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Cross-resistance of topoisomerase I and II inhibitors in neuroblastoma cell lines

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Abstract *Purpose*: We have previously shown that neuroblastoma cell lines established from patients after intensive chemotherapy show sustained resistance to various drugs and especially high resistance to etoposide (up to 51 times higher than a clinically achievable level). To determine whether topoisomerase I inhibitors (topotecan and CPT-11) are effective against etoposide-resistant neuroblastomas, we studied the response to topotecan and the active metabolite of CPT-11 (SN-38) in 19 cell lines with a spectrum of sensitivities to etoposide. *Materials and methods*: The panel included cell lines established at diagnosis and after disease progression either during induction chemotherapy or after myeloablative therapy supported with bone marrow transplantation. Cytotoxicities of topotecan, SN-38, and etoposide were determined using a microplate digital image microscopy (DIMSCAN) assay with a 4-log dynamic range. *Results*: All six etoposide-resistant cell lines were resistant to topotecan and SN-38 (resistance defined as LC_{90} higher then clinically achievable levels for the drug). Significant cross-resistance by Pearson's correlation analysis ($r \geq 0.6$) occurred between topotecan + etoposide, topotecan + SN-38, and etoposide + SN-38. *Conclusions*: Topotecan and CPT-11 do not have significant activity against most etoposide-resistant neuroblastoma cell lines and this suggests that agents other than topoisomerase inhibitors should be explored for the treatment of recurrent neuroblastomas.

Key words Etoposide · Topotecan · CPT-11 · Bone marrow transplantation

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Introduction

Neuroblastoma is an aggressive childhood neoplasm of the sympathetic nervous system. Intensive chemoradiotherapy supported with bone marrow transplantation (BMT) has improved survival for high risk neuroblastoma [19, 32], especially if followed by 13-*cis*-retinoic acid [19, 29], but most patients with stage 4 neuroblastoma diagnosed after 1 year of age still die from progressive disease occurring before or after myeloablative therapy. The semisynthetic epipodophyllotoxin, etoposide (ETOP), is a topoisomerase II inhibitor [8, 16], that is commonly used both for induction and consolidation phases of neuroblastoma therapy. Treatment strategies for recurrent disease also utilize ETOP. Thus, the poor response rate to reinduction chemotherapy in neuroblastoma patients who develop progressive disease on or after therapy might be attributed to the presence of tumor cells that have developed resistance to ETOP. If true, substitution of drugs which are not cross-resistant to ETOP in consolidation or relapse therapies may improve outcome.

Topotecan (TPT) and irinotecan (CPT-11) are semisynthetic derivatives of camptothecin that inhibit topoisomerase I. Phase I and II clinical trials of TPT have been carried out for various malignancies including: small-cell lung carcinoma [25], non-small-cell lung carcinoma [17], germ cell tumors [28], ovarian cancer [23], head and neck cancer [30], pancreatic cancer [22], brain tumors [1, 3], and neuroblastoma [26]. Some clinical

trials of TPT as a single agent have shown responses [26, 30], while other trials have failed to show any objective clinical response [3, 17, 22, 23, 25, 28]. Clinical trials of CPT-11 have shown responses in non-small-cell lung cancer [21] and colorectal cancer [7]. TPT has shown significant antitumor activity in human xenografts from neuroblastomas [37], ependymomas, gliomas, medulloblastomas [11], colon adenocarcinoma, rhabdomyosarcoma, and pediatric brain tumors [12]. CPT-11 is effective against colon adenocarcinoma, rhabdomyosarcoma, and pediatric brain tumor xenografts [12].

Collateral hypersensitivity has been shown in camptothecin-resistant cell lines to topoisomerase II inhibitors [33], and in ETOP-resistant cell lines to camptothecin derivatives in vitro [5]. These findings suggest that TPT or CPT-11 could be effective against neuroblastomas that have failed ETOP treatment.

We have established a panel of cell lines from patients in various phases of treatment (diagnosis, progressive disease during induction therapy, and relapse after intensive chemoradiotherapy and bone marrow transplantation). This panel also includes four pairs of cell lines established from tumors of patients at diagnosis and at disease progression during induction chemotherapy. Cell lines in our panel have demonstrated various levels of sensitivity to ETOP [14, 15]. We used this panel to determine the cross-resistance of camptothecin derivatives in ETOP-sensitive and -resistant cell lines.

