Topoisomerase I and II Inhibitors Control Caspase-2 Pre-Messenger RNA Splicing in Human Cells

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Abstract

We have recently shown that the topoisomerase II inhibitor, etoposide (VP16), could trigger caspase-2 pre-mRNA splicing in human leukemic cell lines. This leads to increased inclusion of exon 9, which is specifically inserted into the short caspase-2S isoform mRNA and absent from the long caspase-2L isoform mRNA. One of the consequences of this alternative splicing is a decrease in the total amount of the mature form of caspase-2L mRNA and protein. In this study, we analyzed the effects of several representative molecules of various classes of cytotoxic agents on caspase-2 pre-mRNA splicing in both U937 leukemic cells and in HeLa cervix carcinoma cells. Very strikingly, both topoisomerase I (camptothecin and homocamptothecin derivatives) and II (VP16, amsacrine, doxorubicin, mitoxantrone) inhibitors induced exon 9 inclusion. DNA intercalating glycosyl indolocarbazole derivatives as well as DNA alkylating agents, such as cisplatin and melphalan, antimetabolites like 5-fluorouracil, and mitotic spindle poisons like vinblastine had no effect. Therefore, both classes of DNA topoisomerases can control pre-mRNA splicing of the caspase-2 transcript. In addition, the splicing reaction brought about by camptothecin was hampered in human CEM/C2 and in murine P388-45R leukemic deficient in topoisomerase I activity. Conversely, VP16 did not trigger caspase-2 alternative splicing in human HL60/MX2 leukemic cells harboring a mutant topoisomerase II. Minigene transfection analysis revealed that topoisomerase inhibitors did not change the splicing profile when *cis*-acting elements in intron-9, reported to control exon 9 inclusion independently of drug treatment, were removed. Rather, our experiments suggest that exon 9 inclusion induced by topoisomerase inhibitors reflects the activity exerted by topoisomerase I or II on proteins that control splicing reactions, or their direct involvement in pre-mRNA splicing.

Introduction

Recent estimates suggest that about 40–60% of pre-mRNAs are subject to alternative splicing, which accounts for proteome diversity under various situations (1, 2). Pre-mRNA splicing requires a multiprotein complex referred to as the spliceosome, which has recently been purified and shown to contain more than 150 polypeptides (3). The activity of splicing factors is controlled at various levels, including subcellular localization and phosphorylation at serine and threonine residues. For example, the activity of the highly conserved Serine-arginine-rich (SR) proteins can be modulated by phosphorylation of serine residues. SR proteins include one or two RNA recognition motifs (RRMs) and a COOH-terminal arginine-serine repeat of varying length (RS domain; for reviews, see Refs. 4 and 5). The RNA recognition motifs mediate recognition of specific RNA determinants known as exonic splicing elements (6, 7), whereas the RS domain is responsible for specific protein-protein interactions (8–10). SR proteins that are bound to exonic splicing elements affect splicing by directly recruiting the splicing machinery through their RS domain and/or by antagonizing the action of nearby silencer elements (7, 10). Topoisomerase I has been shown to regulate this activity by phosphorylation of specific serine residues located within the RS domain (11), leading to reprogramming of pre-mRNA splicing of several genes such as CD44 and Bcl-X (12).[6] However, the large implication of the kinase activity of topoisomerases as a key determinant for gene regulation remains to be determined.

The expression of numerous genes involved in apoptosis regulation is modulated by alternative splicing (13). These genes are transcribed as diverse mRNA species, which encode proteins with sometimes opposite functions. One example is the *CASP-2* gene that codes for a proteolytic enzyme involved in cell dismantling in response to various stimuli such as cytotoxic drugs (14, 15). Alternative splicing of this pre-mRNA generates two major mRNA species (16) including a long caspase-2L isoform that encodes a pro-apoptotic protein and a short

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caspase-2S isoform with anti-apoptotic functions (16, 17). Synthesis of caspase-2L and caspase-2S mRNA species proceeds through two mutually exclusive splicing events (16). Selective inclusion of exon 9, which gives rise to caspase-2S mRNA, depends on a sequence termed In100 present in intron 9 (18). Minigene and *in vitro* splicing analyses have shown that the presence of this sequence blunts exon 9 inclusion, thus favoring caspase-2L mRNA assembly. Several splicing factors can regulate caspase-2 alternative splicing: hnRNPA1 can promote exon 9 inclusion, whereas the SR proteins ASF/SF2 and SC35 favor its exclusion (19). In addition, Polypyrimidine Track-Binding protein can repress exon 9 inclusion on binding downstream on the In100 element, within intron 9 (20).

We have previously shown that etoposide (VP16) triggered a switch between caspase-2L and caspase-2S mRNA species by inducing exon 9 inclusion in human leukemic cells, thus leading to a decrease in caspase-2L protein expression (21). In the present study, we show that caspase-2 mRNA splicing does not depend on apoptosis induction and is a specific consequence of topoisomerases I and II poisoning. Our observations argue for a direct contribution of topoisomerases to pre-mRNA splicing events.

