**In Vivo Treatment with CPT-11 Leads to Differentiation of Neuroblastoma Xenografts and Topoisomerase I Alterations**

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ABSTRACT

Topoisomerase I inhibitors, such as CPT-11, are potent anticancer drugs against neuroblastoma (NB). Differentiating agents, such as retinoids, improve the survival of children with metastatic NB. To characterize the biological effects associated with exposure to CPT-11 in vivo, athymic mice bearing a human NB xenograft, named IGR-NB8 and characterized as an immature NB with poor prognostic markers, were treated with CPT-11. Prolonged stable disease was observed, resulting in an overall tumor growth delay of 115 days. During treatment, tumors differentiated into ganglioneuroblastomas (GGNBs), which reverted into an immature phenotype when treatment was discontinued. In contrast, 13-cis retinoic acid failed to induce differentiation of IGR-NB8 in vivo. Tumor differentiation was associated with decreased N-myc expression, induction of p73 expression in the perinuclear area and cytoplasm, and a dramatic 35-fold decrease in topoisomerase I (topo I) catalytic activity. The full-length $M_\text{t}$, 100,000 topo I protein was present in both pre- and post-treatment immature NB xenografts. In contrast, differentiated GGNBs did not contain the $M_\text{t}$, 100,000 protein but an intense $M_\text{r}$, 48,000 and 68,000 forms to the cytoplasm was observed in differentiated tumors. The same pattern of topo I expression and catalytic activity was observed in NBs and GGNBs obtained from pediatric patients. Our data suggest that prolonged in vivo exposure to CPT-11 induces differentiation of NB xenografts, which is associated with truncation of the topo I enzyme, relocation of the degraded forms to the cytoplasm, and decreased catalytic activity.

INTRODUCTION

Neuroblastoma (NB) is one of the most frequent solid tumors in pediatric patients and accounts for 6–8% of all childhood malignancies. NBs derive from primitive neural crest cells and are typically located in sympathetic nervous tissues, particularly within the adrenal medulla (1). Generally, NBs are usually able to differentiate either spontaneously or after exposure to different compounds, such as anticancer agents. The degree of differentiation of NBs can be established based on a combination of morphological criteria, the proliferation status, and the absence or presence of stromal Schwann cells (2).

Topoisomerase I (topo I) is an abundant nuclear enzyme involved in essential functions, such as DNA replication, transcription, and recombination. Topo I promotes changes in DNA topology. DNA is relaxed by the supercoiled-driven rotation of the DNA helix downstream of the nick around one or more bond in the intact strand. The process has been referred to as “controlled rotation” (3). This is followed by relegation of the nicked DNA strand and the reformation of an intact DNA helix. Topo I is the target of camptothecin and its analogues, which inhibit the relegation reaction. This leads to the accumulation of DNA-topo I complexes called cleavable complexes. These protein complexes may be converted into frank DNA strand breaks by the replication fork or the transcriptional machinery, thereby initiating a series of events which ultimately result in cell death (4).

CPT-11 (Irinotecan) has been shown to exhibit a wide spectrum of antitumor activity against both adult and pediatric xenografts in preclinical studies (5–7), e.g., we demonstrated previously that i.v. treatment with CPT-11 resulted in extensive tumor regression and delayed growth of three different NB xenograft models (6). Similarly, Thompson et al. (7) showed that oral CPT-11 was active against a panel of six NB xenografts. Similar effects have been reported for another topo I inhibitor, topotecan, which induced tumor regression and a significant delay in tumor growth in animals bearing NB xenografts (8).

To characterize the biological effects associated with CPT-11 exposure in vivo, a NB xenograft model was developed in athymic mice treated with CPT-11 according to the clinically used protocol. We now report that in vivo exposure to clinically relevant doses of irinotecan induces reversible differentiation of NB xenografts, which is associated with both qualitative and quantitative alterations of topo I.