Materials and methods

Cell lines

We used a panel of 19 neuroblastoma cell lines obtained from patients at various points of the disease: six at diagnosis (DX), seven at time of progressive disease during induction therapy (PD-Ind), and six derived during relapse after bone marrow transplantation (PD-BMT). DX cell lines were established from patients prior to treatment, PD-Ind cell lines came from patients who developed progressive disease during induction chemotherapy, and PD-BMT cell lines were obtained at relapse after patients had received high-dose myeloablative chemoradiotherapy and BMT. Table 1 lists the topoisomerase II inhibitors used to treat patients prior to establishing the cell lines. None of the tumors that gave rise to the cell lines used in this study had been treated with the topoisomerase I inhibitors, TPT or CPT-11.

All cell lines in the panel have been previously described [15] except CHLA-140 and CHLA-136. The neuroblastoma origin of CHLA-140 and CHLA-136 was confirmed by the expression of the 299-bp cDNA fragment of tyrosine hydroxylase (TH) [20] and the 653-bp fragment of PGP 9.5 [18] by RT-PCR as we have described previously [15]. PGP 9.5 is expressed in neural tumors while TH expression is limited to neuroblastoma (within small round-cell tumors) [15, 34]. The two new neuroblastoma cell lines expressed both PGP9.5 and TH (data not shown).

The cell lines SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE(1), SK-N-BE(2), SMS-SAN, SMS-LHN, and LA-N-6 were cultured in complete medium comprising RPMI-1640 (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, Calif.). The cell lines CHLA-15, CHLA-20, CHLA-140, CHLA-51, CHLA-8, CHLA-79, CHLA-90, CHLA-134, and CHLA-136 were cultured in complete medium comprising Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Walkersville, Md.) supplemented with ≈3 m*M* L-glutamine (Gemini Bioproducts), 5 μg/ml each of insulin and transferrin and 5 ng/ml of selenous acid (ITS Culture Supplement; Collaborative Biomedical Products, Bedford, Mass.) and 20% heat-inactivated FBS. All cell lines used in the study were under passage 30 and were cultured at 37 °C in a humidified incubator containing an atmosphere of 95% air and 5% CO_2 without antibiotics. All cell lines tested negative for mycoplasma. Cell lines were not selected for resistance to ETOP or any other drug in vitro.

Drugs and chemicals

ETOP was obtained from Bristol-Myers Squibb Co., Princeton, N.J., TPT was provided by the National Institutes of Health Bethesda, Md., and SN-38 (an active in vitro metabolite of CPT-11) was a gift from Pharmacia & Upjohn Co, Kalamazoo, Mich. Calcein AM was purchased from Molecular Probes, Eugene, Ore., fluorescein diacetate (FDA) from Eastman Kodak Company, Rochester, N.Y., and eosin Y from Sigma Chemical Co, St. Louis, Mo.

Table 1 Human neuroblastoma cell lines

DX cell lines[a]	PD-Ind cell lines[b]	PD-BMT cell lines[c]
SMS-KAN[d]	SMS-KANR[d]	CHLA-8 (DOX, VCR, VM-26, ETOP)
SMS-KCN[e]	SMS-KCNR[e]	CHLA-51 (DOX, VM-26, ETOP)
SK-N-BE(1)[f]	SK-N-BE(2)[f]	CHLA-79 (DOX, ETOP)
CHLA-15[g]	CHLA-20[g] (VM-26)[h]	CHLA-90 (DOX, ETOP)
SMS-SAN	SMS-LHN	CHLA-134 (DOX, ETOP)
CHLA-42	LA-N-6 (VM-26[i], ETOP[j])	CHLA-136 (DOX, ETOP)
	CHLA-140 (VM-26)	

[a] Cell lines derived from tumors of patients prior to treatment
[b] Cell lines derived from patients who relapsed during induction chemotherapy
[c] Cell lines derived from patients who relapsed after myeloablative chemoradiotherapy and bone marrow transplantation
[d] A DX (SMS-KAN) and PD-Ind (SMS-KANR) pair of cell lines from the same patient
[e] A DX (SMS-KCN) and PD-Ind (SMS-KCNR) pair of cell lines from the same patient
[f] A DX (SK-N-BE(1)) and PD-Ind (SK-N-BE(2)) pair of cell lines from the same patient
[g] A DX (CHLA-15) and PD-Ind (CHLA-20) pair of cell lines from the same patient
[h] In parenthsis are shown epipodophyllotoxins which were used to treat tumors (in patients) prior to those tumors being used to derive the cell lines
[i] Teniposide
[j] Etoposide

