotecan has not been examined previously. We first addressed this putative role using TDP1 modulation studies, which revealed that TDP1 could influence significant changes in CRC response to irinotecan and to a lesser extent, irradiation. Our findings demonstrate that TDP1 overexpression protects CRC cells from CPT-11 mediated cell death. Conversely, TDP1 depletion sensitises CRC cells to irinotecan, an effect that was mitigated by the additional depletion of TOP1. Together, these findings suggest that CRC cancers may benefit from irinotecan and TDP1 inhibitor combination therapy in the clinic. It is however important that patient tumours are characterised for both TDPI and TOP1 since TDPI inhibition is unlikely to have an effect if TOP1 is not present or attenuated. To this end, our CRC cell line data show that TDPI and TOP1 protein levels correlate positively with mRNA levels and furthermore that TDPI protein level correlates positively with TDPI catalytic activity. These positive and significant correlations suggest that both protein (e.g. immunohistochemistry) and mRNA (e.g. microarray analysis) levels for TDPI and TOP1 may be assessed during patient tumour characterisation in a clinical setting.

A prevailing challenge in the clinical use of irinotecan is that some patients do not respond to treatment. Whilst TOP1 levels are generally thought to mediate the response to TOP1 targeting therapies, results from basal tumour TOP1 levels in clinical trial cohorts of patients treated with irinotecan regimens have been contradictory (reviewed in (39)). High TOP1 levels in CRC have been shown to correlate with increased irinotecan sensitivity and also sensitivity to irinotecan containing chemoradiotherapy (9), although a subsequent study by the Dutch Colorectal Cancer Group found no correlation between TOP1 expression and response to irinotecan/5-fluorouracil chemotherapy in 545 patients (38). Identifying additional biomarkers for response may help us to better stratify patients for irinotecan treatment, selecting only those that are likely to respond. Since our TDPI modulation studies show that TDPI can significantly alter irinotecan sensitivity
in CRC cell lines in a TOP1 dependent manner, we questioned whether TDP1 levels correlate with irinotecan response in the clinical samples. We observed no correlation between the levels of basal TOP1, TDP1, or TDP1 levels normalised to TOP1 levels, and the response to irinotecan in our panel of CRC cell lines. We also show that TDP1 expression alone as measured by IHC did not correlate with response to chemoradiotherapy with irinotecan in locally advanced rectal cancer. There may be a number of explanations for these observations. As basal TOP1 levels have been reported to affect subsequent responses (9), it is likely that the contribution of TDP1 additionally depends on TOP1 expression. Notably, removal of the apparent outlier cell line SW48 led to a positive correlation, as would be expected if TDP1 and TOP1 were dominant determinants for irinotecan sensitivity. Although the sample size was small and correlation was not significant, work is ongoing on clinical samples to further elucidate any such relationship. Specifically, future work on this cohort of patients (from the RICE trial) will additionally characterise TOP1 expression and allow for assessment of TDP1/TOP1 ratios. The response in this clinical setting is also a measure of response to irinotecan, capecitabine and radiotherapy, not irinotecan alone. It is worth noting that a role for TDP1 in the response to ionizing radiation has been previously shown in cultured cells and in mouse models (28-31,37,40), and will be the subject of further investigation in a clinical setting. The lack of direct correlation between TDP1 levels alone and irinotecan response is in agreement with our recent study in small cell lung cancer (41), and does not indicate that TDP1 plays a minimal role in this process. Multiple studies have shown that TOP1-DNA breaks are additionally repaired by both redundant and partially overlapping pathways driven by factors such as TDP2, Mus81, XPF-ERCC1, CtIP and MRE11 (24,42-46). Furthermore, the repair rate of DNA double-strand breaks that form as a result of unrepaired TOP1-DNA SSBs may further influence irinotecan response.

In summary, our findings establish TDP1 as a potential therapeutic target for the treatment of CRC and question the validity of TOP1 or TDP1 on their own as predictive biomarkers for irinotecan response.