MATERIALS AND METHODS

Materials. CPT-11 was kindly provided by Rhône Poulenc Rorer SA (Maisons Alfort, France) and dissolved in 0.9% sodium chloride solution immediately before injection on each day of treatment. 13-cis retinoic acid (13-cis RA), phenylmethylsulfonyl fluoride, benzamidine, aprotinine, DTT, and a soybean trypsin inhibitor were purchased from Sigma-Aldrich (Saint Quentin, France).

The Topogen Kit (pHOT1 supercoiled plasmid DNA, relaxed plasmid DNA, 10 x topo I buffer, and 5 x stop buffer) and a human polyclonal antibody (Ab) directed against human topo I and purified from serum obtained from scleroderma patients were supplied by Topogen, Inc. (Columbus, OH). Human poly(ADP-ribose) polymerase (PARP, Ab-2) monoclonal Ab was supplied by Oncogene. Immobilon C membranes were purchased from Amersham Life Science (Les Ulis, France). Ni-NTA agarose column was supplied by Qiagen S.A. (Courtaboeuf, France).

Animals. Female athymic Swiss mice were bred in the animal experimentation unit at the Institut Gustave Roussy (Villejuif, France). Athymic mice, aged 6–8 weeks, were housed in an isolator and fed sterile nutrients and water. All of the experiments were carried out in accordance with the animal protection, and hygiene conditions were established by the European Community (directive 86/609/CEE).

In Vivo NB Xenograft Studies. IGR-NB8, originating from a stage III abdominal NB in a 5-year-old boy, was derived by direct s.c. transplantation of small tumor fragments in athymic mice. The human origin of the xenografts was confirmed by the presence of human lactate dehydrogenase isozymes. The IGR-NB8 model is a typical immature NB characterized by N-myc amplification, human multidrug-resistance-1 overexpression, paradoxiophilidy, and chromosome 1p deletion (6).

Mice bearing a 100–300 mm$^3$ s.c. tumor fragments were randomly assigned to two groups of nine mice (one control and treated group each). Mice in the control group were given NaCl 0.9% i.v. Mice in the treated group received i.v. CPT-11 at a dose of 27 mg/kg/day (67.5% of the higher nontoxic dose) for 5 days (one cycle; Ref. 6). Treatment was repeated every 21 days for four...
consecutive cycles. After tumor regrowth, in vivo passages were performed, and treatment was repeated.

13-cis RA was given daily p.o. in 0.2 ml of sesame oil (Sigma-Aldrich), using a 20-gauge intragastric feeding tube (Popper & Sons, New Hyde Park, NY), 5 days a week for two consecutive weeks, at doses of 90 and 120 mg/kg/day. Mice in the control group received sesame oil.

Two perpendicular diameters were measured in the tumor twice a week, and the volume of each tumor was calculated according to the equation: $V = \frac{d^2 \times D}{2}$, where $d$ and $D$ are the smallest and largest tumor diameters, respectively. The experiments lasted until tumor volumes reached 1500–2000 mm$^3$. Antitumor activity was based on complete regressions defined as a tumor regression beyond the palpable limit (15 mm$^3$) and partial regressions defined as a tumor regression >50% of the initial tumor volume. Complete and partial regressions had to be observed for at least two consecutive tumor measurements to be retained. Tumor growth delay was defined as the difference in the median time to reach a tumor volume that was 5-fold the initial tumor volume between the treated and control groups (6).

Patients. NB tumor fragments were obtained from eight children (six males and two females; range of age: 1–72 months). Cryostat sections of fresh tumor specimens were used to determine histological characteristics.

Histological Analysis. Fresh xenograft tissue specimens were fixed in acetic acid-formalin-ethanol (Carlo-Erba, Milano, Italy) and embedded in paraffin. The paraffin-embedded sections were routinely stained with H&E or saphranine.