Cytotoxicity assay

The cytotoxicity of ETOP, TPT, and SN-38 against neuroblastoma cell lines was determined using the DIMSCAN assay system [10, 27]. DIMSCAN uses digital imaging microscopy to quantify viable cells which selectively accumulate FDA. DIMSCAN is capable of measuring cytotoxicity over a 4–5 log dynamic range, by quantifying total fluorescence per well (which is proportional to viable, clonogenic cells) after eliminating background fluorescence with digital thresholding [27] and eosin Y quenching [10, 15]. Cell lines were seeded at 15,000 cells in 150 µl of complete medium per well into 96-well plates. After overnight incubation, various concentrations of ETOP, TPT, and SN-38 in 100 µl of complete medium were added to each well. The final drug concentration ranges used were: ETOP 0 to 12 µg/ml, TPT 0 to 20 µg/ml, and SN-38 0 to 100 ng/ml. Each condition was tested in 12 replicates. After incubation of cell lines with ETOP, TPT, and SN-38 for 4 days, 150 µl of medium was removed from each well, FDA in 50 µl of medium (final concentration of FDA 8–10 µg/ml) was added, and the plates were incubated for an additional 25 min at 37 °C before adding 30 µl of 0.5% eosin Y [10] to each well. Total fluorescence was then measured using digital image microscopy and the results are expressed as surviving fractions of treated cells compared to control cells.

Data analysis

LC_{90} values (i.e. the drug concentration that was lethal for 90% of the cell population) were calculated using the software "Dose-Effect Analysis with Microcomputers" [6]. A cell line with an LC_{90} value equal to or more than the achievable clinical concentration was considered resistant to that drug. The reported peak concentrations for the active lactone of TPT are 7 to 96 ng/ml [31], and the C_{max} values in patients for SN-38 are 17.3 to 22.6 ng/ml [21]. The continuous steady-state concentration (CSS) for ETOP in patients treated with doses similar to those used in neuroblastoma patients on continuous infusion myeloablative regimens ranged from 2 to 7 µg/ml [2, 4, 9]. In this study, the reference levels for TPT, SN-38 and ETOP were 100 ng/ml, 20 ng/ml, and 5 µg/ml, respectively.

Statistical significance of cross-resistance between the cytotoxic agents was evaluated by the Pearson correlation analysis [38] employing the software SAS (SAS Institute, Cary, N.C.). For each LC_{90}, standardized residuals were used for the test and were calculated as:

$$\log(LC_{90}) - Av[\log(LC_{90})]/SD$$

The $Av[Log(LC_{90})]$ and SD values were calculated for each drug tested. This concept of standardization was developed to optimally detect and analyze cell line response by the National Cancer Institute [24]. Drugs were considered cross-resistant if the correlation coefficient (r) of the test was ≥0.6.

Results

Cytotoxicity studies

We determined the sensitivities of cell lines to ETOP, TPT, and SN-38. The results of cytotoxicity assays are expressed as LC_{90} values for each drug (Table 2) and were compared to ETOP resistance, which was defined as an LC_{90} value ≥5 µg/ml. The LC_{90} values were calculated from the dose response curves of each drug determined for each of the 19 cell lines. The dose response curves of the cell lines are shown in Fig. 1 for ETOP, Fig. 2 for TPT, and Fig. 3 for SN-38.

Three PD-Ind cell lines and all PD-BMT cell lines were derived from patients treated with ETOP or teniposide, another epipodophyllotoxin (Table 1). Etoposide LC_{90} values ranged from <0.1 to 156 ng/ml for DX cell lines, from 0.2 to 24,000 ng/ml for PD-Ind cell lines, and from 24.3 to 255,000 ng/ml for PD-BMT cell lines. The LC_{90} values of ETOP were higher than the reference drug concentration (the clinically achievable

Table 2 Concentrations of drugs killing 90% of cells (*DX* at diagnosis, *PD-Ind* during progressive disease during induction therapy, *PD-BMT* during relapse after bone marrow transplantation)

