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ORIGINAL ARTICLE

**William C. Zamboni · Peter J. Houghton
Jeff L. Hulstein · Mark Kirstein · Jessica Walsh
Pam J. Cheshire · Suzan K. Hanna · Mary K. Danks
Clinton F. Stewart**

Relationship between tumor extracellular fluid exposure to topotecan and tumor response in human neuroblastoma xenograft and cell lines

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Abstract *Purpose*: We have reported a 6-fold difference in the topotecan (TPT) lactone systemic exposure achieving a complete response in the human neuroblastoma xenografts NB-1691 and NB-1643. However, the relationship between tumor extracellular fluid (ECF) exposure to TPT and the antitumor activity in xenograft and in vitro models has not been established. *Methods*: TPT was given i.v. to mice bearing NB-1691 and NB-1643 tumors. Prior to dosing, microdialysis probes were placed in tumors of mice bearing NB-1691 and NB-1643 tumors. Plasma and tumor ECF concentrations of TPT lactone were assayed by high performance liquid chromatography. The inhibitory concentration (IC_{50}) was determined for NB-1691 and NB-1643 cell lines in vitro. *Results*: The TPT AUC_{ECF} values determined for NB-1691 ($n = 10$) and NB-1643 ($n = 11$) were 7.3 ± 0.84 and 25.6 ± 0.76 ng h ml^{-1}, respectively ($P < 0.05$). TPT tumor ECF penetration in NB-1691 and NB-1643 was 0.04 ± 0.04 and 0.15 ± 0.11 ($P < 0.05$), respectively. The IC_{50} values recorded after 6 h of TPT exposure daily for 5 consecutive days for NB-1691 and NB-1643 were 2.7 ± 1.1 and 0.53 ± 0.19 ng/ml, respectively ($P < 0.05$). *Conclusions*: NB-1643 was more sensitive in vitro than NB-1691, and at similar plasma TPT exposures, NB-1643 had a greater degree of TPT tumor ECF exposure and penetration as compared with NB-1691. Potential factors affecting tumor TPT ECF disposition include tumor vascularity, capillary permeability, and interstitial pressure. The clinical importance of this study is underscored by the need to select anticancer agents with a high capacity for tumor penetration and to optimize drug administration to increase tumor penetration.

Key words Topotecan · Tumor exposure · Microdialysis · Neuroblastoma

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W.C. Zamboni · J.L. Hulstein · M. Kirstein
J. Walsh · C.F. Stewart (✉)
Department of Pharmaceutical Sciences,
St. Jude Children's Research Hospital,
332 North Lauderdale, Memphis, TN 38105, USA
e-mail: clinton.stewart@stjude.org, Tel.: +1-901-495-3665;
Fax: +1-901-525-6869

P.J. Houghton · P.J. Cheshire · M.K. Danks
Department of Molecular Pharmacology,
St. Jude Children's Research Hospital,
Memphis, Tennessee, USA

P.J. Houghton
Department of Pharmacology, University of Tennessee,
Memphis, Tennessee, USA

C.F. Stewart
The Center for Pediatric Pharmacokinetics and Therapeutics,
University of Tennessee, Memphis, Tennessee, USA

Introduction

Topotecan, a camptothecin analog, has shown a wide range of antitumor activity against adult and pediatric malignancies [1, 2]; however, factors associated with the maximal antitumor response are incompletely understood. Previously we reported the activity of topotecan against panels of rhabdomyosarcoma, medulloblastoma [3, 4], and neuroblastoma xenografts [5]. In addition, we identified a relationship between topotecan lactone systemic exposure (as measured by the area under the plasma concentration-time curve, AUC) and the tumor response in mice bearing human neuroblastoma xenografts [6]. In that study a 6-fold difference was noted in the dose and systemic exposure associated with a complete response in mice bearing human neuroblastoma xenografts NB-1691 (2.0 mg/kg, 290 ng ml^{-1} h) as compared with NB-1643 (0.36 mg/kg, 52 ng ml^{-1} h). Thus, relative sensitivities between different tumors may be characterized by the topotecan dose and systemic exposure required to achieve a response. However, drug delivery to the tumor is determined not only by plasma concentrations but also by distribution from the plasma

into the extracellular fluid of the tumor [7, 8]. Moreover, solid tumors have several barriers to drug delivery that may limit drug penetration and provide inherent mechanisms of resistance [9]. Thus, plasma concentrations may be only surrogate measures of drug exposure within the tumor.