Results

VP16 and Camptothecin Promote Exon 9 Inclusion Independently of Apoptosis

We have shown previously that a treatment with 50 μM VP16, a DNA topoisomerase II inhibitor, for 4 h was able to trigger exon 9 inclusion into caspase-2 mRNA in human leukemic U937 and HL60 cells (21). This effect was associated with massive apoptosis induction, typically between 40% and 60%. To see whether alternative splicing was a consequence of, or was independent from apoptosis, we surveyed exon 9 inclusion at earlier time points and following treatment with lower doses of the drug. As shown in Fig. 1A, the highest level of splicing product was detected within 1 h of treatment with 50 μM of VP16, a condition that did not induce apoptosis. This also occurred on treatment with a 5-μM dose of VP16 for 4 h, although with a lower magnitude (data not shown), well before any morphological apoptosis was detectable. The alternative splice event increased with drug concentration (Fig. 1B) and was maximum with 50 μM VP16 for 4 h. In addition, in the presence of z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk; 100 μM), a broad-spectrum caspase inhibitor, no apoptosis was detected on treatment with 50 μM VP16, but exon 9 inclusion was fully achieved (Fig. 1C). Similarly, z-Val-Asp-Val-Ala-Asp-fluoromethylketone (z-VDVAD-fmk; 100 μM), a more selective inhibitor of caspase-2, reduced VP16-induced apoptosis by 50% but did not prevent mRNA splicing (Fig. 1D). Lastly, in U937 cells rendered resistant to apoptosis by stable overexpression of the anti-apoptotic Bcl-2 protein (Fig. 1E), and in HeLa cells (Fig. 1F), which do not undergo apoptosis under these conditions, VP16 induced exon 9 inclusion to the same extent as in U937 cells. Hence, VP16 triggers exon 9 inclusion, regardless of ongoing apoptosis and independently of z-VAD- or z-VDVAD-sensitive caspases.

Similar to the effects of VP16, camptothecin treatment (4 μM) strongly induced apoptosis (50%) in U937 cells. However, this effect was blocked in the presence of z-VAD-fmk (100 μM), but exon 9 inclusion was fully attained (Fig. 1G). In U937-Bcl-2 cells and in HeLa cells, no apoptosis was detected even though exon 9 inclusion was obtained (Fig. 1, H and I). Hence, as for VP16, camptothecin induced exon 9 inclusion regardless of ongoing apoptosis and independently of Z-VAD-sensitive caspases.

Both Topoisomerase I and II Inhibitors Trigger Exon 9 Inclusion in U937 and HeLa Cells

To see whether other topoisomerase inhibitors could mimic the effect of VP16, we tested amsacrine, mitoxantrone, and doxorubicin, three intercalating agents that also inhibit topoisomerase II, together with camptothecin, the prototypic topoisomerase I inhibitor. Each of these drugs enhanced exon 9 inclusion (Fig. 2, A and B). Hence, 5 μM of amsacrine, 2 or 5 μM mitoxantrone, and 5–50 μM doses of doxorubicin for 4–6 h, were fully active at promoting alternative splicing (Fig. 2A and data not shown). Exon 9 inclusion was also induced by other camptothecin-derived topoisomerase I inhibitors, such as SN38 (the active metabolite of the anticancer drug irinotecan) and three homocamptothecin (hCPT) derivatives: BN80765 (hCPT), its difluoro derivative BN80915 (also known as diflomotecan, currently undergoing clinical trials), and the analogue BN80927, which also interferes with topoisomerase II function, in addition to its poisoning effect on topoisomerase I. Another dual topoisomerases I and II inhibitor, the indeno-quinoline derivative TAS-103, also induced exon 9 inclusion (Fig. 2B). Finally, co-treatment of U937 cells with sub-optimal doses of camptothecin (4 μM) and VP16 (20 μM) triggered exon 9 inclusion at a higher level than the individual compounds and to a similar extent as that attained with optimal doses of either molecule (data not shown).

In contrast, we found no effect with another class of topoisomerase I poisons, the glycosyl indolocarbazoles, such as dechlorinated rebeccamycin (4 μM, 4 h and 10 μM, 4 h) and the tumor-active compound NB-506 (4 μM, 4 h and 10 μM, 4 h; (Fig. 2B, *lanes 2–5*). This may not be too surprising because these indolocarbazoles exhibit a complex, multitarget mechanism of action and their cytotoxicity is not directly dependent on topoisomerase I inhibition (12, 22). For example, NB-506 also inhibits the kinase activity of topoisomerase I (12) and binds to the neurotrophin TrkA receptor (23). Similar results were obtained with HeLa cells (data not shown).

Exon 9 inclusion in caspase-2 mRNA was always associated with a decrease in mature caspase-2L mRNA (Fig. 2C). A drop in procaspase-2L protein was also attained in response to VP16 and camptothecin, even in the presence of z-VAD-fmk, which reduced caspase-dependent proteolysis (Fig. 2D). Moreover, human CEM/C2 leukemic cells carrying two ponctual mutations of the topoisomerase I gene (Met370Thr and Asn722Ser; Ref. 24) were refractory to induction of exon 9 inclusion when exposed to topoisomerase I inhibitors (Fig. 2E). Similarly, P388-45R mouse leukemic cells with no detectable topoisomerase I activity did not support exon 9 inclusion increase on camptothecin treatment (Fig. 2F). As expected, the lack of activity of topoisomerase I had no influence on the splicing induction activity of VP16. In addition, HL60/MX2 human