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References


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Tables

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Table 1. TDP1 and TOP1 staining in tumours selected from different colorectal anatomical sites. Tissue microarray slides of paraffin embedded colorectal cancer samples with documented tumour demographic and correlated overall survival data were deparaffinised and stained with antibodies for TDP1 (NB100-81642, Novus Biologicals, CO, USA) and TOP1 (NCL-TOPO1, Leica Microsystems, Milton Keynes, UK), both at 1:200 dilution using a Bond Max Autostainer. Sores were classified as none, low, moderate or high for TDP1 and TOP1 staining, with samples recorded if >5% cells stained accordingly.
Figure Legends

Figure 1. TDP1 and TOP1 protein levels correlate positively with TDP1 and TOP1 mRNA levels in CRC cell lines. (A) CRC whole cell extract (40 µg) was separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against TDP1, TOP1 and actin. The bands were quantified using ImageJ software and relative TDP1 (B) and TOP1 (C) protein levels normalised to that obtained for RKO is shown ± STD for 3 independent experiments. mRNA (1 µg) purified from the CRC cell lines was reverse transcribed into cDNA that was used for real-time PCR analysis using primers against exon-crossing regions for TDP1 and TOP1. The relative quantity of mRNA (RQ value) for TDP1 (D) and TOP1 (E) was calculated as $RQ = 2^{\Delta\Delta CT}$ and normalised to that obtained for the RKO cell line, the average from 3 independent experiments ± STD is depicted. A graphical representation of the Pearson’s correlation coefficient (R) between CRC TDP1 protein and mRNA levels is shown in (F) and for TOP1 protein and mRNA levels in (G).

Figure 2. TDP1 and TOP1 protein levels vary widely in CRC patient samples. (A) A representative IHC image for TDP1 (NB100-81642, Novus Biologicals) in normal colon epithelium showing nuclear staining in crypt and in surface epithelium cells. (B) Representative IHC images for high and low TDP1 staining in CRC samples from a CRC Tissue Microarray (TMA). (C) Distribution of staining intensity for TDP1 and TOP1 (NCL-TOPO1, Leica Microsystems) IHC on CRC samples from a TMA. (D) Representative IHC images for high and no TDP1 staining for rectal cancers obtained from the RICE trial. (E) Distribution of staining intensity for TDP1 IHC on rectal cancer samples obtained from the RICE trial.

Figure 3. TDP1 depletion sensitises CRC cell lines to irradiation and to irinotecan treatment in a TOP1-dependent manner. RKO and SW480 cells transfected with non-targeting scrambled siRNA (Scram), a TDP1 siRNA pool (4 sequences) and/or a TOP1 siRNA pool (4 sequences) for 72 hr were subjected to a clonogenic survival assay during which they were continuously treated with CPT-11 for the duration of colony formation (7-12 days). The colonies were fixed, stained and counted and the surviving fraction calculated and depicted as an average of three independent experiments ± SEM for RKO (A) and SW480 (D). TDP1 siRNA treated RKO cells (B) and SW480 cells (E) were subjected to a survival assay for irradiation sensitivity and average surviving fraction of three independent experiments ± STD is shown.
WCE (40 µg) generated from the siRNA treated RKO (C) and SW480 (F) cells was analysed further by 10% SDS-PAGE and Western blotting using antibodies against TDP1, TOP1 and actin. (G) Recombinant human TDP1 (2 pM) and WCE (25 ng, 30 ng and 35 ng) generated from either mock (Scram) or TDP1 siRNA treated RKO cells were subjected to an in vitro TDP1 activity assay and the reaction products separated on a 20% Urea Sequagel prior to imaging at 635 nm. The substrate (3’-PY) and product (3’-P) are indicated by arrows. The bands were quantified using Image J software and the average % cleavage for three independent experiments ± STD is shown in (H). TDP1 depletion was carried out in the RKO cell line using four separate TDP1 siRNA sequences (05 to 08). Cells were subsequently used in a clonogenic CPT-11 survival assay (I) or a gel-based TDP1 activity assay to measure knockdown efficiency (J). The average surviving fraction for two independent experiments ± STD is shown in (I). (K) TDP1 siRNA or mock (scram) treated RKO cells were treated with media containing either DMSO or 50 µM CPT-11 for 1 hr and then subjected to analysis by alkaline comet assay. The data shown is a representative single experiment ± STD for 100 cells per sample. (L) TDP1 siRNA or mock (scram) treated RKO cells on coverslips were treated with media containing DMSO or 1 µM CPT-11 for 1.5 hr and left to recover for 2 hr prior to fixing, permeabilising and staining with a γ-H2AX and a FITC labelled secondary antibody. γ-H2AX foci were visualised and analysed for 36 cells per sample on a Nikon Eclipse e-400 microscope. The average number of γ-H2AX foci per cell for three independent experiments is shown ± STD. Paired Student’s t-test (2-tailed) statistical analysis shown as * = p<0.05 and ** = p<0.01.

