Specific Inhibition of Serine- and Arginine-rich Splicing Factors Phosphorylation, Spliceosome Assembly, and Splicing by the Antitumor Drug NB-506

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ABSTRACT

Specific phosphorylation of serine- and arginine-rich pre-mRNA splicing factors (SR proteins) is one of the key determinants regulating splicing events. Several kinases involved in SR protein phosphorylation have been identified and characterized, among which human DNA topoisomerase I is known to have DNA-relaxing activity. In this study, we have investigated the mechanism of splicing inhibition by a glycosylated indolocarbazole derivative (NB-506), a potent inhibitor of both kinase and relaxing activities of topoisomerase I. NB-506 completely inhibits the capacity of topoisomerase I to phosphorylate, in vitro, the human splicing factor 2/alternative splicing factor (SF2/ASF). This inhibition is specific, because NB-506 does not demonstrate activity against other kinases known to phosphorylate SF2/ASF such as SR protein kinase 1 and cdc2 kinase. Importantly, HeLa nuclear extracts competent in splicing but not splicing-deficient cytoplasmic Si100 extracts treated with the drug fail to phosphorylate SF2/ASF and to support splicing of pre-mRNA substrates containing SF2/ASF-target sequences. Native gel analysis of splicing complexes revealed that the drug affects the formation of the spliceosome, a dynamic ribonucleoprotein structure where splicing takes place. In the presence of the drug, neither pre-spliceosome nor spliceosome is formed, demonstrating that splicing inhibition occurs at early steps of spliceosome assembly. Splicing inhibition can be relieved by adding phosphorylated SF2/ASF, showing that extracts treated with NB-506 lack a phosphorylating activity required for splicing. Moreover, NB-506 has a cytotoxic effect on murine P388 leukemia cells but not on P388CPT5 camptothecin-resistant cells that carry two point mutations in conserved regions of topoisomerase I gene (Gly361Val and Asp709Tyr). After drug treatment, P388 cells accumulated hypophosphorylated forms of SR proteins and polyadenylated RNA in the nucleus. In contrast, neither SR protein phosphorylation nor polyadenylated mRNA distribution was affected in P388 CPT5-treated cells. Consistently, NB506 treatment altered the mRNA levels and/or splicing pattern of several tested genes (Bcl-X, CD 44, SC35, and Sp70) in P388 cells but not in P388 CPT5 cells. The study shows for the first time that indolocarbazole drugs targeting topoisomerase I can affect gene expression by modulating pre-mRNA splicing through inhibition of SR proteins phosphorylation.

INTRODUCTION

Pre-mRNA splicing is an essential step in the expression of most metazoan protein-coding genes that can lead from a single array of coding sequences (exons) to the production of different proteins or, alternatively, to a lack of protein expression by introduction of a premature stop codon (1). Determining how these sometimes subtle changes in mRNA sequences affect protein function is a crucial question in many aspects of developmental and cell biology, including control of apoptosis (2) and tumor progression (3).

The accurate excision of intervening sequences (introns) from pre-mRNAs by two transestification reactions occurs in a large ribonucleoprotein complex called spliceosome (4). The spliceosome formation results from a dynamic series of interactions between the four major snRNPs4 (U1, U2, U4/U6, and U5), non-snRNPs proteins, and the pre-mRNA (4). Several metazoan splicing factors involved in splice site selection at earliest steps of the spliceosome assembly pathway are characterized by RNA recognition motif and RS domains that are essential for their function (1, 5). These include members of SR protein family (SRp20, SF2/ASF or SRp30, SC35 or SRp30b, 9G8, SRp30c, SRp40, SRp55, and SRp75; Refs. 5, 6), U1 snRNP-specific protein U1-70K, and the splice factor U2AF, which comprises two subunits (35K and 65K; Ref. 7). All of these factors can mediate a network of protein-protein interactions through their RS domains, resulting in the stimulation and/or stabilization of complexes assembled at the 5′ or 3′ splice sites (7, 8). These interactions can directly be affected by phosphorylation of the serines at the RS domains (9–14).

Although regulation of SR protein phosphorylation and the functional consequences associated with it are just beginning to be understood, it is established that SR proteins can be phosphorylated by several kinases (15). We have established recently that DNA topoisomerase I, a constitutively expressed nuclear phospho-protein that localizes to active transcription sites and belongs to a super family of DNA topo-isomerases and tyrosine recombinases (16, 17), exhibits a SR protein-phosphorylating activity in addition to its DNA-relaxing activity (18). Although topoisomerase I lacks sequence motifs homologous to known protein kinases (e.g., ATP-binding site), it efficiently binds ATP (18, 19). Photoaffinity labeling combined with mutational analysis showed that the COOH terminal is required for ATP binding (19), whereas the NH2-terminal 174 amino acids are essential for the interaction with the RS domain of SF2/ASF protein in vitro (20).