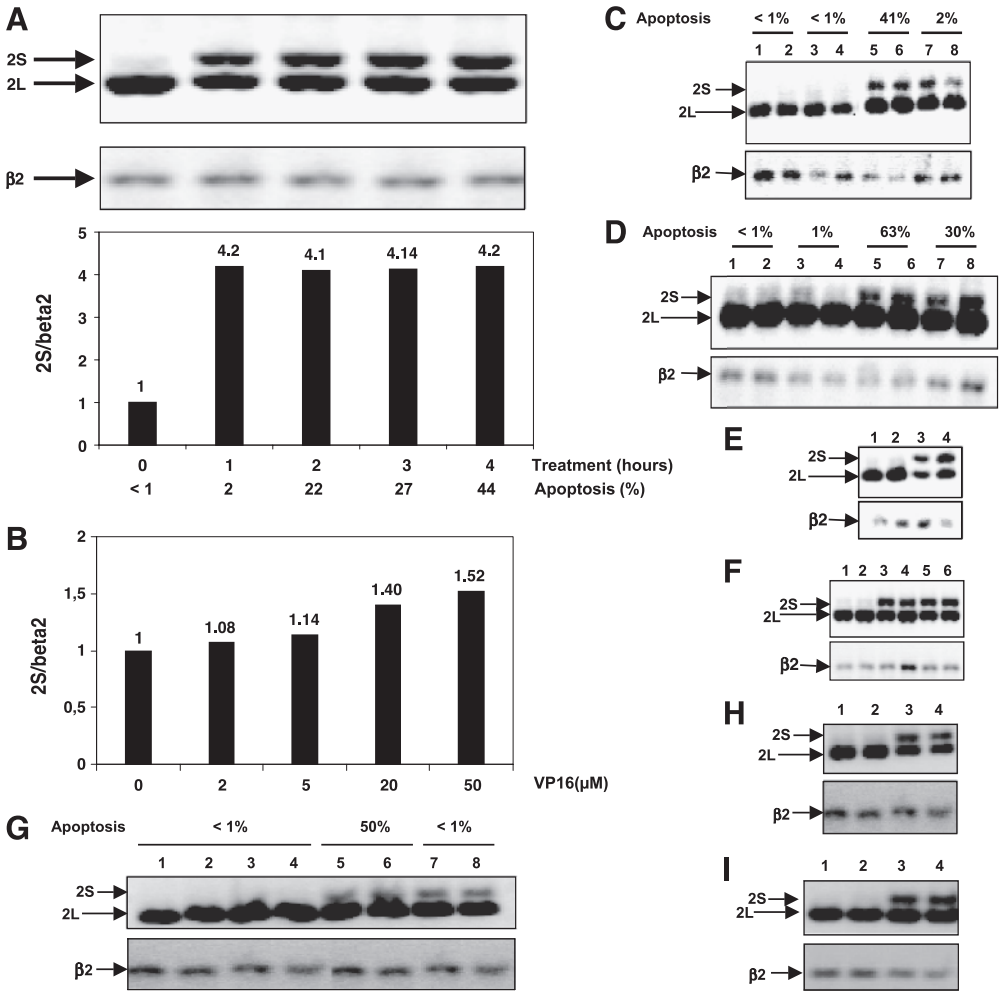

FIGURE 1. **A.** Kinetics of the effect of VP16 (50 μM) on exon 9 inclusion in U937 cells. Cells were treated as indicated, and caspase-2 mRNA was analyzed by reverse transcription-PCR (RT-PCR) using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. β2 Microglobulin (β2) mRNA was used as a standardizing control. **B.** Effect of different doses of VP16 on exon 9 inclusion in U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. β2 mRNA was used as a standardizing control. **C.** Effect of z-VAD-fmk on exon 9 inclusion and apoptosis in VP16-treated U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* z-VAD-fmk (100 μM); *lanes 5* and *6,* VP16 (50 μM); *lanes 7* and *8,* VP16 (50 μM) + z-VAD-fmk (100 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **D.** Effect of z-VDVAD-fmk on exon 9 inclusion and apoptosis in VP16-treated U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* z-VDVAD-fmk (100 μM); *lanes 5* and *6,* VP16 (50 μM); *lanes 7* and *8,* VP16 (50 μM) + z-VDVAD-fmk (100 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **E.** Effect of VP16 on exon 9 inclusion in U937-Bcl-2 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* VP16 (50 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **F.** Effect of VP16 on exon 9 inclusion in HeLa cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* VP16 (50 μM); *lanes 5* and *6,* VP16 (100 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **G.** Effect of z-VAD-fmk on exon 9 inclusion and apoptosis in camptothecin-treated U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′ICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* z-VAD-fmk (100 μM); *lanes 5* and *6,* camptothecin (10 μM); *lanes 7* and *8,* camptothecin (10 μM) + z-VAD-fmk (100 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **H.** Effect of camptothecin on exon 9 inclusion in U937-Bcl-2 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* camptothecin (4 μM, 4 h). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **I.** Effect of camptothecin on exon 9 inclusion in HeLa cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* camptothecin (10 μM, 4 h). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results.

leukemic cells carrying inactivating mutations in the *top II* gene did not show enhanced exon 9 inclusion in response to topoisomerase II inhibitors (Fig. 2G), whereas topoisomerase I inhibitors were as active as in wild-type HL60 cells.

Taken together, these results indicate that topoisomerase inhibitors play a negative role on caspase-2L mRNA assembly and suggest that both families of topoisomerases may be involved in the splicing control of caspase-2 pre-mRNA.