NB morphological features are based on the International Neuroblastoma Pathology Classification (2). NBs were classified into four categories with their subtypes: (a) NB (Schwannian stroma poor), undifferentiated, poorly differentiated, and differentiating; (b) ganglioneuroblastoma, internéstromal (Schwannian stroma rich); (c) ganglioneuroma (Schwannian stroma dominant), maturing and mature; and (d) ganglioneuroblastoma, nodular (composite Schwannian stroma rich/stroma dominant and stroma poor).

The maturing ganglioneuroma subtype is also termed “borderline” ganglioneuroblastoma according to the Joshi classification (9).

Quantitation of N-myc. Frozen tissue samples were cut in slices for histological evaluation of tumor cell content. Nucleic acids were extracted using Qiagen RNA/DNA mini or midi kit according to conditions recommended by the supplier. Quality of DNA and total RNA is then assessed by gel electrophoresis. Quantitation of N-myc was done by real-time quantitative PCR as reported previously (10).

Immunohistochemical Analysis of p73. The p73 protein was detected on frozen and/or acetic acid formalin-fixed, paraffin-embedded sections with the rabbit p73 human polyclonal Ab (1/300) in NB tumors. This Ab recognizes COOH-terminal residues of the protein that are specific to the α form of the p73 protein.

Measurements of Topo I Activity in Human Tissues and Xenografts. Crude extracts were prepared from CPT-11-treated and untreated xenografts and from patient tumors.

All steps during extract preparation were performed at 4°C. Frozen tissues (50 mg) were grossly minced, suspended in buffer A [0.15 NaCl, 1 mM KH$_2$PO$_4$, 5 mM MgCl$_2$, and 1 mM EDTA (pH 6.4)] containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 mM benzamidine, 1 µg/ml aprotinin, and 10 µg/ml soybean trypsin inhibitor) at 50 mg/100 µl, and homogenized with a motor teflon-glass homogenizer. An equal volume of nuclear lysis buffer B (0.55 mM NaCl) was added slowly, and the extract was kept on ice for 1 h.

The suspension was centrifuged at 12,000 rpm for 30 min. The supernatant was assayed for topo I activity. Protein was estimated by the bicinchoninic acid protein assay (Pierce), using BSA as a standard.

Assays of Topo I Catalytic Activity. The topo I catalytic activity of crude extracts was examined by DNA relaxation assay using supercoiled pHOT1 plasmid DNA as the substrate. For each sample, 10 extracts were serially diluted in buffer containing 10 mM Tris-HCl, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 50 µg/ml BSA (pH 7.5). Supercoiled DNA (0.5 µg) was incubated with each diluted extract at 37°C for 30 min in 10 x topo I assay buffer (Topogen, Inc.). DNA topoisomerases were separated by gel electrophoresis in 1.25% agarose and stained with ethidium bromide. One arbitrary unit of topo I activity was defined as the amount of topo I producing relaxation of 0.25 µg of DNA under the above described conditions. Topo I activity was expressed in arbitrary units per milligram of protein.

**Purification of Topo I.** Probond resin was used to purify topo I. Crude extracts were suspended in buffer C [0.5 mM DTT, 10 mM MgCl$_2$, 3 mM MnCl$_2$, 50 mM KCl, and 50 mM HEPES (pH 7.9)] and incubated with 50 µl of Ni-NTA Agarose column (Qiagen) equilibrated with buffer C for 30 min with continuous agitation at 4°C. Unbound and nonspecifically bound proteins were removed by centrifugation at 5000 rpm for 1 min and washed twice with 300 µl of buffer C. Topo I was eluted in the same buffer at pH 7 containing 150 mM imidazole.

