Genome-wide Analysis of Novel Splice Variants Induced by Topoisomerase I Poisoning Shows Preferential Occurrence in Genes Encoding Splicing Factors

Stéphanie Solier¹, Jennifer Barb², Barry R. Zeeberg¹, Sudhir Varma¹, Mike C. Ryan¹, Kurt W. Kohn¹, John N. Weinstein¹, Peter J. Munson⁶, and Yves Pommier¹

Abstract

RNA splicing is required to remove introns from pre-mRNA, and alternative splicing generates protein diversity. Topoisomerase I (Top1), which traps Top1 cleavage complexes (Top1cc), can alter RNA splicing. Here, we tested the effect of Top1 inhibition on splicing at the genome-wide level in human colon carcinoma HCT116 and breast carcinoma MCF7 cells. The RNA of HCT116 cells treated with CPT for various times was analyzed with ExonHit Human Splice Array. Unlike other exon array platforms, the ExonHit arrays include junction probes that allow the detection of splice variants with high sensitivity and specificity. We report that CPT treatment preferentially affects the splicing of splicing-related factors, such as RBM8A, and generates transcripts coding for inactive proteins lacking key functional domains. The splicing alterations induced by CPT are not observed with cisplatin or vinblastine and are not simply due to reduced Top1 activity, as Top1 downregulation by short interfering RNA did not alter splicing like CPT treatment. Inhibition of RNA polymerase II (Pol II) hyperphosphorylation by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) blocked the splicing alteration induced by CPT, which suggests that the rapid Pol II hyperphosphorylation induced by CPT interferes with normal splicing. The preferential effect of CPT on genes encoding splicing factors may explain the abnormal splicing of a large number of genes in response to Top1cc. Cancer Res; 70(20); 8055–65. ©2010 AACR.

Introduction

Alternative splicing is observed with >95% of the genes (1, 2) and constitutes the main source for protein diversity, allowing the generation of different proteins from a given pre-mRNA by the differential use of splice sites (3). Alternative splicing is produced by several mechanisms: exclusion/inclusion of exons, alternative 3′ splice sites, alternative 5′ splice sites, mutual exclusion of exons, unsplicing of an exon, multiple promoters, and multiple poly(A) sites (Fig. 1C and D). Splicing takes place at the pre-mRNA level within spliceosomes, each of which contain around 300 polypeptides and five small nuclear ribonucleoproteins (snRNP; ref. 2). In addition, serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNP) are well-known splicing regulators (2). The SR proteins activate splicing by binding exonic splicing enhancers, whereas hnRNPs repress splicing by binding exonic or intronic splicing silencers (1, 2). In addition to the SR proteins and the hnRNPs that are ubiquitously expressed, splicing regulators like CELF, Nova, nPTB, FOX1, FOX2, ESRP1, ESRP2, and nSR100 are tissue-specific (2).

Splicing consists of two transesterification reactions. First, the hydroxyl of the branch point site attacks the phosphate of the 5′ splice site, generating a free exon and an intermediate lariat. Second, the hydroxyl of the 5′ splice site attacks the phosphate of the 3′ splice site, generating the mature mRNA and a lariat of introns that will be finally eliminated. Over 70% of the splice events change the protein sequence, and 19% generate truncated proteins (4).

DNA topoisomerase I (Top1) is required to remove DNA superhelical tensions generated by DNA replication and transcription (5–8). During transcription, positive and negative DNA supercoiling are produced ahead of and behind the elongating RNA polymerase II (Pol II) complex, respectively (9). Top1 relaxes both positive and negative supercoiling by producing transient Top1 cleavage complexes (Top1cc), which are Top1-linked DNA single-strand breaks (7, 10). Several studies have suggested the implication of Top1 in splicing. First, Rossi and colleagues showed that Top1 can phosphorylate the SR splicing proteins (11, 12) and that two domains of Top1 were implicated in this activity: one as an
ATP binding site in the carboxy-terminal region of Top1 and the other as a binding site for SF2/ASF and a protein kinase domain in the amino-terminal region of Top1 (12). More recently, this kinase activity of Top1 has been confirmed (13), and Top1 has been proposed to shift from its classic DNA relaxation activity to its kinase activity after binding the SR splicing factors (14, 15). SF2/ASF can interact by its two RNA recognition motif (RRM) domains with Top1 and inhibit DNA relaxation by the enzyme (16). Top1 is also important in the regulation of gene expression by its preferential association with transcriptionally active regions (17–19) and by controlling promoter activity independently from its DNA relaxing activities (20, 21).