Although concentrations of anticancer agents in the tumor extracellular fluid (ECF) represent exposure in the immediate vicinity of tumor cells [10], a relationship between topotecan tumor ECF exposure and antitumor activity has not been reported. Studies evaluating the intratumoral concentration of anticancer agents are rare in general, and we are unaware of any study of the penetration of camptothecin analogs into tumor ECF. Data from these studies may also provide information on administration schedules that might increase tumor penetration.

Microdialysis is based on the diffusion of analytes from interstitial tissue into the semipermeable membrane of the microdialysis probe [10, 11]. It has been used for in vivo measurement of anticancer drugs in the interstitium of tumors in xenograft models and in patients with accessible tumors [12–16]. Microdialysis provides a technique enabling one to obtain tumor ECF from which a concentration-time profile can be determined. Moreover, pharmacokinetic model parameters can be estimated to describe drug disposition and penetration into tumor. These parameters provide a means of comparing tumor ECF and plasma systemic exposures within the same animal and between different tumors [10, 11].

The objectives of the present study were to evaluate tumor ECF disposition of topotecan lactone using microdialysis methodology and to determine the relationship between topotecan tumor ECF exposure and the antitumor response in mice bearing a relatively resistant (NB-1691) or sensitive (NB-1643) human neuroblastoma xenograft. A pharmacokinetic model that describes topotecan plasma and tumor ECF disposition is provided. We also used in vitro cell-culture studies of the same human neuroblastoma cell lines (NB-1691 and NB-1643) used in the xenograft studies to evaluate growth-inhibitory concentrations (IC_{50}). The results of this study may have specific use in the development of topotecan treatment strategies for patients with neuroblastoma and in the description of differences in the tumor sensitivity of topotecan, as well as providing a broader application to the treatment of adult and pediatric solid tumors.

Materials and methods

Immune deprivation of mice

Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, Me.) aged 4 weeks were immune-deprived by thymectomy followed 3 weeks later by whole-body irradiation (1,200 cGy) using a ^{137}Cs source. Mice received 3×10^6 nucleated bone marrow cells within 6–8 h of irradiation [17, 18]. Tumor pieces of approximately 3 mm^3 were implanted in the space of the dorsal lateral flanks of the mice to initiate tumor growth.

Both NB-1691 and NB-1643 xenografts were composed of heterogeneous mixtures of viable and necrotic cells throughout the tumor matrix. The morphology of the xenografts differed slightly in that NB-1691 tumors had a more solid consistency than did NB-1643 tumors, but there was no evidence of a necrotic center in either type of xenograft. The histology was similar for the larger (i.e., 3.5–5.0 g) tumors used in this study and the smaller (i.e., 1.5–2.0 g) tumors used in our previous studies [3, 5].

Tumor lines

NB-1691 and NB-1643 human neuroblastoma xenografts were obtained from a previously treated and a previously untreated patient, respectively [6]. In addition, both tumors were of stage D and demonstrated amplification of N-MYC. For chemotherapy studies, all tumors were used within six passages of their engraftment in mice. Each tumor grew routinely in $>95\%$ of recipient mice and were human as determined by karyotype.

Formulation and administration

Topotecan powder (SmithKline Beecham, King of Prussia, Pa.) was dissolved in 0.9% NaCl (0.25 mg/ml). Mice (30–38 g, 4–6 months of age) bearing NB-1691 or NB-1643 neuroblastoma xenografts were given topotecan i.v. (0.05 ml/10 g body weight) in a single dose of 1.25 mg/kg delivered as a short injection (<1 min) into the lateral tail vein.