Phase of therapy[a]	ETOP-sensitive cell lines[b]				ETOP-resistant cell lines[c]			
	Cell line	LC_{90} (ng/ml)[d]			Cell line	LC_{90} (ng/ml)		
		TPT	SN-38	ETOP		TPT	SN-38	ETOP
DX	SMS-KAN	0.2	1.9	156	No ETOP-resistant cell lines at diagnosis			
	SMS-KCN	10.8	<0.1	2.7				
	SK-N-BE(1)	11	1.9	<0.1				
	CHLA-15	1.5	0.1	0.2				
	SMS-SAN	<0.1	0.1	<0.1				
	CHLA-42	41.8	5.4	67.4				
PD-Ind	SMS-KANR	54.2	1.8	1.2	LA-N-6	**148**	**20**	**24,059**
	SMS-KCNR	40.6	<0.1	0.2	CHLA-140	**102**	**23**	**15,242**
	SK-N-BE(2)	12.8	**25**	577				
	CHLA-20	2.6	1.7	691				
	SMS-LHN	14.4	2.7	682				
PD-BMT	CHLA-51	48.7	**38**	24.3	CHLA-79	**801**	**414**	**12,617**
	CHLA-8	4.5	2.6	71.8	CHLA-90	**6,459**	**254**	**51,254**
					CHLA-134	**16,274**	**848**	**255,873**
					CHLA-136	**311**	**46**	**56,722**

[a] Phase of therapy for the patient at the time the tumor specimen was obtained and used to derive the cell line
[b] LC_{90} values for ETOP <5 µg/ml
[c] LC_{90} values for ETOP >5 µg/ml
[d] Results were obtained from data shown in Figs. 1–3. IC_{90} values higher than clinically achievable levels are shown in bold