Top1 is the target of the plant alkaloid camptothecin (CPT), and its clinical derivatives, topotecan and irinotecan, are widely used as anticancer agents (7). CPT and its derivatives are noncompetitive, reversible specific Top1 inhibitors that prevent DNA religation due to the trapping (poisoning) of Top1cc (7, 10). Poisoning of Top1 also occurs under normal conditions when the DNA template contains damaged bases including abasic sites, mismatches, oxidized bases, or carcinogenic adducts (22, 23). The induction of Top1cc
Top1 Affects the Alternative Splicing of Splicing Factors

by CPT has been shown to affect transcription in several ways. RNA elongation is rapidly arrested by Top1cc (24) with reduction of Pol II density at promoter pausing sites (19), activation of low abundance antisense RNAs (17), and rapid hyperphosphorylation of Pol II in response to CPT treatment (25). Pol II hyperphosphorylation is rapidly reversible on CPT removal or cyclin-dependent kinase (CDK) inhibition by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; ref. 25). The induction of Top1cc by CPT has been shown to affect RNA splicing, but published studies have only been done on some specific genes (26–29).

Here, we tested the implication of Top1cc in splicing at the global genome level in human carcinoma cells to determine whether Top1 inhibition selectively affects particular families of genes. For this purpose, we used ExonHit arrays. Unlike arrays that contain only exon probes, the ExonHit arrays also contain junction probes, which allows the detection and quantification of novel splice variants (30).

Materials and Methods

Chemicals and cells
CPT, cisplatin, vinblastine, and DRB were obtained from Sigma-Aldrich. Human HCT116 and MCF7 cell lines were obtained from American Type Culture Collection and grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products) at 37°C in 95% air and 5% CO2.

Western blotting and antibodies
Western blotting was performed according to standard protocols (31). The C21 Top1 mouse monoclonal antibody was a kind gift from Dr. Yung-Chi Cheng (Yale University, New Haven, CT). The other primary antibodies used were anti-PoII (Santa Cruz Biotechnology), anti-P2-PoII (Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling).

Short interfering RNA
For Top1 downregulation, cells were transfected with a short interfering RNA (siRNA) duplex (Qiagen) against the sequence AAGGACTCCATCAGATACTAT from the Top1 mRNA. A negative control siRNA duplex was obtained from Qiagen (target DNA sequence, AATTCTCCGAACGTGT-CACGT). Cells were seeded in six-well plates at a density of 150,000 cells per well at 16 hours before transfection (31).

Reverse transcription-PCR
Cells were washed in PBS. RNA extraction was performed with the “Nucleospin RNA II” kit (Macherey-Nagel). The “OneStep RT-PCR” kit (Qiagen) was used under the following conditions: 1× buffer, 400 μmol/L of each deoxyribonucleotide triphosphate, 0.6 μmol/L of each primer, 2 μL of enzyme mix, and 1 μg of template RNA in a total volume of 50 μL. RNA was reverse-transcribed for 30 minutes at 50°C; the initial PCR step was activated by heating for 15 minutes at 95°C before PCR (1-minute denaturation at 94°C, 1-minute annealing at 60°C, 1-minute extension at 72°C, 32 cycles) using a MJ research PTC-200 Thermo Cycler (MJ Research). The PCR products were analyzed on agarose gels. The amplified DNA fragments were stained with ethidium bromide, and fluorescence was detected by video camera imaging using the Quantity One software (Bio-RAD). Primer sequences are listed in Supplementary Table S1.

Probe set annotations and data normalization
ExonHit probe sequences were aligned to known variants in the SpliceMiner database (32). Exons were numbered consecutively according to genomic position, accounting for strand. Background subtraction and quantile normalization of the probes were done using Partek statistical software (Partek, Inc.). The median polish step was done using R. A probe is designated “body” or “junction” depending on whether it interrogates a known exon or an exon-exon boundary. A junction probe is designated “J1” if it interrogates consecutive exons and “J2” if it interrogates a junction which skips one or more intervening exons.