Figure 4. TDP1 overexpression protects irinotecan responsive CRC cells from irinotecan mediated cell death. CRC cell lines transfected with the indicated amounts of pCI-neo-myc vector or with pCI-neo-myc vector encoding human TDP1 were subjected to a clonogenic survival assay during which they were continuously treated with CPT-11 for the duration of colony formation (7-12 days). The colonies were fixed, stained and counted and the surviving fraction calculated and depicted as an average of three independent experiments ± STD for the CRC cell lines SW480 (A), RKO (C) and DLD1 (E). WCE (40 µg) generated from the SW480 (B), RKO (D) and DLD1 (F) cell lines overexpressing either pCI-neo-myc vector or that encoding human TDP1 was analysed by 10% SDS-PAGE and Western blotting using antibodies against TDP1, TOP1 and actin. The bands relating to overexpressed Myc-tagged TDP1 (TDP1-
myc) and endogenous TDP1 (TDP1-end) are indicated. (G) TDP1 activity assay reactions containing Cy5.5 labelled substrate and WCE for SW480 (25 ng), RKO (10 ng) and DLD1 (25 ng) overexpressing indicated amounts of pCI-neo-myc vector or that encoding human TDP1 were separated on a 20% Urea Sequagel and imaged at 635 nm. A recombinant human TDP1 (4 pM) control was included. The substrate (3’-PY) and product (3’-P) are indicated by arrows. The bands were quantified using Image J software and the average % cleavage for three independent experiments ± STD is shown in (H). (I) RKO cells containing pCI-neo-myc empty vector or that encoding human TDP1 were treated with either DMSO or 50 µM CPT-11 for 1 hr prior to analysis by alkaline comet assay. Data shown is of three independent repeats ± STD. (J) RKO cells overexpressing pCI-neo-myc vector or that encoding human TDP1 plated on coverslips were treated with DMSO or 1 µM CPT-11 for 1.5 hr and left to recover for 2 hr prior to fixxing, permeabilising and staining with a γ-H2AX and a FITC labelled secondary antibodies. γ-H2AX foci were visualised and analysed on a Nikon Eclipse e-400 microscope. The average number of γ-H2AX foci per cell for three independent experiments is shown ± STD. Paired Student’s t-test (2-tailed) statistical analysis shown as *= p<0.05 and **= p<0.01.

Figure 5. In vitro TDP1 catalytic activity correlates positively with TDP1 protein levels in CRC cell lines. (A) Diagram depicting TDP1 processing of a 5’-Cy5.5 labelled oligonucleotide substrate harbouring a 3’-phosphotyrosine modification (3’-PY) to form a Cy5.5 labelled oligonucleotide product harbouring a 3’-phosphate (3’-P) that is smaller in size. (B) 10 uL reaction volumes containing Cy5.5 labelled substrate and the indicated amounts of recombinant human TDP1 (2 pM) or CRC WCE (ng) were separated on a 20% Urea Sequagel and imaged at 635 nm. The substrate (3’-PY) and product (3’-P) are indicated by arrows. (C) Reaction products were quantified using ImageJ software and the average % cleavage for three independent experiments ± STD is shown. A graphical representation of the Pearson’s correlation coefficient (R) between CRC TDP1 protein levels and % cleavage for 30 ng WCE (D) or 60 ng WCE (E).