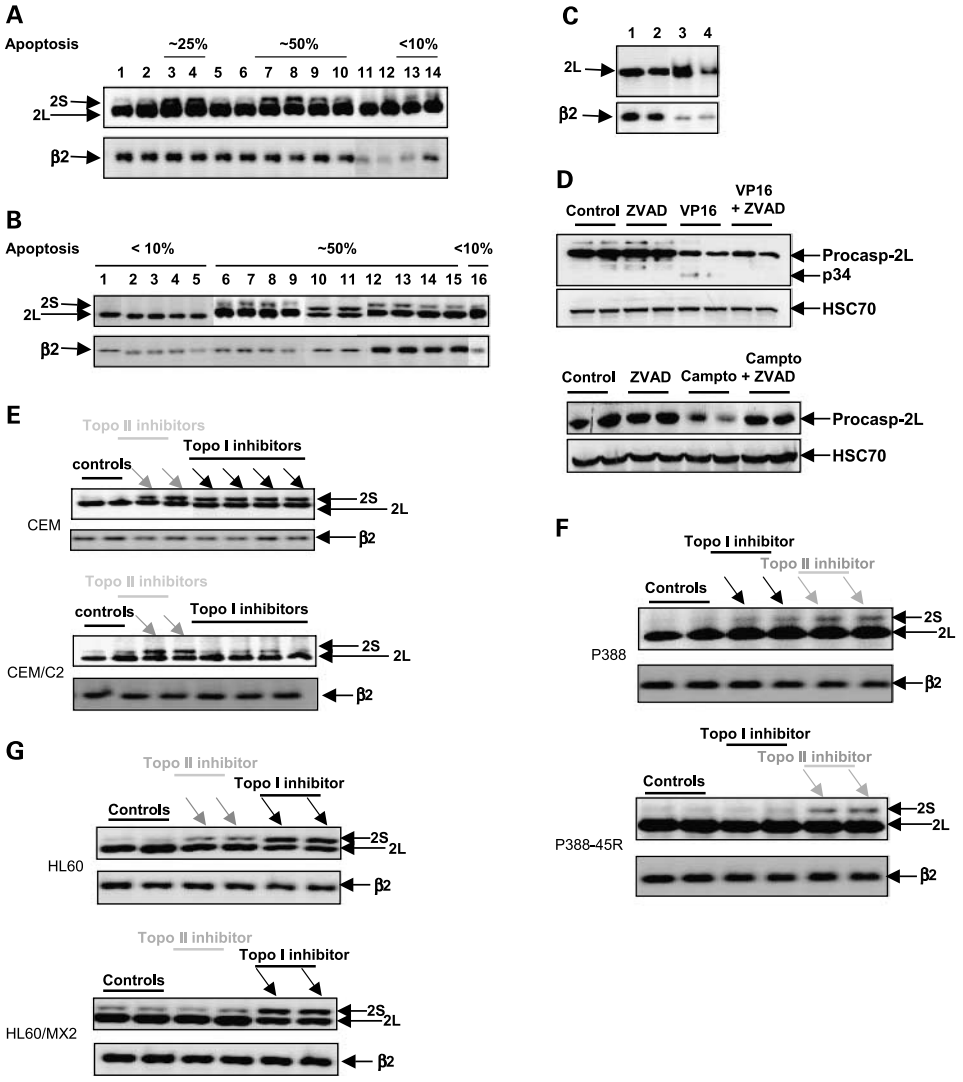

FIGURE 2. **A.** Effect of intercalating agents (topoisomerase II inhibitors) on exon 9 inclusion and apoptosis in U937 cells. Cells were treated for 4 h as indicated and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* amsacrine (5 μM); *lanes 5* and *6,* control; *lanes 7* and *8,* mitoxantrone (2 μM); *lanes 9* and *10,* mitoxantrone (5 μM); *lanes 11* and *12,* control; *lanes 13* and *14,* doxorubicin (5 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **B.** Effect of topoisomerase I inhibitors on exon 9 inclusion and apoptosis in U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lane 1,* control; *lane 2,* NB-506 (4 μM); *lane 3,* NB-506 (10 μM); *lane 4,* dechlorinated rebeccamycin (4 μM); *lane 5,* dechlorinated rebeccamycin (10 μM); *lane 6,* hCPT (4 μM); *lane 7,* hCPT (10 μM); *lane 8,* SN38 (4 μM); *lane 9,* SN38 (10 μM); *lane 10,* camptothecin (4 μM); *lane 11,* camptothecin (10 μM); *lane 12,* BN80915 (4 μM); *lane 13,* BN80915 (10 μM); *lane 14,* BN80927 (4 μM); *lane 15,* BN80927 (10 μM); *lane 16,* TAS103 (10 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **C.** Effect of topoisomerase inhibitors on mature caspase-2L mRNA in U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 1Scasp2l and 3′hICH-1 primers. Control cells received vehicle alone. *Lane 1,* control; *lane 2,* VP16 (50 μM); *lane 3,* control; *lane 4,* camptothecin (10 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **D.** Effect of topoisomerase inhibitors on procaspase-2L protein in U937 cells. Cells were treated for 4 h as indicated, and caspase-2 protein was analyzed by Western blotting. Control cells received vehicle alone. *Lanes 1* and *2,* control; *lanes 3* and *4,* z-VAD-fmk (100 μM); *lanes 5* and *6,* VP16 (50 μM) or camptothecin (10μM); *lanes 7* and *8,* VP16 (50 μM) or camptothecin (10μM) + z-VAD-fmk (100 μM). HSC70 was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **E.** Effect of topoisomerase inhibitors on exon 9 inclusion in wild-type CEM and in CEM/C2 topoisomerase I mutant derivatives. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Arrows* indicate the positive effects on alternative splicing. *Lanes 1* and *2,* controls; *lane 3,* VP16 (10 μM); *lane 4,* VP16 (50 μM); *lane 5,* hCPT (BN80765, 4 μM); *lane 6,* hCPT (10 μM); *lane 7,* SN38 (4 μM); *lane 8,* SN38 (10 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **F.** Effect of topoisomerase inhibitors on exon 9 inclusion in P388 cells and in P388-45R topoisomerase I-deficient derivatives cells. Cells were treated as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′mICH-1 and 3′mICH-1 primers. Control cells received vehicle alone. *Arrows* indicate positive effects on alternative splicing. *Lanes 1* and *2,* controls; *lanes 3* and *4,* camptothecin (10 μM, 4 h); *lanes 5* and *6,* VP16 (50 μM, 4 h). β2 mRNA was used as a standardizing control. The experiment is representative of three independent analyses with similar results. **G.** Effect of topoisomerase inhibitors on exon 9 inclusion in HL60 and in HL60/MX2 topoisomerase II mutant derivatives. Cells were treated as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Arrows* indicate positive effects on alternative splicing. *Lanes 1* and *2,* controls; *lanes 3* and *4,* VP16 (50 μM, 4 h); *lanes 5* and *6,* camptothecin (10 μM, 4 h). β2 mRNA was used as a standardizing control. The experiment is representative of three independent analyses with similar results.