Principal component analysis
The study design consisted of two controls (c4h, c20h) collected at 4 and 20 hours and five CPT (10 μmol/L) treated samples (1h, 2h, 4h, 15h, and 20h) collected at 1, 2, 4, 15, and 20 hours, respectively. The RNA intensity values for both the body and junction probes were subjected to principal component analysis (PCA). A biplot of PC1 versus PC2, accounting for 69% of the total variability, revealed three clusters composed of control samples (c4h, c20h), early treated (1h, 2h, and 4h), and late treated samples (15h, 20h).

Statistical model
The ExonSVD analysis modifies the traditional three-way ANOVA model with a new parameter for probe set responsiveness. Unlike the ANOVA model, which is prone to give false-positive signals for alternative splicing in the presence of “dead” or unresponsive probes, the ExonSVD model includes an explicit parameter, D, for probe set responsiveness. Also, the ExonSVD model can handle body and junction probes directly without further elaboration. The body probes primarily determine the differential expression, whereas the junction probes, which are sensitive to the varying levels of exon-exon junctions, are the primary source of information regarding alternative splicing. The E effect of the model (alternative splicing effect) is determined as the residual after differential expression effects are accounted for and is tested for significance to determine whether alternative splicing has occurred. This new model alleviates the need for prefitering to eliminate dead and unresponsive probe sets. The P values for E were calculated using the F distribution where the degrees of freedom, which depend on the number of exons, were determined by fitting rational polynomials to the expected sum of squares in a numerical simulation.

Significance index and event type
We developed a “significance index” calculated as the negative log10(P) for alternative splicing plus two times the
maximum absolute value of the $E$ term for each gene. Using this significance index, one can order the entire set of genes according to evidence for alternative splicing.

For the genes with evidence of alternative splicing, we define events of four types (Exon gained early, Exon lost early, Exon gained late, and Exon lost late) compared with the control condition. For each interior exon, we interrogate the junctions between it and its preceding (J1-1st) or its following exon (J1-2nd) and the junction joining the preceding to the following exon (J2). In some cases, the J2 probe was not available. In others, other J1 probes were not available, so a determination of the event could not be made. An event of certain type was declared if corresponding residuals from the ExonSVD model satisfied a set of conditions involving the signs of differences of $E_{ik}$. For example, for “exon loss early,” $[E(\text{early}, J2) - E(\text{c}, J2)] > 0$, $[E(\text{early}, J1-1st) - E(\text{c}, J1-1st)] < 0$ and $[E(\text{early}, J1-2nd) - E(\text{c}, J1-2nd)] < 0$. We also required that the magnitude of one of the differences be $>2$-fold. Not all of the significant genes contained one of these four types of events. Some had multiple events, and some had both gain and loss of different exons.

**High-Throughput GoMiner (HTGM)**

High-Throughput GoMiner (HTGM) leverages the Gene Ontology (GO) to identify “biological processes” represented in a list of genes. The HTGM (33) used here is an enhancement of GoMiner that efficiently processes an arbitrary number of such gene lists. The gene lists obtained from AnovaSVD were sorted in decreasing order with respect to the significance index. We submitted the top 998 genes for HTGM analysis. The HTGM parameters are listed in Supplementary Table S2.

A GO category is considered to be enriched if the number of changed genes that HTGM assigned to it is greater than the number expected by chance. The enrichment of a category is considered to be statistically significant if its false discovery rate (FDR) is less than or equal to a given threshold (typically 0.10). See ref. 33 for details.
Genesis clustering program
Clustered image maps (CIM) were produced in our studies by the Genesis program (33). We chose the Euclidean distance metric and average linkage for hierarchical clustering. Large generic categories were removed from all CIMs to facilitate visualization.

Results

Genome-wide analysis of splicing alterations induced by Top1 poisoning
The ExonHit array allows the analysis of 138,636 splicing events among 20,649 genes. The probes are designed to mostly recognize two kinds of splicing events: exon skipping and novel exon (Fig. 1A). The array uses a combination of exon and junction probes that are targeted inside exon sequences (probes F, T, and B) and at junctions between two neighboring exons (probes C, D, and E), respectively (Fig. 1B). A novel exon (exon gain) is characterized by an increase in the B, C, and D probes and a decrease in the E probe (Fig. 1C). A skipped exon (exon loss) produces an increase in the E probe and a decrease in the B, C, and D probes (Fig. 1D).