Figure 6. TDP1 and TOP1 protein levels do not correlate with CRC irinotecan sensitivity. (A) Adhered CRC cells seeded on 10 cm dishes were subjected to CPT-11 treatment for the duration of colony formation (7 to 12 days). Colonies were fixed, stained and counted and the surviving fraction calculated and depicted as an average of three independent experiments ± STD for each cell line. The Pearson’s correlation coefficient (R) between CRC surviving fraction at
0.5 μM (B) or 1.0 μM (C) CPT-11 treatment and TDP1 protein levels, TOP1 protein levels and TDP1/TOP1 protein level ratio is shown. For the latter, a correlation lacking data for SW48 cell lines (triangle) was also carried out (grey dotted line) (D) Irinotecan/radiotherapy response for rectal cancers obtained from the RICE trial was measured as non-responsive or responsive if pathologic complete remission (pCR) or residual tumour (micro-foci positive) was observed and the distribution of response for none/low TDP1 rectal cancers or moderate/high TDP1 rectal cancers is shown.
Figure 1

A. IB: TDP1
IB: TOP1
IB: Actin

B. TDP1 protein
C. TOP1 protein

D. TDP1 mRNA
E. TOP1 mRNA

F. TDP1 mRNA vs. TDP1 protein
R= 0.884

G. TOP1 mRNA vs. TOP1 protein
R= 0.870
A. Normal colon epithelium (TDP1 IHC)

B. CRC (TMA; TDP1 IHC)

C. CRC samples (TMA)

0 10 20 30

Top1 staining (n=57) TDP1 staining (n=59)

High
Moderate
Low
None

35.1 % 23.7 %
42.1 % 45.8 %
22.8 % 27.1 %
3.4 %

D. Rectal cancer (RICE trial; TDP1 IHC)

E. Rectal cancer samples (RICE trial)

0 20 40 60 80

High
Moderate
Low
None

47.2 % 25.6 %
23.2 %
47.2 %
Figure 3

A. Surviving Fraction vs [CPT-11] µM for RKO cells

B. Surviving Fraction vs IR (Gy) for RKO cells

C. IB: TDP1, TOP1, Actin

D. Surviving Fraction vs [CPT-11] µM for SW480 cells

E. Surviving Fraction vs IR (Gy) for SW480 cells

F. IB: TDP1, TOP1, Actin

G. RKO WCE (ng) 25, 30, 35

H. % Cleavage vs RKO WCE (ng)

I. Surviving Fraction vs CPT-11 (µM) for RKO cells

J. RKO WCE (30 ng)

K. Tail moment vs CPT-11 (µM)

L. γ-H2AX foci per cell vs CPT-11 (µM)
Figure 4

A. Surviving Fraction as a function of [CPT-11] concentration for SW480 cells with different treatments.

B. Western blot analysis showing IB: TDP1, IB: TOP1, and IB: Actin for different treatments.

C. Surviving Fraction as a function of [CPT-11] concentration for RKO cells with different treatments.

D. Western blot analysis showing IB: TDP1, IB: TOP1, and IB: Actin for different treatments.

E. Surviving Fraction as a function of [CPT-11] concentration for DLD1 cells with different treatments.

F. Western blot analysis showing IB: TDP1, IB: TOP1, and IB: Actin for different treatments.

G. Gel electrophoresis showing 3'-PY and 3'-P for different treatments.

H. % Cleavage for different treatments.

I. Tail moment for different concentrations of CPT-11.

J. γ-H2AX foci per cell for different concentrations of CPT-11.
Figure 5

A. 

B. 

C. 

D. 

E. 

R = 0.937

R = 0.895
Figure 6

A. [CPT-11] µM vs. Surviving Fraction for various cell lines: RKO, SW480, SW48, Caco2, DLD1, and LS417T.

B. Correlation between TDP1 and TOP1 protein levels and Surviving Fraction for 0.5 µM CPT-11:
- TDP1: R = -0.377
- TOP1: R = -0.169
- TDP1/TOP1: R = -0.270

C. Correlation between TDP1 and TOP1 protein levels and Surviving Fraction for 1.0 µM CPT-11:
- TDP1: R = -0.504
- TOP1: R = -0.284
- TDP1/TOP1: R = -0.278

D. Comparison of RICE trial rectal cancers:
- Moderate/High TDP1
- None/Low TDP1

Non-responsive vs. pCR/micro-foci positive.
Molecular Cancer Therapeutics

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Cornelia Meisenberg, Duncan C Gilbert, Anthony Chalmers, et al.

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