**Separation of Nuclear and Cytoplasmic Protein Fractions.** Nuclear and cytoplasmic protein fractions were prepared from patient tumors. All steps during extract preparation were performed at 4°C. Frozen tissues were minced and suspended in PBS. Suspension was filtered with Spectra/Mesh membrane (Fisher Bioblock Scientific), allowing a macro-filtering of the cells. The cell suspension was centrifuged for 5 min at 1300 rpm. The cellular pellet was suspended in buffer 1 [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT] containing protease inhibitor cocktail tablets (complete mini; Roche) and incubated for 15 min. The homogenate was then centrifuged for 5 min at 4000 rpm to produce cytoplasmic supernatant and a nuclear fraction as the pellet. The pellet was then suspended in buffer 2 [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% glycerol, and 0.6% NP40] containing protease inhibitor cocktail tablets. The suspension was incubated for 30 min in the permanent agitation and then centrifuged for 10 min at 10,000 rpm. The supernatant of nuclear fraction was recovered.

**Analysis of Topo I Degradation.** To demonstrate a selective degradation of topo I, we carried out a Western blot analysis of PARP. HL-60 cells treated with Etoposide at 68 µmol/l, a concentration shown to induce apoptotic cell death (11), was used as a control. Nuclear extracts from patient tumors and crude extract from HL-60-treated and nontreated cell extracts were prepared as described previously.

**Electrophoresis-SDS Polyacrylamide Gel.** Samples of fresh crude and nuclear/cytoplasmic extracts (50–100 µg protein/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels. The separated proteins were transferred onto a nitrocellulose paper (400 mA) at 4°C in a model TE50 transphor electrophoresis unit (Hoefer Scientific, San Francisco, CA). The nitrocellulose blots were blocked in 5% fat-free powdered milk in PBS × 1 for 2 h at room temperature in blocking buffer. The blots were washed with PBS × 1, 0.1% Tween 20 and incubated with polyclonal Ab directed against human topo I for 2 h at room temperature in blocking buffer or with PARP (Ab2) Ab overnight at 4°C. The blots were washed three times with PBS × 1, 0.1% Tween 20 and incubated with appropriate secondary Ab for 2 h at room temperature in blocking buffer. After washing three times with PBS × 1, 0.1% Tween 20, the blots were submitted to autoradiography.

Molecular weight standards used were phosphorylase b (109,000), BSA (80,000), ovalbumin (51,400), carbonic anhydrase (34,000), soybean trypsin inhibitor (27,000), and lysozyme (16,600).

**RESULTS**

**CPT-11 Induces Transient Regression of IGR-NB8 Xenografts.** The influence of CPT-11 treatment on the growth of IGR-NB8 xenografts is shown in Fig. 1. The volume of untreated tumor controls increased rapidly with a doubling time of ~7 days. Strikingly, the first cycle of CPT-11 treatment induced complete tumor regression in half of the animals and partial regression in the other half. However, tumor regressions were transient, and after four cycles of CPT-11, the residual tumor volume remained consistently stable. After discontinuation of treatment, tumors remained stable for ~1 month, and then growth resumed with an average tumor volume doubling time of 9.4 days.

Xenografts passed after ~125 days received further CPT-11 treatment according to the same protocol and exhibited the same pattern of tumor growth. Indeed, transient regression after one cycle was followed by prolonged tumor stabilization observed throughout the four cycles of CPT-11 and further tumor regrowth after discontinuation of treatment.
CPT-11 Induces Neuronal Differentiation in IGR-NB8 Xenografts.

Fresh xenograft tumor tissues were collected from untreated animals at day 20 (Fig. 1A) as well as from treated animals during tumor stabilization on day 77 (Fig. 1B) or during tumor regrowth on day 125 (Fig. 1C). Untreated IGR-NB8A xenografts displayed the histological features of a poorly differentiated NB composed of undifferentiated neuroblastic cells (small uniform rounded cells) containing a high fraction of mitotic figures and very scant schwannian stroma (Fig. 2A). IGR-NB8B xenografts collected at day 77 exhibited the features of a maturing ganglioneuroma composed of maturing ganglion cells (enlarged, eccentric nucleus with vesicular chromatin and a well-defined nucleolus), mature ganglion cells, and abundant schwannian stroma (Fig. 2B). IGR-NB8C xenografts collected at day 125 exhibited the histological features of a poorly differentiated NB (Fig. 2C) that were similar to that observed for untreated xenografts (Fig. 2A). In vivo treatment with CPT-11 clearly induced reversible differentiation of IGR-NB8 xenografts. Interestingly, histological analysis of NBs from eight pediatric patients (not treated with CPT-11) showed that four had a phenotype almost analogous to that observed in the untreated xenografts (Fig. 2A) containing poorly differentiated NB cells with very limited schwannian stroma and, at most, 5% of cells with morphological features of differentiation toward ganglion cells. The other four tumors exhibited a more mature phenotype with abundant schwannian stroma and morphological features of both ganglioneuroblastomas and ganglioneuroma (results not shown). Therefore, both morphological phenotypes found in xenografts correspond to that observed in the clinical setting.