Other Anticancer Agents Do Not Trigger Caspase-2 Alternative Splicing

To determine whether anticancer agents that do not target topoisomerases could also induce alternative splicing, we tested vinblastine, a mitotic spindle poison, 5-fluorouracil (5FU), an anti-metabolite, and melphalan and cisplatin, two DNA alkylating drugs. None of these compounds triggered changes in the alternative splicing of caspase-2 pre-mRNA (Fig. 3) in U937 cells, and they did not induce a strong apoptosis of the tested cell line over the 4-h treatment (data not shown). These results suggested that caspase-2 pre-mRNA splicing induction was a specific response to topoisomerase I and II inhibitors, at least the main ones, rather than a general response to cytotoxic drug poisoning. Similar results were obtained with HeLa cells (data not shown).

Cis-Acting Elements Involved in Caspase-2 Exon 9 Inclusion Induced by Topoisomerase Inhibitors

An intronic sequence termed In100 present in intron 9 of the Caspase-2 gene was previously shown to be responsible for exon 9 skipping, by its ability to behave as a decoy 3′ splice acceptor site (18). Removal of this 100-bp element was sufficient to increase exon 9 inclusion in caspase-2S mRNA (Fig. 4B, see C3 and C4), as reported previously (18). We used caspase-2 minigenes in transient transfection experiments to determine if the effects of VP16 and camptothecin could depend on the In100 sequence. Our results show that VP16 induced exon 9 inclusion, as can be seen from the increase in caspase-2S over caspase-2L ratio with all constructs, regardless of the presence of In100 (Fig. 4C). A similar observation was made with camptothecin (Fig. 4D). However, the induction of exon 9 inclusion in mRNA encoded by the minigene by either VP16 or camptothecin was rather modest, making it difficult to conclude on the role of In100. As a control, no splicing effect was induced by cisplatin in transfected HeLa cells (Fig. 4E). Taken together, these results suggest that the splicing reaction that leads to either inclusion or exclusion of caspase-2 exon 9 depends on DNA sites normally present within the region and possibly not on In100.

Discussion

This study demonstrates that most of the cytotoxic agents that target DNA topoisomerase I or II induce a strong and rapid shift toward caspase-2 exon 9 inclusion, thus reversing the balance between caspase-2L and caspase-2S mature mRNAs, at the expense of the caspase-2L isoform. Human and mouse leukemic cells that do not have topoisomerase I activity do not show exon 9 inclusion in response to camptothecin derivatives, whereas the response to VP16 is fully maintained. Conversely, cells deficient in topoisomerase II activity do not respond to VP16 but respond normally to camptothecin. These results suggest that topoisomerases I and II can act independently to mediate the observed splicing event. Remarkably, cytotoxic agents that do not target topoisomerases have no effect on the alternative splicing of caspase-2 pre-mRNA. In addition, cells resistant to apoptosis are fully responsive to the splicing triggers. Our results also demonstrate that caspase-2 pre-mRNA splicing occurs earlier than the onset of apoptosis in sensitive leukemic cells, providing a negative signal on the death pathway.

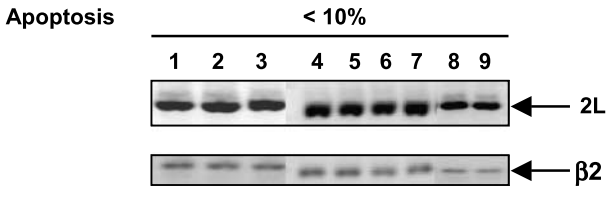

FIGURE 3. Effect of selected anticancer drugs on exon 9 inclusion in U937 cells. Cells were treated for 4 h as indicated, and caspase-2 mRNA was analyzed by RT-PCR using 5′hICH-1 and 3′hICH-1 primers. Control cells received vehicle alone. *Lane 1,* control; *lane 2,* vinblastine (0.05 μM); *lane 3,* vinblastine (1 μM); *lane 4,* control; *lane 5,* 5FU (1.5 μM); *lane 6,* control; *lane 7,* melphalan (100 μM); *lane 8,* control; *lane 9,* cisplatin (67 μM). β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results.

The ability of topoisomerase inhibitors to affect caspase-2 mRNA splicing suggests a role of topoisomerases in the control of the splicing machinery, as previously proposed (11). The mechanism by which this effect is exerted could include posttranslational modifications of proteins involved in formation of the spliceosome, including some yet uncharacterized proteins from among the 150-member set recently discovered (3). DNA topoisomerase I carries a specific kinase activity that can influence mRNA splicing on phosphorylation of SR proteins (25). A potent inhibitor of both kinase and DNA relaxing activities of topoisomerase I, NB506, has been shown to modulate the splicing of several pre-mRNAs, including Bcl-X and CD44, through inhibition of SR protein phosphorylation (12). However, in the present study, NB506 had no effect on exon 9 inclusion into caspase-2 mRNA, and dechlorinated rebeccamycin, which does not target the kinase activity of topoisomerase I (26), but still inhibits its DNA relaxing activity, had no effect either on exon 9 inclusion. These observations suggest that neither the kinase nor the DNA relaxing activity of topoisomerase I has a strong influence on caspase-2 pre-mRNA splicing induced by topoisomerases poisons. In addition, caspase-2 mRNA splicing is not uniformly affected by topoisomerase inhibitors, which could indicate that the effect of these drugs is also sequence-specific.