To study the effect of Top1 poisoning on splicing, we purified total RNA from human colon carcinoma HCT116 cells treated with 10 μmol/L CPT for 1, 2, 4, 15, and 20 hours (Fig. 2A) and performed ExonHit array analysis for each sample. Control samples were analyzed at 4 and 20 hours following DMSO treatment (0.1%; the solvent used to dissolve CPT).

The data were analyzed using a new ExonSVD model (see Materials and Methods; all the data are in GEO; accession number GSE23677; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23677). Figure 2B shows the PCA organization of the results with the controls, the early CPT treatment time points (1h, 2h, and 4h), and the later time points (15h and 20h) grouping separately. This shows a time-dependent effect on splicing and a correlation between splicing alterations and CPT treatment. Nine hundred ninety-eight genes among all the genes analyzed (around 5%) had a significance index of 6 or greater, indicative of statistically significant and/or large changes in splicing in response to CPT. The type of alternative splicing event could be determined for 50% of the genes (434 genes) and corresponded to novel exon or exon skipping (Fig. 2C). For the other 50% of the genes, determination was not possible. 

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Validation of the ExonHit results in HCT116 cells treated with CPT (10 μmol/L, 20 h). A, example of the EIF2S2 gene. Top, schematic gene representation and position of the ExonHit probes (B, C, D, E, F, T) and of the primers used for the reverse transcription PCR (RT-PCR; black arrows). Middle, log2 difference for each of the probes depending on CPT treatment normalized to the untreated controls. Bottom, RT-PCR showing the effect of CPT on exons 4 and 5 exclusion. EIF2S2 mRNA was analyzed by RT-PCR using EIF2S2 E2s and EIF2S2 E6as primers. B, example of the PNN gene. Top, schematic gene representation and position of the ExonHit probes (B, C, D, E, F, T) and of the primers used for the RT-PCR (black arrows). Middle, log2 difference for each of the probes depending on CPT treatment normalized to the untreated controls. Bottom, RT-PCR showing the effect of CPT on exon 5 exclusion. PNN mRNA was analyzed by RT-PCR using PNN E4s and PNN E7as primers. Control cells received vehicle alone. β2 microglobulin (β2) mRNA was used as a standardizing control.
due to the complexity of the apparent event or because the array did not probe the relevant splice junctions. The appearance of novel exon was both an early as well as a late event (Fig. 2C). On the other hand, exon skipping, in most cases, was only detectable at the late time treatments with CPT (15 or 20 hours; Fig. 2C). Next, we wanted to know whether the splice events occurred in specific regions of the transcript. We divided the 434 genes with splicing alterations into three groups according to the number of exons contained in each gene [based on the publicly available Genbank information; 1–10 (103 genes), 11–20 (164 genes), >20 exons (167 genes)]. Splice events occurred all along the transcript and tended to increase with the length of the transcripts (Fig. 2D). Multiple events on a same transcript concerned 22% of the short transcripts, 35% of the medium transcripts, and 45% of the long transcripts. Together, these results