Molecular Markers of NB Differentiation in Xenografts Treated with CPT-11.

We examined the relationship of N-myc expression to the in vivo differentiation of IGR-NB8 induced by CPT-11 by real-time quantitative PCR. A 3.2-fold decrease in the level of N-myc expression was observed in differentiated IGR-NB8B xenografts as compared with undifferentiated IGR-NB8A before treatment (data not shown). After discontinuation of treatment and tumor regrowth, the level of N-myc expression in undifferentiated IGR-NB8C was equivalent to pretreatment level.

The expression of p73, a p53 homologue (12), was shown to correlate with neuronal differentiation (13, 14). Immunohistochemical analysis of p73 using a polyclonal Ab directed toward the protein COOH terminus was used to quantify p73 expression. The p73 protein was detected in IGR-NB8A, IGR-NB8B, and IGR-NB8C xenografts. Immunohistochemical analysis showed low p73 expression localized in the nucleus of untreated IGR-NB8A xenografts (Fig. 3A), whereas higher p73 expression was observed in differentiated IGR-
NB8B xenografts, localized in the perinuclear area and cytoplasm (Fig. 3B). In undifferentiated IGR-NB8C, p73 expression was mainly localized in the nucleus and less intense in the perinuclear area, indicating reversible differentiation of IGR-NB8 (Fig. 3C).

**Topo I Catalytic Activity Varies in IGR-NB8 Xenografts and Tumors from Pediatric Patients According to CPT-11 Treatment and Neuronal Differentiation.** Crude cellular extracts were prepared from NB xenografts obtained from either untreated controls (Fig. 1A) or CPT-11-treated animals and collected during tumor stabilization on day 77 (Fig. 1B) or during tumor regrowth on day 125 (Fig. 1C). The poorly differentiated NB phenotype (A and C) was associated with high topo I catalytic activity. In contrast, the more differentiated ganglioneuroblastomas (B) exhibited 35-fold lower topo I activity (Fig. 4). A similar trend was observed in tumors from pediatric patients. Crude extracts prepared from poorly differentiated NBs exhibited high topo I catalytic activity, whereas 10-fold lower topo I activity was observed in tumor samples presenting a more mature phenotype with morphological features of both ganglioneuroblastomas and ganglioneuromas (Fig. 4).

**Qualitative Alterations of Topo I in NBs from Xenografts and Patients.** The amounts and molecular weight of topo I from tumor samples were characterized using an affinity-purified polyclonal Ab directed against human topo I obtained from scleroderma patients. Western blot analysis of crude extracts prepared from untreated IGR-NB8 xenografts (three tumors analyzed) showed three major bands with a molecular weight of M₁ = 100,000; M₂ = 54,000; and M₃ = 48,000 (Fig. 5A, Lanes 1 and 2). A similar pattern was observed in xenografts from treated animals collected at day 125 (three tumors analyzed), except that an additional M₄ = 68,000 band was present (Fig. 5A, Lanes 5 and 6). In contrast, crude extracts prepared from IGR-NB8 xenografts collected at day 77 (two tumors analyzed) showed a very intense M₃, 48,000 and weak M₄, 54,000 band (Fig. 5A, Lanes 3 and 4).