The control of caspase-2 exon 9 inclusion has been shown to involve several of the major pre-mRNA splicing proteins (18, 20). Using Caspase-2 minigene constructs, the ability of some of these proteins to drive either inclusion or exclusion of exon 9 was shown to depend partially on their activity toward the intron 9 In100 element, the deletion of which promotes exon 9 inclusion (18). We show here that topoisomerase inhibitor-induced exon 9 inclusion from the Caspase-2 minigene remained limited, as compared to the strong efficacy observed on the endogenous gene. Hence, the In100 sequence and the proteins that interact with this sequence to modulate splicing events may not be the target for topoisomerase inhibitors. This observation suggests that other, more remote, sequences are required or that the normal genomic context is important.

In addition to their DNA relaxation activity, topoisomerases are involved in the control of DNA replication and transcription (27). Various factors modulate these biological activities, including the subcellular localization of the enzymes

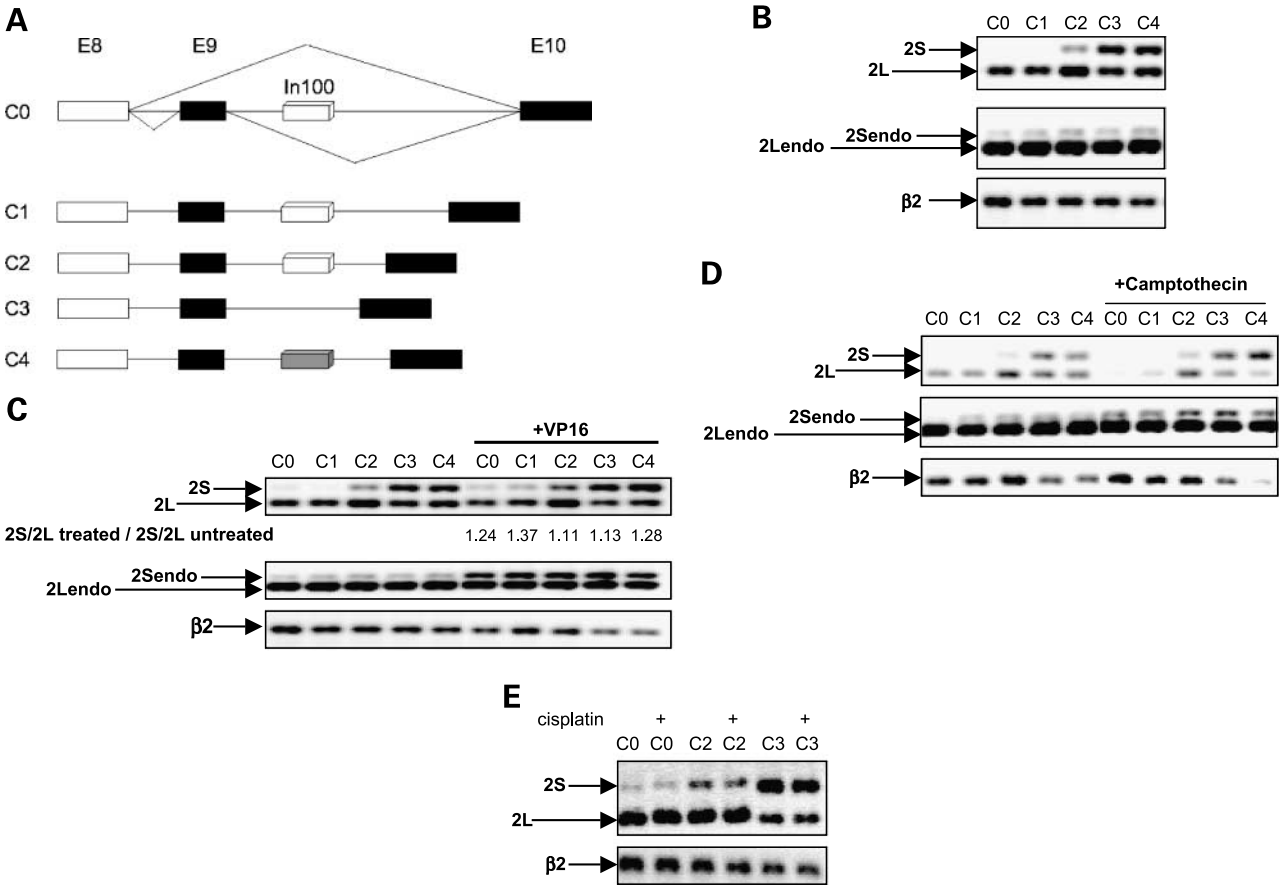

FIGURE 4. **A.** Schematic representation of mouse *CASP-2* minigenes. The constructs have been described previously by one of the authors (J.W.;18). **B.** Exon 9 inclusion following transfection of *CASP-2* minigene constructs in HeLa cells. Cells were transiently transfected with *CASP-2* minigenes (see **A**) and caspase-2 mRNA was analyzed by RT-PCR. Primers used for minigene analysis were 5′mICH-1 and 3′mICH-1. Primers used for the endogenous gene were 5′hICH-1 and 3′hICH-1. Control cells received vehicle alone. β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **C.** Effect of VP16 (50 μM, 4 h) on exon 9 inclusion from *CASP-2* minigene constructs transfected in HeLa cells. Cells were transiently transfected with *CASP-2* minigenes (see **A**) and treated or not with VP16, and caspase-2 mRNA was analyzed by RT-PCR. Primers used for minigene analysis were 5′mICH-1 and 3′mICH-1. Primers used for the endogenous gene were 5′hICH-1 and 3′hICH-1. Control cells received vehicle alone. β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **D.** Effect of camptothecin (10 μM, 4 h) on exon 9 inclusion from *CASP-2* minigene constructs transfected in HeLa cells. Cells were transiently transfected with *CASP-2* minigenes (see **A**) and treated or not with camptothecin, and caspase-2 mRNA was analyzed by RT-PCR. Primers used for minigene analysis were 5′mICH-1 and 3′mICH-1. Primers used for the endogenous gene were 5′hICH-1 and 3′hICH-1. Control cells received vehicle alone. β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results. **E.** Effect of cisplatin (67 μM, 4 h) on exon 9 inclusion from *CASP-2* minigene constructs transfected in HeLa cells. Cells were transiently transfected with *CASP-2* minigenes (see **A**) and treated with cisplatin, and caspase-2 mRNA was analyzed by RT-PCR. Primers used for minigene analysis were 5′mICH-1 and 3′mICH-1. Primers used for the endogenous gene were 5′hICH-1 and 3′hICH-1. Control cells received vehicle alone. β2 mRNA was used as a standardizing control. The experiment is representative of more than three independent analyses with similar results.