DNA topoisomerase I is also the nuclear target for a number of anticancer agents derived from the plant alkaloid CPT and for indolocarbazole derivatives (16) such as the antibiotic rebeccamycin, the antitumor agent NB-506, which is undergoing clinical trials (21), and its regio-isomer J-109,382 (22). Although NB-506 bears a structural analogy with the specific PKC inhibitor staurosporine, it has no significant effect on PKC (23) but inhibits both relaxation and kinase activities of topoisomerase I (shown here). In this study, we show that NB-506 has the capacity to block, in vitro, spliceosome assembly and splicing through inhibition of SR protein phosphorylation. NB-506 leads also to specific inhibition of SR protein phosphorylation in

4 The abbreviations used are: snRNP, small nuclear ribonucleoprotein; RS, arginine-serine rich; SF2/ASF, splicing factor 2/alternative splicing factor; CPT, camptothecin; PKC, protein kinase C; RT-PCR, reverse transcription-PCR; SRPK1, SR protein kinase 1; NE, nuclear extract; oligo(dT), oligodeoxynucleotidylic acid.

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8676

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cultured cells and modulates gene expression by changing the splicing pattern of protein-encoding genes. To our knowledge, NB-506 is the first antitumor drug shown to interfere with the spliceosome assembly pathway.

MATERIALS AND METHODS

Drugs. NB-506 and J-109,382 were kindly provided by Dr. Tomoko Yoshinari (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan). Their chemical synthesis has been reported (21, 24). Rebeccamycin, purified as described (23), was provided by Dr. Michelle Prudhomme (SEESIB, Aubière, France). Drugs were dissolved in DMSO at 10 mg/ml. Fresh dilutions (in 10% DMSO) were immediately used before use. The final DMSO concentration never exceeded 0.5% (v/v), a concentration at which DMSO (also present in the controls) does not affect the spliceosome 1 activity.

Cell Cultures, Drug Treatment, and mRNA Visualization. P388 murine leukemia cell line was obtained from the tumor bank of the National Cancer Institute (Bethesda, MD). P388CPT5 cell line resistant to CPT was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage (25). Both cell lines were grown in RPMI 1640 medium containing 0.1% 2-mercaptoethanol, 10 mm l-glutamine, 10% (v/v) FCS, 100 IU/ml penicillin, 2 µg/ml streptomycin, 50 µg/ml gentamicin, and 50 µg/ml nystatin at 37°C in a humidified atmosphere containing 5% CO2.

To localize polyadenylated mRNA, exponentially growing cells, either untreated or treated for 24 h with 1 µM NB-506, were harvested by centrifugation at 600 rpm/min on coverslips coated with gelatin. The cells were fixed and hybridized to CY3 (Amersham, Arlington Heights, IL) fluorophore-labeled probes. The cells were fixed at 600 rpm/min on coverslips coated with gelatin. The cells were fixed and hybridized to CY3 (Amersham, Arlington Heights, IL) fluorophore-labeled probes. The cells were fixed and hybridized to CY3 (Amersham, Arlington Heights, IL) fluorophore-labeled probes.

Labeling, Purification, and Two-dimensional Gel Analysis of SR Proteins. To achieve in vivo efficient labeling of SR proteins, P388 and P388CPT5 cells were seeded at 5 x 105 cells/ml in medium supplemented with 5% (v/v) FCS and exposed to 2.5 mCi/liter carrier-free [H3]32PO4/H9262 [except that 0.5 M MATERIALS AND METHODS pathway. The first antitumor drug shown to interfere with the spliceosome assembly pathway.]

Splicing and Spliceosome Assembly Assays. The single-intron human β-globin construct pSPHB-3S was described (28). Radiolabeled β-3S RNA was synthesized by in vitro transcription in the presence of 20 units of SP6 RNA polymerase (Boehringer), 1 µg of pSPHB-3S plasmid linearized with BamHI, and 5 µM [α-32P]UTP (800 Ci/mmole) in 25-µl reactions according to the manufacturer’s conditions. The splicing reactions were done under standard conditions for 1 h in a total volume of 20 µl containing 50 fmol labeled pre-mRNA and 6, 8, or 10 µl of HeLa NE complemented with buffer D (27). Splicing products were analyzed by electrophoresis on denaturing 6% polyacrylamide gels and revealed by autoradiography.