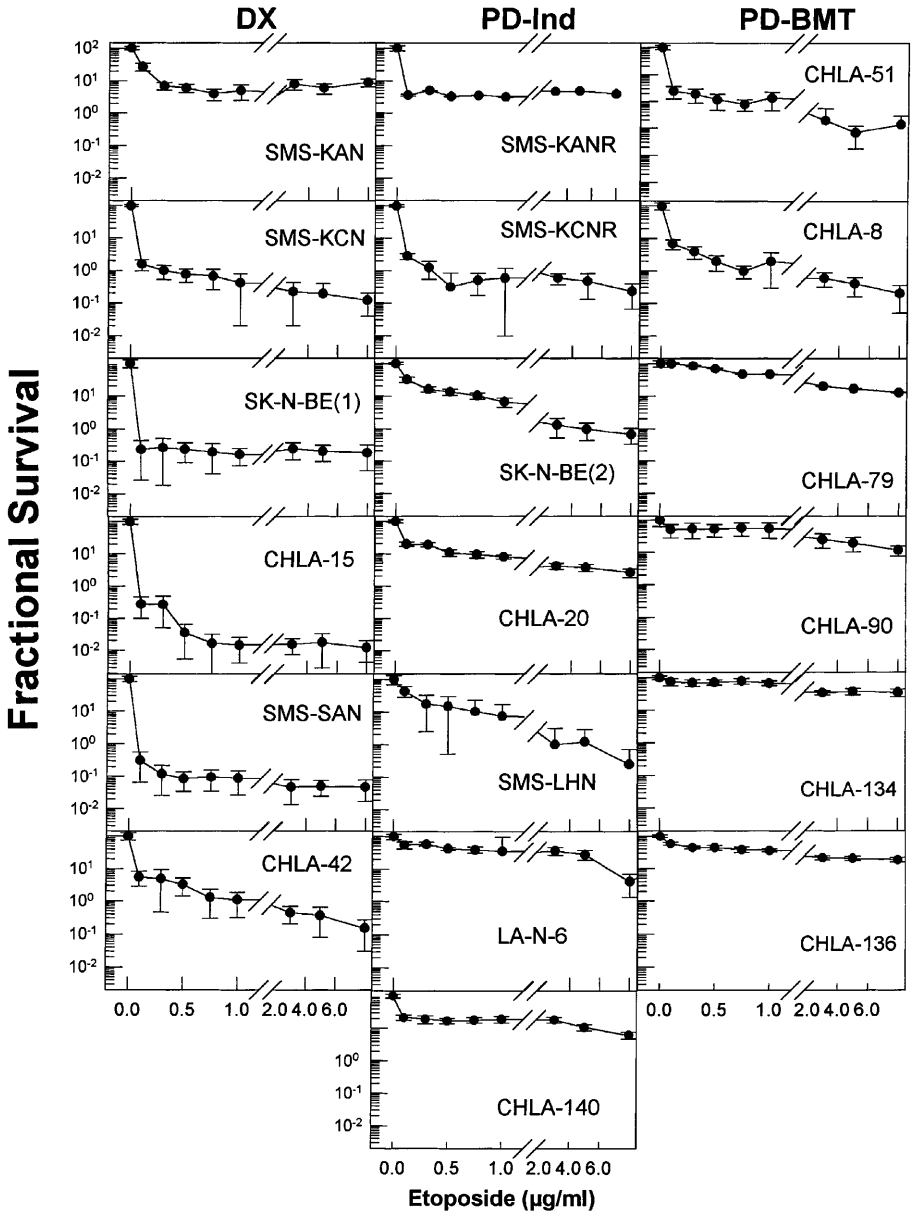

Fig. 1 Dose-response curves for ETOP obtained by DIMSCAN analysis of neuroblastoma cell lines treated over a concentration range of 0–8 µg/ml; 5 µg/ml was considered a clinically achievable level (*DX* cell lines established from patients at the time of diagnosis, *PD-Ind* cell lines derived from patients with progressive disease on conventional chemotherapy, *PD-BMT* cell lines derived from patients after myeloablative chemoradiotherapy followed by BMT)

CSS for high-dose ETOP therapy) for none of six DX cell lines, two of seven PD-Ind cell lines, and four of six PD-BMT cell lines. Resistance to ETOP was seen in most cell lines derived from patients treated with ETOP: LA-N-6, CHLA-140, CHLA-79, CHLA-90, CHLA-134, and CHLA-136. However, two PD-BMT cell lines (CHLA-8 and CHLA-51, derived from ETOP-treated patients) were sensitive to the drug.

TPT LC_{90} values ranged from <0.1 to 42 ng/ml for DX cell lines, from 2.6 to 148 ng/ml for PD-Ind cell lines, and from 4.5 to 16,000 ng/ml for PD-BMT cell lines. The LC_{90} values of TPT were higher than the reference drug concentration (the reported peak concentration) for none of six DX cell lines, two of seven PD-Ind cell lines, and four of six PD-BMT cell lines. All six ETOP-resistant cell lines were also resistant to TPT while all 13 ETOP-sensitive cell lines were sensitive to TPT.

LC_{90} values for SN-38 ranged from <0.1 to 5.6 ng/ml for DX cell lines, from <0.1 to 23 ng/ml for PD-Ind cell lines, and from 2.6 to 850 ng/ml for PD-BMT cell lines. The SN-38 LC_{90} values were higher than the reference drug concentration of this metabolite for none of six DX cell lines, four of seven PD-Ind cell lines, and five of six PD-BMT cell lines. Five of six ETOP-resistant cell lines were resistant to SN-38, while 11/13 ETOP-sensitive cell lines were sensitive to SN-38.

Although overall there was cross-resistance between ETOP, TPT, and SN-38, TPT and SN-38 had far greater activity than ETOP against CHLA-20 (Figs. 1–3, Table 2), a PD-Ind cell line that showed a modest level of ETOP resistance.