(28, 29), the p53 status of the cells (30) and, concerning topoisomerase I, interaction with RNA splicing proteins (31). In addition, topoisomerases can relay subcellular transduction signals by virtue of their ability to become phosphorylated following direct interaction with specific kinases. For example, it has been shown recently that the atypical PKCζ isoform was able to interact with topoisomerase IIβ, leading to its phosphorylation and to a decrease in its catalytic activity (32). By which mechanism topoisomerase I or II poisoning elicits the observed effects on caspase-2 mRNA alternative splicing requires further investigation.

One of the biological consequences of caspase-2 mRNA switch is a decrease in the overall amount of the procaspase-2L protein. In view of the role of caspase-2L in apoptosis induction in response to various cytotoxic agents, including those that trigger exon 9 inclusion, this effect could limit further apoptosis. Therefore, although the primary cell response to these agents is to undergo apoptosis, the result might be partially mitigated by a "safety" mechanism that would limit cell damage. The ability of cytotoxic drugs to simultaneously activate signals that contribute to cell death and others that antagonize the death pathways has now been largely demonstrated (33). For example, many anticancer agents activate lipid second messengers that include ceramide, a pro-apoptotic molecule, and diacylglycerol, which prevents cell death (34). Another example includes the simultaneous activation of caspases and the

transcription factor NF-κB that stimulates expression of caspase inhibitors (35). The balance between caspase-2 isoforms, if stabilized at the expense of caspase-2L, could decrease cell sensitivity to pro-apoptotic drugs that require caspase-2 activity to exert their cytotoxic effects, and this alternative splicing could account for some antagonistic effects between topoisomerase poisons and other cytotoxic drugs (36, 37).

Determination of exon 9 inclusion could also be proposed as a simple assay to evaluate the ability of a cytotoxic agent with an unknown mechanism of action to target one or the other of topoisomerase I and II classes. Lastly, because increased inclusion of caspase-2 exon 9 may limit the pro-apoptotic activity of topoisomerase poisons, molecules that specifically prevent this effect would theoretically sensitize tumor cells to topoisomerase inhibitors.

Materials and Methods

Cell Lines and Culture

The human leukemic U937, U937-Bcl-2, CEM, CEM/C2, HL60, and HL60/MX2 cell lines were obtained from the American Type Culture Collection (Rockville, MD), and were maintained in RPMI 1640 (RPMI, BioWhittaker, Fontenay-sous-bois, France) supplemented with 2 mM L-glutamine (Invitrogen, Cergy-Pontoise, France) and 10% (v/v) FCS (BioWhittaker). The CEM/C2 and HL60/MX2 cell lines carry inactivating mutations in the topoisomerase I and II genes, respectively. The U937-Bcl-2 cell line was obtained in our laboratory following stable transfection of Bcl-2 cDNA. The mouse P388 leukemic cell line was used to isolate a clonal population of camptothecin-resistant cells, which gave rise to the P388-45R cell line that does not display any detectable topoisomerase I activity (38). Both murine cell lines were maintained in RPMI 1640 supplemented with 10% (v/v) decomplemented FCS and with 0.01 mM β-mercaptoethanol (Carlo Erba, Val de Reuil, France). The HeLa cell line was obtained from the ECACC (Salisbury, United Kingdom) and was maintained in EMEM (BioWhittaker) supplemented with 10% (v/v) FCS and 2 mM L-glutamine. The cells were cultivated in the presence of 95% air and 5% CO_2. A mixed trypsin/EDTA solution in Hanks' balanced salt solution (BioWhittaker) was used to harvest adherent cells. Absence of *Mycoplasma* was determined with the "mycotech" kit (Invitrogen).

Chemicals

To ensure exponential growth, cells were resuspended in fresh medium 24 h before each treatment performed at the cell density of 10^6/ml. The characteristics of the drugs used in this study are indicated in Table 1. Actinomycin D, amsacrine, camptothecin, cisplatin, doxorubicin, VP16, 5FU, melphalan, and vinblastine were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The hCPT derivatives BN80765, BN80915, and BN80927 were kindly provided to one of us (C.B.) by Dr. D.C.H. Bigg (Institut Henri Beaufour, Les Ulis, France). Similarly, the glycosyl indolocarbazole derivatives dechlorinated rebeccamycin and NB506 were provided (to C.B.) by Dr. M. Prudhomme (Université Blaise Pascal, SESIB, UMR 6504, Aubière, France) and Dr. H. Arakawa (Banyu Pharmaceuticals Co., Ltd., Tokyo, Japan). A sample of the indeno-quinoline derivative TAS-103 was provided (to C.B.) by Dr. N. Osheroff (Vanderbilt University, Nashville, TN). Mitoxantrone was from Laboratoires Léderlé (France). The caspase inhibitors z-VAD-fmk and z-VDVAD-fmk were obtained from R&D Systems (Minneapolis, MN). For each compound, a stock solution (generally 5 or 10 mM) was prepared in DMSO (except particular cases for compounds soluble in water or drugs requiring special handling such as cisplatin, dissolved in NaCl) and subsequent dilutions were made in the same solvent. In all experiments, the final concentration of DMSO did not exceed 0.5% (v/v), a concentration which was not toxic to the cells.