Kinetics of phosphorylation in HeLa NEs or S100 extracts were performed as described (29). Aliquots (5 µl) from the various reactions treated with heparin (2 mg/ml) were mixed with 1 µl of 97% glycerol, 1% bromophenol blue and resolved directly on a 4% nondenaturing polyacrylamide gel (acrylamide:bis-acrylamide weight ratio of 80:1) in 50 mM tri-glycine (pH 8.3).

Determination of Splicing Patterns Using RT-PCR Assay. Cytoplasmic mRNAs were purified from P388 and CPT5 cells before and after a 24-h treatment with 1.0 µM NB-506. First-strand cDNA was synthesized from 5 µg of DNase-treated RNA with the Amersham cDNA synthesis kit. For analysis of SC35, CIK/Str, CD44, and Bcl-X alternatively spliced mRNA isoforms, one tenth of the reaction was amplified with Gold Taq polymerase (Hybaid) with the following forward and reverse primers (name, sequence is given 5’ → 3’):

SC35 (forward), CAAGTCTCCAGAAGAAGAGG; SC35 (reverse), GCATCTCAACTGTCACAC; CIK/Str (forward), GCCAGGAGAGAGCATGCATCGTGTG; and CIK/Str (reverse), TCTCTGCCACGCTCTCTCC.

The PCR cycle number was kept to a minimum to maintain linearity. After denaturation for 2 min at 94°C, the PCR regimen were 1 min at 94°C, 1 min at 56°C (SC35 and CIK/Str), 58°C (CD44), or 54°C (Bcl-X), and 1 min (SC35, CIK/Str, and Bcl-X) or 1 min 30 s (CD44) at 72°C for 25 cycles, followed by a 10-min extension at 72°C in a Hybaid PCR Sprint machine. PCR products were separated on 1.5% (SC35, CIK/Str, and Bcl-X) or 1.0% (CD44) agarose gels containing ethidium bromide were visualized under UV light.

RESULTS AND DISCUSSION

Specific Inhibition of The Kinase Activity of Topoisomerase I by NB-506. We have shown previously (18) that recombinant human DNA topoisomerase I, overexpressed in insect cells using the baculovirus system, has the same characteristics as the native HeLa enzyme. It binds ATP efficiently and specifically phosphorylates in vitro SR protein-splicing factors at short RS domain regions (19, 20). To investigate the inhibition of kinase activity by the indolocarbazole derivative NB-506, the purified recombinant topoisomerase I was used in kinase assays containing increasing concentrations of drug, and kinase activity was detected by its ability to phosphorylate bacterially expressed recombinant SF2/ASF with [γ-32P]ATP. Analysis of the phosphorylated products by SDS-PAGE and detection by autoradiography (Fig. 1) revealed that, under conditions where the concentration of [γ-32P]ATP was constant, the phosphorylation of SF2/ASF is markedly inhibited in the presence of NB-506 (Fig. 1, 265).
in vitro and cdc2 kinase (Fig. 1, panel SRPK1), and p34 cdc2 kinase (panel Cdc2) is as described in “Materials and Methods” with increasing concentration of the drug: 1 μM, 10 μM, and 50 μM (Lanes 1–3, 4–6, or 7–9, respectively). Ctr, the control reaction with 10% DMSO alone instead of NB-506. The labeled SF2/ASF was analyzed on a 12% SDS-polyacrylamide gel and revealed by autoradiography. The position of SF2/ASF was localized by staining the gel with Coomassie Blue.

panel TOPO I). The kinase activity of topoisomerase I is reduced by 40% in the presence of 1 μM drug concentration (Fig. 1, panel TOPO I, Lane 3), whereas a drug concentration of 50 μM is required to block the phosphorylation of SF2/ASF to more than 90% (Lane 5). IC50 for inhibition of SF2/ASF phosphorylation by NB-506 was found equal to 1.5 μM. The specificity of the NB-506 inhibitory effect was further confirmed by using two other kinases, SRPK1 (Fig. 1, panel SRPK1) and cdc2 kinase (Fig. 1, panel Cdc2 kinase), which have been shown previously (20) to phosphorylate in vitro the RS domain of SF2/ASF. No inhibition of the phosphorylation of SF2/ASF by either kinase was observed with NB-506, even when concentrations as high as 50 μM were used (Fig. 1, both panels, Lane 5). Given that NB-506 is also inactive toward PKC (23) and that indolocarbazole compounds like staurosporine usually interact with the ATP-binding site, it can be concluded that inhibition of the kinase activity of topoisomerase I does not involve the binding of NB-506 to its ATP-binding site. Consistent with this conclusion, staurosporine does not inhibit the kinase activity of topoisomerase I, and binding studies performed with fluorescently labeled ATP revealed that NB-506 does not compete with the binding of ATP (data not shown).