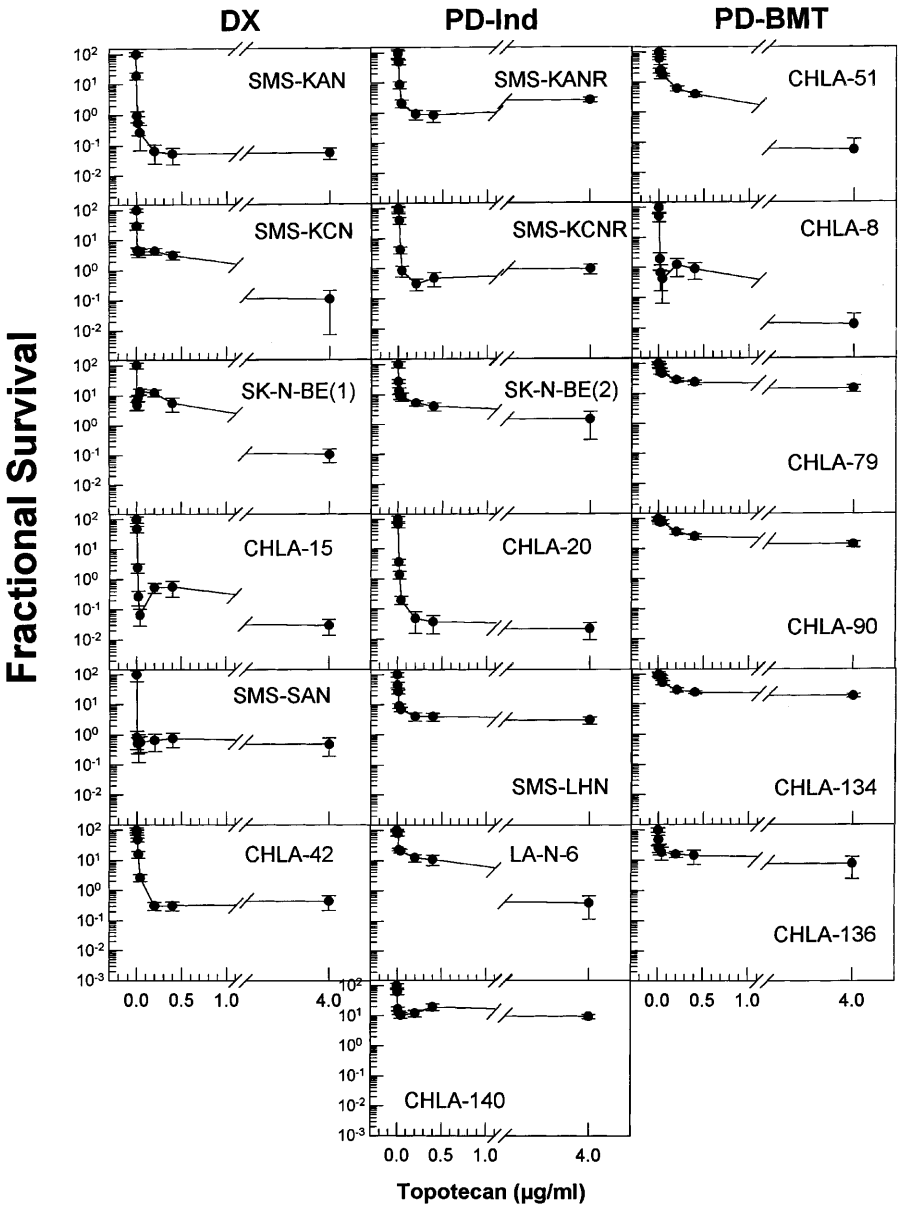

Fig. 2 Dose-response curves for TPT obtained by DIMSCAN analysis of neuroblastoma cell lines treated over a concentration range of 0–4 µg/ml; 100 ng/ml was considered a clinically achievable level (*DX* cell lines established from patients at the time of diagnosis, *PD-Ind* cell lines derived from patients with progressive disease on conventional chemotherapy, *PD-BMT* cell lines derived from patients after myeloablative chemoradiotherapy followed by BMT)

Cross-resistance patterns

Statistical significance of cross-resistance was evaluated for neuroblastoma cell lines by Pearson's correlation (Table 3). Significant cross-resistance was found between ETOP and TPT ($r = 0.67$, $P = 0.002$, 95% confidence interval 0.31–0.86), TPT and SN-38 ($r = 0.79$, $P < 0.001$, 95% confidence interval 0.52–0.92), and ETOP and SN-38 ($r = 0.84$, $P < 0.001$, 95% confidence interval 0.62–0.94).

Discussion

Myeloablative chemotherapy supported with BMT has improved the event-free survival of high-risk neuroblastoma patients [19, 29, 32], but the majority of stage 4 patients diagnosed after 1 year of age still develop recurrent disease refractory to additional treatment. We have shown that sustained drug resistance occurs in neuroblastoma cell lines and that resistance is highest in cell lines established after myeloablative therapy [14, 15]. Development of chemotherapy-resistant disease appears to be the result of selection for tumor cells resistant to those drugs employed in therapy [15]. Identification of drugs that are not cross-resistant from among those drugs in current use for neuroblastoma may overcome treatment failure in patients who develop chemotherapy-refractory disease, and could form the basis of new consolidation chemotherapy regimens which may improve clinical outcome.