Western blot analysis of tumor tissues obtained from patients showed similar results. Topo I from patients with poorly differentiated NB was predominantly present in the M₁, 100,000 form corresponding to a full-length enzyme. In addition, a minor M₃, 54,000 band was present in all samples (Fig. 5B, Lane 1). In marked contrast, most of topo I from patients with more differentiated ganglioneuromas was present in a M₄, 48,000 form (Fig. 5B, Lane 2).

**Purification of Topo I from Xenografts to Identify Topo I Peptides.** To determine which topo I peptides were derived from the NH₂-terminal part of the molecule, xenograft extracts were partly purified by Ni-NTA agarose column chromatography. This is possible because the NH₂-terminal part of topo I is rich in histidine, enabling high affinity binding of this part of the molecule to a Ni-NTA agarose column compared with that of peptides derived from other parts of the molecule which do not possess a similar histidine-rich domain. Ni-NTA agarose column chromatography of crude extracts from NB xenografts (Fig. 5C, Lane 1) showed that none of the three bands of M₁, ~70,000 nor the M₄, 54,000 band was able to bind to the Ni-NTA agarose column (Fig. 5C, Lane 2), unlike the M₃, 48,000 and 100,000 bands, which bound with high affinity and could only be eluted with concentrations of imidazole > 100 mM (Fig. 5C, Lanes 3 and 4). These results strongly suggest that only the M₃, 48,000 peptide is derived from the NH₂-terminal part of topo I.

**Subcellular Redistribution of Topo I in Differentiated Ganglioneuroblastoma.** Danks et al. (15) reported a degradation of topo I and concomitant relocation of the M₁, 68,000 form to the cytoplasm after camptothecin treatment of anaplastic astrocytoma cells. To verify the cellular localization of topo I, a separation of the nuclear and cytoplasmic fractions from patient tumors was performed (Fig. 6). In agreement with precedent results, topo I from poorly differentiated NB was predominantly present in the M₁, 100,000 form, and this full-length enzyme was exclusively present in the nucleus. In contrast, in differentiated ganglioneuroblastoma, both the M₃, 48,000 and 68,000 forms were present in the nucleus and cytoplasm. This result suggests that the differentiation state was associated with both topo I proteolysis and redistribution of the M₃, 48,000 and 68,000 forms to the cytoplasm.

**Selective Degradation of Topo I.** PARP is a nuclear protein involved in the response to DNA damage. The cleavage of this M₃,
116,000 enzyme into two fragments of $M_0 \sim 90,000$ and 26,000 is considered as an early marker of apoptosis (16). In differentiated ganglioneuroblastoma, topo I was present as a $M_0 48,000$ form. This proteolysis can be attributable to a significant level of apoptosis in the differentiated tumors; under those circumstances, protease inhibitors could not protect effectively topo I from degradation. To demonstrate a selective degradation of topo I, PARP was used as control. Western blot analysis of tumor tissue obtained from patients showed that in both poorly differentiated NB and differentiated ganglioneuroblastoma, the full-length enzyme ($M_0 116,000$) was present (Fig. 7). This result suggested that in differentiated ganglioneuroblastoma, the topo I degradation was selective.

No Effect of 13-cis RA on Tumor Growth and Morphological Features of IGR-NB8. The influence of treatment with 13-cis RA on the in vivo growth of IGR-B8 xenografts is shown in Fig. 8. After 2 weeks, 13-cis RA caused mainly severe cutaneous toxicity (dryness and peeling of skin), weight loss, and diarrhea. The volume of untreated IGR-NB8 (control) and IGR-NB8-treated xenografts with 90 or 120 mg/kg/day 13-cis RA increased rapidly, thus indicating that 13-cis RA had no effects on IGR-NB8 growth. At the histological level, IGR-NB8 xenografts collected during 13 cis RA treatment exhibited the histological features of a poorly differentiated NB composed of undifferentiated neuroblastic cells and very scant schwannian stroma. In conclusion, 13-cis RA did not induce differentiation of IGR-NB8 xenografts.