NB-506 Prevents SF2/ASF Complete Phosphorylation from HeLa NE But Not from S100 Extract. To address whether NB-506-mediated kinase inhibition affects pre-mRNA splicing, we determined whether inhibition of SF2/ASF phosphorylation can be reproduced using bacterially expressed SF2/ASF and HeLa NE or cytoplasmic S100 extracts as sources of protein kinases. The recombinant SF2/ASF was designed to contain a NH2-terminal His-tag to facilitate purification over an immobilized metal ion affinity column (His-bind resin) and detection with an anti-his tag antibody. A time course analysis of SF2/ASF phosphorylation was performed under splicing conditions in extracts from HeLa cells, and SF2/ASF was revealed by Western blots with the anti-his tag antibody (Fig. 2). In this assay, cold ATP and creatine phosphate to regenerate ATP are included, and changes in the phosphorylation status are qualitatively reflected by changes in electrophoretic mobility. After 120 min of incubation in NE (Fig. 2A, Lane 7) or S100 (Fig. 2B, Lane 7), the majority of SF2/ASF demonstrated a lower mobility (P-SF2/ASF) compared with unphosphorylated SF2/ASF (Fig. 2, A and B; compare Lane 1 and Lane 7), implying that kinases from either extracts can phosphorylate SF2/ASF on multiple sites. The difference in the observed mobility was entirely attributable to phosphorylation of SF2/ASF, as judged by phosphatase treatment of samples from 120 min of incubation (data not shown). Treatment of S100 extracts with concentrations of NB-506 as high as 100 μM did not affect the mobility shift of SF2/ASF at any time points (Fig. 2B, Lanes 8–17) showing that kinases contained in S100 extracts were refractory to inhibition by the drug. In sharp contrast, the drug caused an accumulation of both unphosphorylated and partially phosphorylated forms when NB-506 (50 μM and 100 μM) was added to NE (Fig. 2A, Lanes 8–17). Inhibitory concentrations of NE phosphorylation also correlate with those measured with the kinase activity of purified topoisomerase I. However, phosphorylation of SF2/ASF in NE was not affected by 100 μM of CPT (Fig. 2A, Lanes 19–22), which does not directly inhibit the kinase activity of topoisomerase I. Thus, the data indicate that NB-506-mediated inhibition is selective for a kinase activity of NEs, which could correspond to that of topoisomerase I, a major nuclear phospho-protein.

NB-506 Inhibits Splicing. Accurate phosphorylation and dephosphorylation within the RS domain of SR proteins is important for their activities in splicing (9–15). Given that both hyperphosphorylation and hypophosphorylation of SR proteins inhibit splicing (13) and that NB-506 prevents SF2/ASF complete phosphorylation in NE, we were particularly interested to know whether the drug would affect the splicing activity of NE. To assess the effect of NB-506 on pre-mRNA splicing, we used a model β-globin pre-mRNA substrate (β-3S1) that has three copies of a high-affinity binding site for SF2/ASF established by systematic evolution of ligands by exponential enrichment (SELEX) analysis (28), inserted 20 bases downstream of the first β-globin intron. Because of these sequences, the β-3S1 substrate was efficiently spliced with low levels of HeLa NE (Fig. 3A, Lanes 1 and 9). When this preparation of HeLa NE was supplemented with increasing amounts of NB-506 (25–100 μM), splicing was readily inhibited in a dose-dependent manner (Fig. 3A, Lanes 2–4). Consistently, the inhibitory effect of the drug was reduced when higher concentrations of NE were used (10 μl of NE; Fig. 3A, Lanes 6–8).