Sample processing and HPLC assay

Heparinized blood samples ($\sim$1 ml) were collected (from three animals per time point by cardiac puncture after methoxyflurane anesthesia) prior to treatment and at 0.25, 1, 2, 4, and 6 h after drug administration. Because of limited blood volume, studies of topotecan plasma and tumor ECF disposition were performed in separate groups of mice. Microdialysis samples were obtained during in vitro and in vivo studies (see below). All plasma samples were handled and processed as previously described [19–22]. For microdialysis samples, 20 µl of dialysate sample was added to 80 µl of cold (-30 °C) methanol. The methanolic mixture was vortexed for 10 s and then centrifuged for 2 min at 10 000 rpm. The supernatant was decanted into a screw-top tube and stored at -70 °C. The injection loop size for plasma samples was 200 µl and for microdialysis samples it was 50 µl. An isocratic high-performance liquid chromatography (HPLC) assay with fluorescence detection was used to determine topotecan lactone plasma and ECF concentrations [22]. The lower limit of assay sensitivity was 0.25 ng/ml.

Microdialysis methodology and tumor ECF sample collection

The principles of microdialysis sampling have previously been reviewed in detail [10, 11, 16]. In brief, a short length of hollow dialysis fiber is continuously perfused with a physiologic solution. The presence of the analyte of interest in the ECF and its absence in the perfusate leads to a concentration gradient across the dialysis membrane. The analyte diffuses through the dialysis membrane and is collected for analysis. This process is performed in vivo through the use of a microdialysis probe that is implanted into tissue and continuously perfused with a physiologic solution at a low flow rate (0.5–10 µl/min). After the probe has been implanted into tumor tissue, substances are filtered by diffusion from the extracellular space through the semipermeable membrane into the perfusion medium and are carried via microtubing into the collection vials.

In the present study a commercially available microdialysis probe (CMA 20, Stockholm, Sweden) with a molecular cutoff of 20 kDa, a membrane length of 4 mm, and an outer diameter of

0.5 mm was used. The probe was perfused by a microdialysis microperfusion pump (CMA 102, Stockholm, Sweden), with Ringer's solution (USP) being used as the perfusion fluid. Dialysate samples were collected by a microfraction collector (CMA 142, Stockholm, Sweden).

In vitro assessment of probe recovery

To characterize the transfer rate, the relative recovery, and the optimal flow rate of the probe we assessed the in vitro recovery of topotecan. The microdialysis probe was placed in a 250-ml glass beaker that contained topotecan at 10 ng/ml in Ringer's solution (USP). The topotecan in vitro solution was changed after the evaluation of every two flow rates, and the concentration of topotecan in this solution was determined prior to and after the evaluation of every two flow rates. The probe was perfused at flow rates of 0.5, 1, 2, 3, 8, and 16 μl/min, and dialysate samples were collected every 25 min. The probe was allowed to reach equilibrium prior to sample collection at each flow rate. Samples ($n = 4$) for each flow rate were analyzed separately by HPLC. In vitro recovery was calculated as follows:

$$\text{In vitro recovery} = \frac{\text{Perfusate conc}_{\text{OUT}}}{\text{In vitro solution conc}} \quad . \tag{1}$$

In vivo *assessment of probe recovery*

The in vitro recovery may be substantially different from the in vivo (i.e., tumor ECF) recovery [10, 11, 23]. Thus, we assessed the in vivo recovery for each probe in each tumor according to the retrodialysis method [10, 11, 23, 24]. Retrodialysis quantification of in vivo recovery is based on the principle that the diffusion process across the microdialysis semipermeable membrane is equal in both directions. Therefore, topotecan can be included in the perfusion medium and its disappearance from the perfusate into the tumor ECF is used as an estimation of in vivo recovery. In vivo recovery is calculated as follows [23, 24]:

$$\text{In vivo recovery} = \frac{\text{Perfusate conc}_{\text{IN}} - \text{perfusate conc}_{\text{OUT}}}{\text{Perfusate conc}_{\text{IN}}} \quad . \tag{2}$$