Cell Death Analysis

Plasma membrane integrity was studied by trypan blue exclusion. Apoptosis was identified by staining the cells with Hoechst 33342 (Sigma-Aldrich). Briefly, 100,000 cells were collected by centrifugation at 500 × *g* for 5 min and

Table 1. Drugs Used and Their Known Molecular Mechanism of Action (MMOA)

Drugs	MMOA	Effects on Topoisomerases	References
Camptothecin		Topo I poison	(39)
SN38		Topo I poison	(40)
hCPTs			(41)
BN80765 (hCPT)		Topo I poison	
BN80915 (diflomotecan)		Topo I poison	
BN80927		Topo I poison and Topo II catalytic inhibitor	
Indolocarbazoles	DNA intercalators	Topo I poison	(26)
Dechlorinated rebeccamycin			(42)
NB506			
Amsacrine	DNA intercalator	Topo II poison	(43)
Doxorubicin	DNA intercalator	Topo II poison	(44)
Mitoxantrone	DNA intercalator	Topo II poison	(45)
TAS-103	DNA intercalator	Topo I + II poison	(46)
VP16		Topo II poison	(47)
5FU	Antimetabolite		(48)
Cisplatin	DNA alkylator		(49)
Melphalan	DNA alkylator		(50)
Vinblastine	Mitotic spindle poison		(51)

Table 2. Primer Sequences

Name	Sequence (5′→3′)	T_m (°C)	PCR Cycle Number
5′hβ2	TCTTTTTCAGTGGGGGTGAA	58	32
3′hβ2	CTCACGTCATCCAGCAGAGA	62	32
5′hICH-1 (exon 8)	AACTGCCCAAGCCTACAGAA	60	32
3′hICH-1 (exon 10)	GCGTGGTTCTTTCCATCTTGTTGGTCA	65	32
1Scasp2l (exon 1)	ATGGCCGCTGACAGGGGACGC	67	29
3′mβ2	CAGTCTCAGTGGGGGTGAAT	62	32
5′mβ2	ATGGGAAGCCGAACATACTG	60	32
5′mICH-1 (exon 8)	ATGCTAACTGTCCAAGTCTACAGAAC	52	32
3′mICH-1 (exon 10)	GTCTCATCTTCATCAACTCCTCTTTGCC	61	32

Note: 5′hICH-1, 3′hICH-1, 1Scasp2l, 5′hβ2, and 3′hβ2 are human sequences. 5′mICH-1, 3′mICH-1, 5′mβ2, and 3′mβ2 are mouse sequences.

resuspended in 500 μl Hoechst-containing PBS (final concentration 10 μg/ml). After a 30-min incubation in the dark at 37°C, cells were centrifuged at 500 × *g* for 10 min, suspended in 20 μl PBS, and analyzed using a fluorescence microscope.

RT-PCR

Cells were washed in PBS. The RNA extraction was performed with the "Nucleospin RNA II" kit (Macherey-Nagel, Hoerd, France). The "OneStep RT-PCR" kit (QIAGEN, Courtaboeuf, France) was used under the following conditions: 1× buffer, 400 μM of each dNTP, 0.6 μM 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 min at 50°C, the initial PCR step was activated by heating for 15 min at 95°C before PCR (1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C, 29 or 32 cycles) using a GeneAmp PCR System 2400 (Perkin-Elmer Instruments, Les Ulis, France). 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 Photomat software (Microvision Instruments, Evry, France). Primer sequences and use are listed in Table 2.

Western Blotting

Cells were washed twice in PBS and lysed in boiling buffer (1% SDS, 1 mM Na_3VO_4, 10 mM Tris pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 10 μg/ml aprotinin (Sigma-Aldrich), 2.5 μg/ml pepstatin (Sigma-Aldrich), 2.5 μg/ml trypsin inhibitor (Sigma-Aldrich), and 5 μg/ml leupeptin (Sigma-Aldrich) for 10 min at 4°C. Equal amounts of proteins were boiled in Laemli buffer for 5 min, separated by SDS-PAGE using 12% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Ivry-sur-Seine, France). The membranes were incubated overnight at 4°C with an anti-human caspase-2 monoclonal antibody (PharMingen, Pont de Claye, France) or with an anti-human Heat Shock Constitutive 70 kDa Protein (HSC70; Santa Cruz/Tebu, Le Perrey en Yvelines, France) as a loading control. The blots were revealed using an Enhanced Chemiluminescence detection kit (Amersham, Orsay, France) by autoradiography.

Transient Transfections

HeLa cells were seeded in six-well plates at a density of 10^6 cells/well 16 h before transfection with the "Superfect Reagent" kit (QIAGEN). Cells were cotransfected with pRSV-β-galactosidase reporter vector (20 ng/10^5 cells) as a control for transfection efficiency. Drugs were added to the cells 24 h after transfection, and the cells were harvested 4 h later.

Plasmid Vectors

The constructs used in transfection experiments (Fig. 4A) have been reported previously (18). C0 corresponds to the wild-type sequence of the mouse *CASP-2* minigene (encompassing exon 8 to exon 10). C1, C2, C3 contain internal deletions of intron 9. C4 contains a 100-bp spacer of foreign sequence to replace the In100 sequence.

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