Fig. 2. Kinetics of phosphorylation of recombinant SF2/ASF expressed and purified from E. coli (eSF2/ASF) in either NEs (A) or S100 cytoplasmic extracts (B) from HeLa cells. Recombinant protein was incubated under splicing conditions in the indicated extracts in the absence (Lanes 1–3) or presence of either 50 μM (Lanes 8–12) and 100 μM (Lanes 13–17) of NB-506 or 100 μM of CPT (Lanes 18–22). Aliquots of these reactions terminated at the indicated times were fractionated by SDS-PAGE and analyzed by Western blotting with an anti-His tag antibody. Lane 1, recombinant proteins without added extracts. Lane 2, extracts without added recombinant proteins.
No inhibition was observed with either the antibiotic rebeccamycin, a naturally occurring indolocarbazole structurally similar to NB-506 and the regio-isomer J-109,382, or CPT, which does not inhibit phosphorylation of SF2/ASF by topoisomerase I (Fig. 3A, Lanes 9–16 and 17–24, respectively). However, splicing was also inhibited by J-109,382 (22), which was as efficient as NB-506 at inhibiting the kinase activity of topoisomerase I (data not shown), demonstrating a requirement for a specific structure of the indolocarbazole for the inhibition of splicing. Furthermore, J-109,382 did not bind DNA (data not shown) and interacts only weakly with DNA \textit{in vitro} (22), indicating that splicing inhibition is not attributable to mere nonspecific binding to pre-mRNA.

Interestingly, purified recombinant SF2/ASF expressed in a baculovirus system (bSF2/ASF), where the phosphorylation of recombinant proteins is expected to take place, but not unphosphorylated version expressed in bacteria (eSF2/ASF), restored full splicing activity of NB-506-treated HeLa NEs (Fig. 3B; compare Lanes 5 and 9 with Lanes 7 and 10). The amount of purified bSF2/ASF added was of the same order of magnitude of that contained in our preparation of HeLa NE, as judged from Western blot analysis performed with an antibody specific for SF2/ASF (data not shown). A similar quantity of unphosphorylated eSF2/ASF was rather detrimental for splicing (Fig. 3B, Lanes 2 and 5). Thus, the data support the notion that NB-506 mediates splicing inhibition by preventing complete phosphorylation of the SF2/ASF SR protein.

**NB-506 Inhibits Spliceosome Assembly.** SR proteins are known to promote the earliest stages of the spliceosome assembly (5, 7). Therefore, we tested whether NB-506 could interfere with the spliceosome formation. \(^{32}\)P-labeled \(\beta\)-3S1 was incubated with HeLa NE treated with various concentrations of NB-506, and assembled ribonucleoprotein complexes were analyzed by native gel electrophoresis (29). As shown in Fig. 4A, the untreated control extracts show the characteristic pattern of spliceosome assembly pathway. Two heparin-resistant complexes corresponding to complex A and complex B are formed in an ATP-dependent fashion at early time points, in addition to a fast migrating nonspecific complex H (Fig. 4A, Lane 3). The complex A formation involves the stable binding of U2 snRNP to the pre-mRNA sequences near to the 3' splice site (pre-spliceosome). Subsequent binding of a tri-snRNP particle U4/U5/U6 rather than that of the individual snRNPs gives rise to the B complex (spliceosome). Therefore, at later time points (10–30 min), the level of complex A gradually decreased with parallel increase in the amount of complex B (Fig. 4A, Lanes 4–6). A conformational change probably involving disruption of base-paired U4 and U6 snRNAs appears to be a critical step in the initiation of splicing reactions by the assembled B complex (4, 15). Thus, upon longer incubation (60 min), the B form was converted to a faster migrating complex containing the products of the splicing reaction (complex X; Fig. 4A, Lane 6). In sharp contrast, neither complex A nor complex B was formed after 5-min incubation in extracts treated with NB-506 concentrations varying from 25 to 100 \(\mu\text{M}\) (Fig. 4A, Lanes 8, 14, and 19). At low concentrations of the drug, only a low level of complex B is still formed after 30-min incubation (Lane 10), whereas at higher concentrations, the formation of both A and B complexes was completely abolished at any time points (Fig. 4A, Lanes 14–17 and Lanes 19–22). The complexes assembled in the presence of the drug were similar to the complexes formed in the absence of ATP (compare Lanes 2, 7, 13 and 18 with Lanes 14–17 and 19–22), indicating that the inhibitory effect does not reflect a reduction in the kinetics of spliceosome assembly. Thus, NB-506 inhibits an early step in the spliceosome assembly pathway. If correct, this conclusion predicts that the drug will not have any effect in splicing if the spliceosome is allowed to form. Results shown in Fig. 4B are consistent with this prediction. When NB-506 was added to the splicing assay after a 15-min (Lanes 8, 11, and 14) or 30-min (Lanes 9, 12, and 15) preincubation, splicing products were still observed like in the control reaction (Lane 4). Parallel analysis confirmed that, at 15-min time point, spliceosome assembly (data not shown) and the first catalytic step but not the second catalytic step of splicing (Lane 2) have occurred.