Table 1. List of the best GoMiner categories

<table>
<thead>
<tr>
<th>Go categories</th>
<th>FDR</th>
<th>No. spliced genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006397_mRNA_processing</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>GO:0000398_nuclear_mRNA_splicing__via spliceosome</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>GO:0000375_RNA_splicing__via_transesterification_reactions</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>GO:0016071_mRNA_metabolic_process</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>GO:0008830_RNA_spinging</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>GO:0026213_ribonucleoprotein_complex_biogenesis</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>GO:0007052_mitotic_spindle_organization</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>GO:000278_mitotic_cell_cycle</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>GO:000245_spliceosome_assembly</td>
<td>0.01</td>
<td>8</td>
</tr>
<tr>
<td>GO:0015850_organic_alcohol_transport</td>
<td>0.01</td>
<td>4</td>
</tr>
<tr>
<td>GO:000279_M_phase</td>
<td>0.01</td>
<td>23</td>
</tr>
<tr>
<td>GO:0007051_spindle_organization</td>
<td>0.01</td>
<td>7</td>
</tr>
<tr>
<td>GO:000280_nuclear_division</td>
<td>0.01</td>
<td>17</td>
</tr>
<tr>
<td>GO:0007067_mitosis</td>
<td>0.01</td>
<td>17</td>
</tr>
<tr>
<td>GO:0048285_organelle_fission</td>
<td>0.02</td>
<td>17</td>
</tr>
<tr>
<td>GO:0000087_M_phase_of_mitotic_cell_cycle</td>
<td>0.02</td>
<td>17</td>
</tr>
<tr>
<td>GO:0026218_ribonucleoprotein_complex_assembly</td>
<td>0.03</td>
<td>9</td>
</tr>
<tr>
<td>GO:007049_cell_cycle</td>
<td>0.05</td>
<td>46</td>
</tr>
<tr>
<td>GO:0006281_DNA_repair</td>
<td>0.05</td>
<td>21</td>
</tr>
<tr>
<td>GO:0032259_methylation</td>
<td>0.08</td>
<td>8</td>
</tr>
<tr>
<td>GO:0043141_bipolymer_methylation</td>
<td>0.08</td>
<td>8</td>
</tr>
<tr>
<td>GO:0022402_cell_cycle_process</td>
<td>0.08</td>
<td>36</td>
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<tr>
<td>GO:0006730_one_carbon_metabolic_process</td>
<td>0.09</td>
<td>8</td>
</tr>
<tr>
<td>GO:002403_cell_cycle_phase</td>
<td>0.1</td>
<td>26</td>
</tr>
</tbody>
</table>

NOTE: The table displays the name of the categories with lowest FDR concerning the genes having a significance index of 6 or more, i.e., 998 genes.
suggest that splicing alteration occurred both on short and long transcripts but tended to increase with the length of the transcript.

To validate the ExonHit genome-wide results, we took 12 genes which ranked near the top of the list of the 998 genes, according to significance index (Supplementary Table S4) and performed RT-PCR with gene-specific primers. The RT-PCR results were in agreement with the ExonHit results for >90% of the splice alterations analyzed (see Supplementary Table S4). Figure 3 showed two examples of exon skipping for the EIF2S2 and PNN genes. CPT induced the skipping of exons 4 and 5 in the EIF2S2 transcript. The ExonHit data showed an increase of the E probe (that links exon 3 to exon 6), and the RT-PCR showed the decrease of the long transcript (containing exons 4 and 5) following CPT treatment and the appearance of the short transcript (without exons 4 and 5). For the PNN transcript, CPT induced the skipping of exon 5 with an increase of the E probe (that links exon 4 to exon 6) in the ExonHit analysis. Accordingly, RT-PCR showed a decrease of the long transcript (containing exon 5) and the appearance of the short transcript (without exon 5) following CPT treatment. Exon gain was also validated in response to CPT for the caspase-2 gene (Supplementary Fig. S1).

Splicing alterations are enriched in genes coding for splicing factors

Next, we tested whether CPT affected the alternative splicing of specific gene families. The 998 differentially spliced genes were analyzed by GoMiner software (http://discover.nci.nih.gov/gominer/index.jsp). The categories of genes that tended to be preferentially affected are listed in Fig. 4 and Table 1 and include those related to splicing, mitosis, and methylation. Splicing categories were the most affected by CPT treatment. Individual splicing events were then further analyzed for the genes encoding splicing-related factors. The splicing alterations induced by CPT on the RBM8A, ZRANB2, BAT1, and SF1 genes were further examined (Supplementary Table S4; Fig. 5). Kinetic experiments were performed for the RBM8A and SF1 genes not only in HCT116 cells but also in human breast carcinoma MCF7 cells treated with CPT. By RT-PCR, the effect on splicing for RBM8A, which is the skipping of exon 3, was detected early, beginning 1 hour after CPT treatment in both cell lines (Fig. 5A). Splicing of SF1, which corresponds to the skipping of exon 4, was detectable later, at 15 hours of CPT treatment (Fig. 5B). Together, these results show preferential splicing alterations in the genes encoding splicing factors by Top1 poisoning.
Pol II hyperphosphorylation is associated with Top1-induced splicing alterations

To determine the specificity of CPT in inducing altered splicing, other anticancer agents that do not target Top1 were tested. Neither cisplatin, a DNA alkylating drug, nor vinblastine, a mitotic spindle poison, affected the splicing of RBM8A or SF1 (Fig. 6A). To test whether the effect of CPT was related to Top1cc or due to Top1 depletion by its sequestering in Top1cc, we downregulated Top1 using a siRNA (Fig. 6B, right). Downregulation of Top1 had no effect on alternative splicing of the RBM8A or SF1 genes (Fig. 6B, left), indicating that the generation of Top1cc rather than Top1 inactivation is necessary to induce splicing.