DISCUSSION

NB is a malignant tumor composed of embryonic nerve cells originating in the sympathetic nervous system derived from the neural crest. NBs may differentiate into benign ganglioneuroma either spontaneously or during chemotherapy (1). Different chemical compounds, such as retinoic acid (17), sodium phenylacetate (18), and polypropenoic acid (19), can induce differentiation of NB cell lines in vitro. The morphological differentiation observed with these compounds is characterized by the formation of pseudoganglia and neurite extension. The same morphological changes have been described for human NB cell lines treated with cisplatin in vitro (20). Interestingly, morphological differentiation has also been shown to occur in patients undergoing chemotherapy (21).

The present study shows that CPT-11 induces reversible differentiation of NB xenografts in vivo. To our knowledge, this is the first study to evidence reversible differentiation in an in vivo experimental model. Assessment of differentiation was based on morphological criteria, as defined by the International Neuroblastoma Pathology Committee (2, 22). After administration of four cycles of CPT-11, we observed differentiation of NB into a more mature form. After discontinuation of treatment (67 days after the first CPT-11 injection), tumors reverted to an undifferentiated state with the same morphological aspect as the untreated xenografts.

N-myc is a member of the myc family of proto-oncogenes involved in initiation and progression of tumors. Although c-myc, the most characterized member of the family, is well known for its role in cellular proliferation and apoptosis, the function of N-myc in differentiation and proliferation remains unclear. Thiele et al. (23) showed morphological differentiation and decreased N-myc expression in NB cells treated with retinoic acid. We examined the N-myc expression during CPT-11-induced differentiation of IGR-NB8, and we observed a decreased level of N-myc expression. Zhu et al. (24) showed that in NB, N-myc gene modulates differentiation of IGR-NB8, and we observed a decreased level of N-myc expression. It has been reported that increased p73 levels in NB cells induced to differentiate by retinoic acid (13). Using p73 expression as a neuronal differentiation marker (13), we observed a weak specific p73 expression mainly in the nucleus of undifferentiated neuroblasts in xenografts (IGR-NB8A and IGR-NB8C). By contrast, after CPT-11 treatment, strong p73 expression was localized in the perinuclear area and cytoplasm of differentiated IGR-NB8B (ganglioneuroblastoma). p73 belongs to the p53 family of proteins. They share strong similarities in structural sequence and functions, such as the suppression of cell growth and apoptosis. p73 may play an important role in differentiation. Douc-Rasy et al. (14) showed that p73 is located in the nucleus in undifferentiated and differentiated
NBs and predominantly in the perinuclear and cytoplasmic areas, respectively.

The differentiated features may play an important role in resistance to chemotherapy. It is frequently observed that, after an initial, often short-lived response to treatment with cytotoxic agents, tumor resistance ensues, and relapses are observed. The emergence of resistant cells is often associated with histological differentiation (21). In our study, CPT-11 induced tumor response attaining 100% after the first cycle of treatment. Additional treatment failed to induce any further tumor response, indicating that differentiated NB xenografts had become refractory to CPT-11. In addition, the xenograft was further passed in vivo and treated with CPT-11 to establish a resistance model. During this process, neither complete or partial tumor regression nor any tumor stabilization was observed.7

CPT-11-induced differentiation of NB xenografts was associated with both quantitative and qualitative alterations of topo I. Our results show that the differentiated phenotype is associated with a dramatic decrease in topo I catalytic activity, a selective degradation of the Mr 100,000 form of topo I and redistribution of the Mr 48,000 and 68,000 forms to the cytoplasm. In contrast, dedifferentiation of the tumor was associated with elevated catalytic topo I activity and the reappearance of the full-length form of the enzyme in the nucleus. In a previous study, we showed that the catalytic activity of topo I is higher in immature NBs than in ganglioneuroblastoma and adrenal glands (8). This is consistent with the findings in this study which clearly demonstrate that the maturation of ganglioneuroblastomas into ganglion-euromas is associated with loss of the full-length enzyme. Topo I alterations have been reported previously in several other human cancers, such as carcinomas of the colon, prostate, and ovary. Giovannella et al. (25) evidenced elevated topo I protein levels in human colon adenocarcinoma and colon xenografts compared with that of normal colonic tissues. Husain et al. (26) reported that the catalytic activity of topo I in crude extracts from colorectal and prostate tumors was higher than in the corresponding normal tissues. Finally, van der Zee et al. (27) found that the catalytic activity of topo I was elevated in malignant epithelial ovarian tumors compared with more benign ovarian tumors.