In summary, the data show that NB-506, a potent inhibitor of
Topoisomerase I extracted from P388CPT5 cells was found to be biochemically resistant to the inhibitory effect of CPT (25). To address the effect of NB506 on the kinase activity of the mutant topoisomerase I, topoisomerase I proteins were isolated (18) from P388 and P388CPT5 cells and their abilities to phosphorylate SF2/ASF were examined. Surprisingly, the specific activity of the kinase was five times higher (10 nmol of phosphate transferred min\(^{-1}\), mg\(^{-1}\)) in P388CPT5 cells as compared with P388 cells (2 nmol of phosphate transferred min\(^{-1}\), mg\(^{-1}\)), whereas both enzymes were equally sensitive to inhibition by NB506 (data not shown). This finding suggested that the mutant topoisomerase I from P388CPT5 might adopt an altered conformation that increases the kinase activity of the enzyme and reduces its capacity to form cleavable complexes in the presence of either CPT or NB506.

If the kinase activity of topoisomerase I is brought into play to phosphorylate SR proteins in vivo, it can be expected that NB-506 be more active at inhibiting SR protein phosphorylation in P388 than in P388CPT5 cells. We attempted to test this possibility by comparing the levels of phosphorylation of SR proteins purified from NB506-treated cells versus those from untreated cells. To evaluate the level of SR protein phosphorylation, they were metabolically labeled in vivo with \(^{32}\)P inorganic phosphate and isolated as described (6). Comparison of \(^{32}\)P-labeled SR proteins purified from P388 and P388CPT5 untreated cells and analyzed in SDS PAGE showed no discernible difference between protein patterns (Fig. 6A; compare Lanes 1 and 4). In agreement with previous results (6, 18), five proteins with sizes consistent with those of SR proteins were detected by autoradiography (Fig. 6A, right panel, Lanes 1 and 4) and Coomassie Blue staining (Fig. 6A, left panel, Lanes 1–6). They reacted with mAb 104 antibody (data not shown) and correspond, therefore, to SRp20, SRp30, SRp40, SRp55, and SRp75 characterized previously (6). After 6 h of drug treatment, P388 cells showed a different pattern. Although SRp30 and SRp55 bands are still detected (Fig. 6A, right panel, Lanes 2, 6, and 8), NB-506 treatment enhanced radioactivity contained in the less phosphorylated variants of SRp75, SRp55, and SRp30 from P388.

topoisomerase I, blocks pre-mRNA splicing in vitro by specifically preventing spliceosome assembly.

NB-506 Differentially Affects The Phosphorylation Status of SR Proteins in P388 Leukemia Cells and P388CPT5 Cells Resistant to CPT. To investigate the in vivo effects of NB-506, P388 and P388CPT5 murine leukemia cell lines sensitive and resistant to CPT, respectively, were used. NB-506 proved to have a very toxic effect on P388 cells (30), whereas it exhibited little or no effect on the growth of P388CPT5 cells. Similar results (30) were found for other indolocarbazole derivatives of rebeccamycin. P388CPT5 cells showed marked cross-resistance to these derivatives with relative resistance indexes included between 8.5 and 58, to be compared with a 87.5 relative resistance index for CPT, the drug used to select the resistant cells (31). A recent report (30) indicated that different CPT-resistant cell lines present partial and variable resistance to NB-506 and suggested that NB-506 may have additional targets besides topoisomerase I. A basis for such differences in NB-506 resistance between these cell lines and P388CPT5 may be related to the location of the different point mutations in the topoisomerase I sequences. Sequencing of the entire topoisomerase I cDNA with specific primers revealed that topoisomerase I polypeptide from P388CPT5 cells harbors two point mutations in highly conserved residues (Fig. 5A): a mutation in the core domain changing codon 361 from a GGG Gly-codon to a GTG Val-codon and another mutation in the linker domain changing codon 709 from a GAT Asp-codon to a TAT Tyr-codon. Although the mechanism by which these two mutations confer drug resistance was not addressed here, it is important to recall that residues in both the core and the linker region are important for CPT action (Fig. 5B and Refs. 16, 32).