Thus, the estimated topotecan concentration in the tumor ECF is calculated as follows [23, 24]:

$$\text{Estimated tumor ECF conc} = \frac{\text{Measured microdialysis sample}}{\text{In vivo recovery}} \quad . \tag{3}$$

Microdialysis study procedure

All procedures were approved by the Animal Care and Use Committee at St. Jude Children's Research Hospital. Methoxyflurane anesthesia was used prior to surgical incisions. A 4-cm incision was made along the mid-back, and tissue was retracted laterally to expose the flank tumor. In single-probe studies (NB-1643, $n = 8$; NB-1691, $n = 7$) a microdialysis probe was placed 6 mm into the upper right corner, that is, within 3–5 mm of the surface of the tumor growing in the flank (1.5–2.0 cm^2, 3.5–5.0 g). Dual-probe studies ($n = 3$ for each tumor line) were performed to evaluate the variability of topotecan disposition in the ECF of a single tumor. In dual-probe studies, microdialysis probes were placed 6 mm into the upper right and left corners of the tumor, that is, within 3–5 mm of the surface.

After probe placement a short period (~45 min) was allowed for probe and tumor ECF equilibration prior to the start of retrodialysis calibration [10, 11, 16]. Retrodialysis calibration was performed using a dialysis solution (Ringer's solution, USP, with topotecan at 10 ng/ml) perfused through the probe at 1 μl/min, and dialysate samples were collected by a microfraction collector every 25 min for four samples. After retrodialysis a washout period was performed for 1.7 h, during which the perfusate solution (Ringer's solution) without topotecan was infused at 1 μl/min. During the washout period, dialysate samples were collected every 25 min for four samples. Then, topotecan at 1.25 mg/kg was injected i.v. (< 1 min) into the lateral tail vein. Perfusate solution (Ringer's solution) was infused through the probe at 1 μl/min. Dialysate samples were collected by a microfraction collector every 25 min for 5 h. All samples were processed as described above. At the end of the 5-h collection period the mouse was killed by cervical dislocation after methoxyflurane anesthesia. The tumor was removed and sagittal sections (30 μm) of the tumor were taken to confirm proper probe placement.

Pharmacokinetic analysis

A three-compartment model using maximum-likelihood estimation was fit to topotecan plasma and tumor ECF concentration versus time data (ADAPT II Fig. 1). Plasma concentrations ($n = 3$ per time point) measured in mice bearing NB-1691 or NB-1643 tumors were modeled simultaneously with the tumor ECF concentrations determined for each NB-1691 or NB-1643 xenograft, respectively [25, 26]. Estimated three-compartment model parameters included the volume of the central compartment (V_{c1}), the elimination rate constant (k_{10}) and the intercompartmental rate constants (k_{12}, k_{21}, k_{31}), the maximal rate of topotecan penetration into the tumor ECF (V_{max}), and the plasma concentration occurring when the rate of penetration was at one-half the maximal rate of penetration (K_m). The volume of the tumor compartment (V_{c3}) was set at 5 ml/m^2 as estimated by volume displacement. Pharmacokinetic parameters calculated from these estimates included systemic clearance (CL_{SYS}) and clearance from the tumor ECF (CL_{ECF}) [26, 27]. Areas under the plasma (AUC_{PL}) and tumor ECF (AUC_{ECF}) concentration-time curves were calculated from zero to 5 h and from zero to infinity [26, 27]. Tumor ECF penetration was calculated as the ratio of AUC_{ECF} to AUC_{PL}.

Cell lines

The human neuroblastoma cell lines NB-1643 and NB-1691 were derived from the same tumors described in Tumor lines and were