In the present work we compared the sensitivity/resistance patterns of neuroblastoma cell lines to topoisomerase I inhibitors (TPT and SN-38), with the

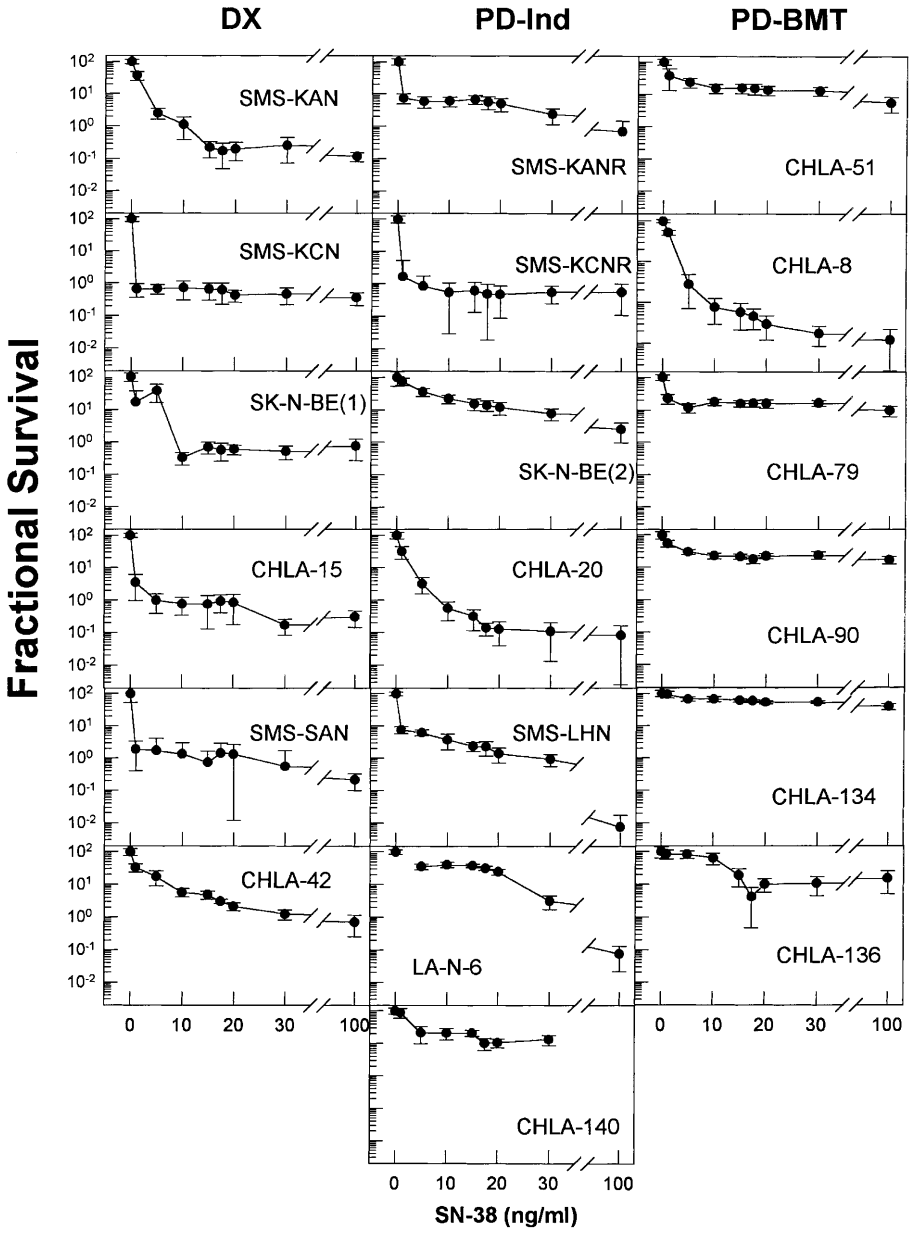

Fig. 3 Dose-response curves for SN-38 obtained by DIMSCAN analysis of neuroblastoma cell lines treated over a concentration range of 0–100 ng/ml; 20 ng/ml was considered a clinically achievable level (*DX* cell lines established from patients at the time of diagnosis, *PD-Ind* cell lines derived from patients with progressive disease on conventional chemotherapy, *PD-BMT* cell lines derived from patients after myeloablative chemoradiotherapy followed by BMT)

topoisomerase II inhibitor, ETOP, a commonly used agent in neuroblastoma. The cell lines utilized in this study were established from patients at various points of treatment and were not selected for resistance in vitro. This cell line panel demonstrated stable drug resistance in the absence of selecting agents, which correlated with the agents and the intensities of therapies to which the tumors were exposed in vivo [14, 15].