Figure 6. CPT-induced alternative splicing is Top1cc dependent and linked to Pol II hyperphosphorylation. A, HCT116 cells were treated with CPT (10 μmol/L, 20 h), cisplatin (CDDP, 50 μmol/L, 20 h), or vinblastine (0.1 μmol/L, 20 h) and RBM8A and SF1 were analyzed by RT-PCR using RBM8A E2s/RBM8A E4as and SF1 E3s/SF1 E6as, respectively. Control cells received vehicle (DMSO) alone. β2 microglobulin (β2) mRNA was used as a control. The percentage of cells in sub-G1 (37) is indicated at the bottom. B, downregulation of Top1 does not induce the alternative splicing of RBM8A or SF1. HCT116 cells were transfected with a negative control siRNA or a siRNA against Top1. Left, RBM8A or SF1 mRNA was analyzed by RT-PCR using RBM8A E2s/RBM8A E4as and SF1 E3s/SF1 E6as, respectively. β2 microglobulin (β2) mRNA was used as a standardizing control. exp, experiment. Right, Western blotting showing the efficiency of Top1 downregulation by siRNA. Top1 was analyzed by Western blotting. GAPDH was used as a loading control. C, the CDK inhibitor DRB suppresses the effect of CPT on alternative splicing. HCT116 cells were pretreated with DRB (100 μmol/L, 1 h) before treatment by CPT (10 μmol/L, 2 h). RBM8A splicing was analyzed by RT-PCR using RBM8A E2s and RBM8A E4as primers. Control cells received vehicle (DMSO) alone. β2 microglobulin (β2) mRNA was used as a standardizing control. D, DRB prevents the Pol II hyperphosphorylation induced by CPT. HCT116 cells were pretreated with DRB as in C. Pol II was analyzed by Western blotting. Pol IIa (hypophosphorylated) and Pol IIo (hyperphosphorylated) forms are indicated. GAPDH was used as a loading control.
Because Pol II hyperphosphorylation has recently been reported in response to CPT-induced Top1cc (25) and Pol II hyperphosphorylation has been implicated in alternative splicing (34, 35), we tested the relationship between CPT-induced Pol II hyperphosphorylation and splicing alterations. HCT116 cells were pretreated with DRB, an inhibitor of CDK (36), before exposing the cells to CPT. DRB pretreatment completely abrogated the CPT-induced splicing of RBM8A (Fig. 6C) and caspase-2 (Supplementary Fig. S2). Under these conditions, DRB abrogated CPT-induced Pol II hyperphosphorylation (Fig. 6D; ref. 25). These results suggest a relationship between CPT-induced Pol II hyperphosphorylation and splicing alterations.

Discussion

This genome-wide analysis study shows that Top1cc alters the splicing of a large number of genes with a preference for the genes encoding splicing or splicing-related factors. Previous studies on the splicing effects of Top1 inhibitors focused on single genes (26–29, 38) and did not reveal the important contribution of the splicing factor genes as preferential splicing targets for Top1 poisons.

The ExonHit human splice array with its junction probes enabled the detection of early splice alterations with higher sensitivity than arrays that display only exons probes (for instance, the Affymetrix GeneChip Human Exon 1.0 ST array). To detect a new splice event, we find the junction probes to be more sensitive than the exon probes, allowing the detection of events with high sensitivity and selectivity (30). The use of exon probes is sufficient to compare expression levels of alternative transcripts in stable cell lines where transcripts are stably produced; however, to study the changes in splicing following acute treatments, it is mostly the junction probes that allowed the detection of new variants because of the long half-life of most basal transcripts (median half-life = 10 hours; ref. 39). Effectively, in the analysis of our ExonHit data, the junction probes were critical to identify the appearance of new splice events by the detection of change (mostly an increase) in E or C and D probes (see Fig. 1).