Western blot analyses of undifferentiated IGR-NB8 xenografts and pediatric tumors also showed the presence of other bands (several in malignant epithelial ovarian tumors compared with more benign Zee activity of topo I in crude extracts from colorectal and prostate tumors were relocalized in cytoplams in differentiated ganglioneuroblastoma. The differentiation-induced decrease in topo I catalytic activity, a selective degradation of the NH2-terminal region or were not derived from topo I.

Other inhibitors of topo I and chemical agents have been shown to induce differentiation and modification of topo I activity in vitro. Chou et al. (31) demonstrated that camptothecin induced differentiation of human and mouse myeloid leukemia cells. 10 hydroxy-camptothecin also induced differentiation of a parental human promyelo-cyctic leukemia cell line (HL60) and resistant subline and reduced DNA topo I activity in mature cells compared with undifferentiated controls (32). Shayo et al. (33) showed a rapid decrease in topo I activity when differentiation was induced by 12-myristate 13-acetate, N,N'hexamethylenbisacetamide, and retinoic acid, differentiating agents that are not specific inhibitors of topo I. These different studies and our data showed a decrease in topo I activity when differentiation was induced, suggesting that this decrease in topo I activity is a consequence rather than cause of differentiation. The differentiation induced by CPT-11, a specific inhibitor of topo I, could be independent of its effects on this enzyme. Our study evidenced the presence of a Mr 48,000 protein by Western blot when differentiation was induced by CPT-11. This indicates an alteration of the topo I protein and suggests the involvement of a post-translational mechanism.

Retinoids are known to induce differentiation of NB cell lines in vitro. In addition, 13-cis retinoid acid was shown to improve the survival of children with metastatic NB after treatment with conventional or high-dose chemotherapy (34). The effects of retinoic acid are mediated via two classes of nuclear receptors, the RA and retinoid X receptors. An interaction between retinoid receptors and other nuclear proteins may be determinants of retinoid responses in NB cells (35). Furthermore, recently, a correlation was reported between N-myc gene amplification and resistance to an all-trans retinoic acid effect in NB cells (36). In our model, which amplifies the N-myc gene, 13-cis RA failed to induce differentiation and inhibition of tumor growth at clinically relevant doses. This suggests that CPT-11 is able to induce differentiation via molecular mechanisms that are different from that of retinoic acid. Additional studies are required to identify these mechanisms.

In conclusion, we have demonstrated that the DNA topo I inhibitor CPT-11 is able to induce reversible differentiation in a human NB xenograft in vivo. This differentiation was associated with loss of the capacity to proliferate, several phenotypic changes, and alterations of topo I, its nuclear target. The differentiation process is complex. Additional studies are warranted to elucidate the mechanism of CPT-11-induced differentiation. Because differentiating agents have been shown to improve the survival of children with metastatic NB, our findings may be relevant for the development of CPT-11 in children with NB.

ACKNOWLEDGMENTS

We thank all of the nurses and physicians at the Gustave-Roussy Institute (Villejuif, France) who devoted their care to the patients included in this study. We also thank Patrice Ardonin and the staff in the Animal Experimentation Unit, Institut Gustave-Roussy, for the care of the animals. Finally, we thank Loma Saint-Ange for editing this manuscript.

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Cancer Res 2004;64:3223-3229.