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5 J. F. Riou, unpublished data.
cells (Fig. 6B, left bottom panel) compared with the same proteins from P388CPT5 cells (right bottom panel). With the exception of SRp40, the detection of which in P388 cells was completely abolished, inhibition of the other SR protein phosphorylation by NB-506 was not complete, implying that other protein kinase(s) not affected by NB-506 are also active in vivo.

In keeping with this finding, SR proteins were less phosphorylated in HeLa cells treated with topotecan, a tumor-active analogue of CPT used in cancer chemotherapy (18). Because topoisomerase I appears to be a privileged cellular target of these drugs, it can be assumed that the observed hypophosphorylation of SR proteins in P388-treated cells is a consequence of inactivation of the kinase activity of topoisomerase I.

**Cellular Effects Associated with NB-506 Treatment.** In living cells, spliceosome assembly and splicing are known to occur during ongoing transcription elongation, and inefficient splicing impairs release of RNA from the site of transcription (33). To evidence the effect of NB-506 on pre-mRNA splicing in P388 and P388CPT5 cells, fluorescence in situ hybridization was used to detect the distribution of polyadenylated RNA (34). Treated and untreated cells were hybridized under nondenaturing conditions with fluorescent oligo(dT) (Fig. 7). As expected, polyadenylated RNA species were detected both in the cytoplasm and the nucleus of P388 (panel b) and P388CPT5 (panel j) untreated cells. After 24 h of NB-506 treatment, the cytoplasm of P388 cells was devoid of any hybridization signal (panel f), whereas the nuclear signal was still detected. This result is consistent with the finding that unprocessed RNA species fail to be exported from the nucleus. In contrast, both cytoplasmic and nuclear staining were still visible in the P388CPT5-treated cells (panel n), indicating that a significant proportion of mRNAs in these cells was still correctly processed and exported.

To determine whether NB-506 induces changes in the splicing profile and/or mRNA levels of proteins that play key roles in apoptosis and tumor progression, RNA recovered from treated and untreated cells were analyzed by RT-PCR using oligo(dT) for priming the first-strand DNA synthesis and specific primers to either Bcl-X or CD 44 mRNAs (Fig. 8). Bcl-X is a member of the bcl-2 gene family that can either promote or prevent apoptosis and exists in several isoforms generated by alternative splicing (Ref. 35 and Fig. 8, Bcl-X, left panel). Only the large isoform, Bcl-XL, which protects cells against apoptosis, but not short isoform Bcl-XS, was detected in both P388 and P388CPT5 cells (Fig. 8, Bcl-X, right panel, Lanes 1 and 3).

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**Fig. 5.** A, schematic representation of the topoisomerase I. The different domains as well as the region corresponding to the SF2/ASF-binding domain (20) are depicted. Mutations identified in the murine P388CPT5 cells are shown (Mu), and their correspondence within the human topoisomerase I is indicated (Hu). B, comparison with different mutations identified in topoisomerase I mutants expressed in other CPT-resistant cell lines (16).

**Fig. 6.** NB-506 influences the level of SR protein phosphorylation in vivo. A, one-fourth of the total amount of metabolically 32P-labeled SR proteins isolated from P388 cells (Lanes 1–3) or P388CPT5 cells (Lanes 4–6) untreated (Lanes 1 and 4) or treated with 0.5 μM (Lanes 2 and 5) and 1 μM (Lanes 3 and 6) of NB-506 was analyzed in 10% SDS-PAGE and detected by autoradiography (right panel) or by Coomassie Blue staining (left panel). B, three times the amount of total proteins contained in the samples described in A, Lanes 1 (top left panel), 3 (bottom left panel), 5 (top right panel), and 6 (bottom right panel) were analyzed by two-dimensional gel electrophoresis using Immobiline DryStrip (Amersham Pharmacia Biotech). 32P-labeled SR protein variants were detected by autoradiography. The electrophoretic mobility of each variant from each SR protein was determined in comparison with mobilities of cold hnRNP proteins that were analyzed in the same gel and stained with Coomassie Blue.
In both cases, treatment with NB-506 did not change the splicing profile of Bcl-X gene (Fig. 8, Bcl-X, right panel, Lanes 2 and 4) but induced a large decrease of Bcl-X L mRNA levels in P388 cells (Lane 2). By contrast, the abundance of Bcl-X L mRNA was not significantly affected in P388CPT5-treated cells (Fig. 8, Bcl-X, right panel, Lane 4). This finding is in complete agreement with the observation that, after drug treatment, P388 cells were more sensitive to apoptosis than P388CPT5 cells (data not shown). In P388-treated cells, NB-506 also led to a decrease in mRNA levels of three related isoforms of the CD44 cell surface glycoprotein family (Fig. 8, CD44, right panel, Lane 2) that has been implicated in extracellular matrix attachment and tumor metastasis (36). Not all of the isoforms that derive from the differential splicing of a single CD44 gene (Fig. 8, CD44, left panel) were affected to the same extent by NB-506 treatment. The larger isoform carrying several alternative exons (I1) disappeared after treatment of P388 cells with NB-506, whereas the two smaller isoforms (I2 and I3) were readily detected (compare Lanes 1 and 2). Both untreated and treated P388CPT cells showed identical splicing patterns, and the various isoforms accumulated to similar levels (Fig. 8, CD44, right panel; compare Lanes 3 and 4), implying that NB-506 did not affect the overall CD44 gene expression in P388CPT5 cells. This finding strongly suggests that NB-506-mediated inhibition of topoisomerase I may be responsible for alterations of processing events that are crucial for the accumulation of specific splice variants.