Among the splicing factors that were abnormally spliced in response to CPT, three of them exemplify the functional effect of CPT treatment. RBM8A is a RNA-binding motif protein present in intermediate-containing spliceosomes. RBM8A is a component of a splicing-dependent multiprotein exon junction complex deposited at splice junction on mRNA (40, 41). It influences downstream processes including nuclear mRNA export, subcellular mRNA localization, translation efficiency, and nonsense-mediated mRNA decay. The RBM8A polypeptide contains a 90-amino acid RRM that binds single-stranded RNA and contains two highly conserved short sequences, RNPI and RNPD, that are crucial for RNA binding (42). In response to CPT, the skipping of exon 3 removes the RNPD domain, resulting in a truncated RRM (without RNPD) that is predicted to inactivate RBM8A. Similarly, ZRANB2 (43), which interacts with the U2 small mRNP auxiliary factor and SNRP70, has its zf-RanBP (zinc finger Ran binding protein) domain missing due to the skipping of exon 4 after CPT treatment. We also found that BAT1 (44), a splicing factor required for the association of U2 snRNP with pre-mRNA and mRNA export from the nucleus to the cytoplasm, has its DEAD domain truncated due to the skipping of exons 3 and 5 after CPT treatment. These results illustrate how CPT, by affecting key functional domains of splicing factors that are important for their binding with RNA or other splicing-related factors, can inactivate these splicing factors and consequently affect in “trans” the splicing of other genes.

The exposure of HCT116 cells to 10 μmol/L CPT during 20 hours does not generate a significant amount of apoptosis. The level of apoptosis measured by Hoechst staining was below 10%. Moreover, we previously observed that pretreatment, by the broad caspase inhibitor, Z-VAD-fmk, does not suppress the effect of CPT on the splicing of caspase-2 (29). Also, many of the changes observed at 20 hours were already readily detectable at early time (1 hour). Consequently, the changes observed concerning alternative splicing do not mainly reflect the cells going through the process of dying.

Top1 poisoning by CPT and anticancer drugs, abasic sites, base mismatches, oxidized bases, carcinogenic adducts, and strand breaks can deplete Top1 activity by sequestering Top1 in the cleavage complexes (7, 22, 23). However, it is well
established that the anticancer cytotoxic effects of CPT are not due to the depletion of Top1 activity but rather to the trapping of Top1cc and subsequent effects of DNA replication and transcription (8, 45). Similarly, our Top1 depletion experiments with siRNA show that the splicing effects of CPT are Top1cc-dependent but are probably unrelated to the depletion of Top1 catalytic activity. We recently reported the rapid induction of Pol II hyperphosphorylation in response to CPT-induced Top1cc (25). In this study, we confirmed Pol II hyperphosphorylation after CPT treatment. Pretreatment with DRB, which inhibits CDK (25) and suppresses Pol II hyperphosphorylation (see Fig. 6; ref. 25), abrogated the splicing effects of CPT. It is plausible that CPT alters splicing in response to Pol II hyperphosphorylation (see Fig. 7), which has been proposed to affect Pol II pausing and the selection of splicing sites (34). Similarly, Munoz and colleagues showed that UVC affects splicing by inducing Pol II hyperphosphorylation and slowing down Pol II elongation (35), which can favor the use of weak splice sites (34, 46). The poisoning of Top1cc by CPT can affect splicing by such a "kinetic coupling model" (47). However, it is also possible that hyperphosphorylation of the carboxy-terminal domain of Pol II affects its interaction with and recruitment of splicing factors as proposed in the "recruitment coupling model" (48, 49). In summary, transcription could regulate alternative splicing by modulation of Pol II elongation rates (kinetic coupling; ref. 47) and by association of splicing factors to the transcribing polymerase (recruitment coupling; ref. 50). Our finding that the genes encoding splicing factors are preferentially altered can explain the effect of Top1cc on the splicing of a large variety of genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

25. Sordet O, Larochelle S, Nicolas E, et al. Hyperphosphorylation of RNA polymerase II in response to topoisomerase I cleavage...
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Genome-wide Analysis of Novel Splice Variants Induced by Topoisomerase I Poisoning Shows Preferential Occurrence in Genes Encoding Splicing Factors

Stéphanie Solier, Jennifer Barb, Barry R. Zeeberg, et al.

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