ETOP is used during induction and consolidation therapy for most high-risk neuroblastoma patients. The cell lines established from patients at the time of diagnosis prior to chemotherapy exposure were sensitive to this agent. Higher LC_{90} values were demonstrated for cell lines isolated from patients who relapsed during induction chemotherapy, and the LC_{90} values increased even further in cell lines obtained from patients who relapsed after intensive myeloablative chemotherapy and BMT. The LC_{90} values for four of six PD-BMT cell lines were 2.5, 10, 11, and 51 times higher than the clinically achievable CSS for ETOP. Such high resistance to ETOP may contribute to drug-related toxicities without effectively contributing to tumor cell kill in many patients, and identifying non-cross-resistant

Table 3 Cross-resistance among topoisomerase inhibitors. Standardized residuals were utilized as described in Materials and methods. The values shown are Pearson correlation coefficients (values ≥0.6 were considered to indicate significant cross-resistance)

	TPT	SN-38	ETOP
TPT	1		
SN-38	0.79 ($P < 0.001$)	1	
ETOP	0.67 ($P = 0.002$)	0.85 ($P < 0.001$)	1

agents to substitute for ETOP in consolidation therapy may lead to therapy with greater antitumor efficacy.

The camptothecins are a new class of cytotoxic agents that target topoisomerase I [13], a nuclear enzyme that catalyzes the relaxation of supercoiled DNA by introducing transient DNA single-strand breaks. Interestingly, collateral hypersensitivity to topoisomerase II inhibitors has been seen in cell lines from human lung and colon cancer selected in vitro for resistance to camptothecin [33]. In the same model, hypersensitivity to topoisomerase II inhibitors is accompanied by elevated levels of topoisomerase II mRNA and protein, and increased enzymatic activity [33]. In a study using SW480 human colon cancer xenografts, TPT treatment was associated with increased topoisomerase IIα levels and sensitivity to ETOP [35]. It has been suggested that increased sensitivity to topoisomerase II inhibitors in the CPT-resistant models is due to topoisomerase II compensating for the decreased topoisomerase I activity. It is based on this concept that a lack of cross-resistance between topoisomerase I and II inhibitors might be expected.

TPT has been shown to have activity against xenograft models derived from pediatric tumors such as rhabdomyosarcoma, neural tumors, and neuroblastoma in several studies [11, 12, 36]. This agent induces complete and partial remissions in advanced neuroblastoma xenograft models derived from newly diagnosed as well as heavily treated patients at systemic exposures similar to those tolerable in children [37]. In our study, the activity of TPT and SN-38 correlated with that of ETOP. Generally, sensitivity of neuroblastoma cell lines to these agents decreased with therapy: cell lines established from patients prior to their exposure to chemotherapy were sensitive to both camptothecin derivatives, while resistance was greater in cell lines established after induction chemotherapy, and it was highest in cell lines established from patients after intensive chemoradiotherapy. Importantly, resistance to TPT and SN-38 correlated with resistance to ETOP – all six ETOP-resistant cell lines were considered resistant to TPT (LC_{90} value higher than the clinically achievable level) and five of six were resistant to SN-38. Cross-resistance was demonstrated between all three drugs ($r > 0.6$, $P < 0.05$). The CHLA-20 cell line showed a modest level of resistance to ETOP (higher than that of the CHLA-15 cell line which was established at diagnosis from the same patient), but in the CHLA-20 cell line, TPT and SN-38 were more active than ETOP. This suggests that in spite of cross-resistance occurring between topoisomerase I and II inhibitors likely being a common occurrence, tumors with only modest ETOP resistance may show good responses to topoisomerase I inhibitors.

The cross-resistance observed between ETOP and the topoisomerase I inhibitors suggests that TPT and CPT-11 may display a similar pattern of cross-resistance in ETOP-treated neuroblastoma patients. These findings suggest that for patients who have developed high levels of ETOP resistance, TPT and CPT-11 may also be ineffective and that other non-cross-resistant agents should be developed for such patients. Although high-level ETOP resistance was common in cell lines derived after myeloablative therapy, there were some such lines (i.e. CHLA-8 and CHLA-51) that were sensitive to all the topoisomerase inhibitors tested and the tumors that gave rise to those cell lines had been previously treated with ETOP. Thus, it will be important to identify the mechanisms of high-level ETOP resistance so that molecular markers for resistance may be used in the future to stratify patients and avoid the use of relatively ineffective drugs in a setting of significant cross-resistance. Even without such markers, our findings would suggest that resistance to topoisomerase I inhibitors will be common in neuroblastoma patients developing recurrent disease after myeloablative regimens employing ETOP.

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