To test this hypothesis more directly, we considered other genes that are regulated by alternative splicing. The SC35 gene encodes the SR protein SC35, which autoregulates its expression by promoting splicing events that destabilize its mRNAs (37). To detect the various splicing isoforms of SC35, the 3' untranslated region of SC35 transcripts, which is responsible for SC35 mRNAs destabilization, was amplified by RT-PCR using specific primers (Fig. 8, SC35, left panel). In agreement with previous results, PCR products corresponding to the major (2.0-kb mRNA) and the minor (1.6-kb mRNA) transcripts were detected in both P388 and P388CPT5 cells (Fig. 8, SC35, right panel, Lanes 1 and 3, respectively). Interestingly, after NB-506 treatment, a novel PCR product corresponding to the 1.7-kb mRNA was amplified from P388 but not from P388CPT5 cells, implying that inhibition of topoisomerase I led to changes in the splicing pattern of the SC35 primary transcripts. Treatment of P388 with NB-506 also affected the splicing pattern of the Clk/Sty mRNA, encoding a member of the dual specificity kinase family that has the ability to autophosphorylate on serine, threonine, and tyrosine residues (38). Alternative splicing of the primary sty transcripts (Fig. 8, Clk/Sty, left panel) generates mRNAs encoding full-length catalyt-

Fig. 7. NB-506 affects cellular localization of polyadenylated RNA in P388 (panels e-h) but not in P388CPT5 (panels m-p) cells. In situ hybridization of fluorescently labeled oligo(dT) probe to endogenous RNAs was carried out as described in “Materials and Methods” with untreated (panel b) and treated P388 (panel f) or untreated (panel j) and treated (panel p) P388CPT5. Polyadenylated RNA species were detected with the red fluorophore CY3. Nuclei of the same untreated (panel c) and treated (panel g) P388 cells or untreated (panel k) and treated (panel o) P388CPT5 cells were counterstained with 4',6-diamidino-2-phenylindole. The Normarski interference-contrast of the same cells are shown (panel a) for untreated and (panel e) for treated P388 cells and (panel i) for untreated and (panel m) for treated P388CPT5 cells.
SPLICING INHIBITION BY NB-506 DRUG

Fig. 8. NB-506 affects alternative splicing events in P388 but not in P388CPT5 cells. Alternative splicing patterns reported for Bcl-X (35), CD44 (36), SC45 (37), and Clk/Sty (38) primary transcripts are depicted on the left. Because of multiple CD44 alternative splice variants, splicing patterns are depicted by ---. Coding and alternative exons are indicated by \( \square \) and \( \bullet \) respectively. Alternative intronic sequences within the SC35 3′ untranslated region are shown (\( \square \)). The position of primers and expected sizes (in nucleotides) of the RT-PCR products corresponding to the different mRNA isoforms are indicated. Right, representative agarose gels of RT-PCR products corresponding to Bcl-X, CD44, SC35, and Clk/Sty splice variants expressed in P388 (Lanes 1 and 2) and P388CPT5 (Lanes 3 and 4) cells before (Lanes 1 and 3) and after a 24-h exposure to 1.0 \( \mu \)M NB-506 (Lanes 2 and 4). The position of size markers (in bp) is shown on the right, and the various splicing isoforms are named on the left of each panel.

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SPLICING INHIBITION BY NB-506 DRUG


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