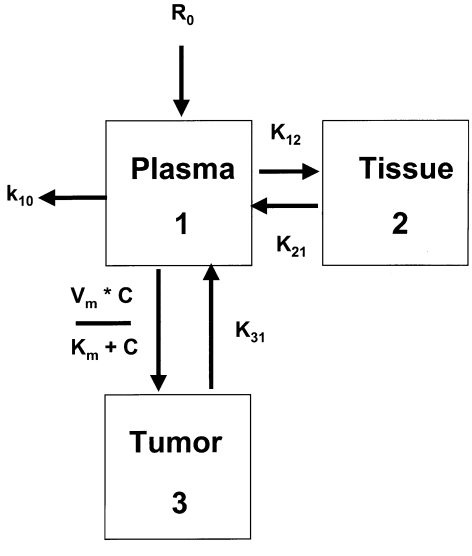

Fig. 1 Three-compartment model describing topotecan plasma and tumor ECF disposition. Model parameters include the volume of the central compartment (V_{c1}), the elimination rate constant (k_{10}) and the intercompartmental rate constants (k_{12}, k_{21}, k_{31}), the volume of the tumor compartment (V_{c3}), the maximal rate of penetration into tumor ECF (V_{max}), and the plasma concentration at one-half V_{max} (K_m)

obtained from the Pediatric Oncology Group cell bank. These cell and xenograft lines were identical as determined by phenotype. The cells were cultured in RPMI-1640 media (BioWhitaker, Walkersville, Md.) supplemented with 10% fetal calf serum (HyClone, Logan, Utah) and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

In vitro growth-inhibition assays

This method has been reported elsewhere [28]. In brief, for determination of the sensitivity of each cell line in vitro to topotecan, cells were plated into 35-mm tissue-culture dishes at a density of 5×10^4 cells/well and were allowed to attach overnight. Stock solutions of topotecan in 10 m*M* TRIS-HCl (pH 4) were prepared and allowed to equilibrate at 4 °C overnight. Drug was added to the tissue-culture medium of NB-1691 and NB-1643 cell lines to achieve a final topotecan total concentration ranging from 0.001 to 10,000 ng/ml. Cells were exposed to topotecan for a single exposure period of 6 h and were exposed for 6 h daily for 5 consecutive days. The cells were allowed to grow for 18 h after the last exposure. Cells were counted, and the concentration of drug producing 50% growth inhibition as compared with controls (IC_{50}) was calculated using Statistica software (StatSoft, Inc. Tulsa, Okla.).

Growth-inhibition studies of NB-1691 and NB-1643 cells were performed in culture medium at a pH of 7.4. At pH 7.4, results of in vitro studies of non-protein-containing buffer solutions of topotecan show that approximately 30% of the drug is in the active-lactone form [28]. In addition, we have conducted in vitro studies in culture medium containing protein (10% fetal bovine serum) at a pH of 7.4, where topotecan lactone accounts for approximately 30% of the total drug content (unpublished observations). Thus, the concentration of topotecan lactone associated with growth inhibition was calculated as 30% of the total topotecan concentration added to the medium.

Statistical analysis

Topotecan plasma and tumor pharmacokinetic parameters are reported as mean values ± standard deviation and median values (range). The concentration-time profile was modeled through a gamma-shape curve, and model parameters were examined by the application of linear regression to log concentration [29]. R-squared was used to examine the model fitness. Student's *t*-test was used to compare IC_{50} values between NB-1691 and NB-1643 cell lines [30]. Since both NB-1691 and NB-1643 were grown in opposite flanks of the same animal we used the Wilcoxon paired-sample test to compare the V_m/K_m ratio, AUC_{ECF}, and tumor ECF penetration between NB-1691 and NB-1643 xenograft lines [30]. The a priori level of significance was set at 0.05.

Results

In vitro and in vivo probe recovery

The difference observed in topotecan concentration in the in vitro solution between the pre- and posttreatment samples was less than 10% for each cohort of two flow rates. The average in vitro topotecan concentration obtainted from the pre- and posttreatment samples was used as the value for calculation of the in vitro recovery. The relative in vitro recovery, depicted as the perfusate infusion rate versus the percentage of recovery, is depicted in Fig. 2. From the results of this experiment we chose a perfusate flow rate of 1 µl/min and a sample time of 25 min for the remainder of the microdialysis studies. This was the lowest flow rate that achieved an adequate recovery (35%) and sample volume (20 µl) for use in our HPLC assay (i.e., lower limit of quantitation 0.25 ng/ml).

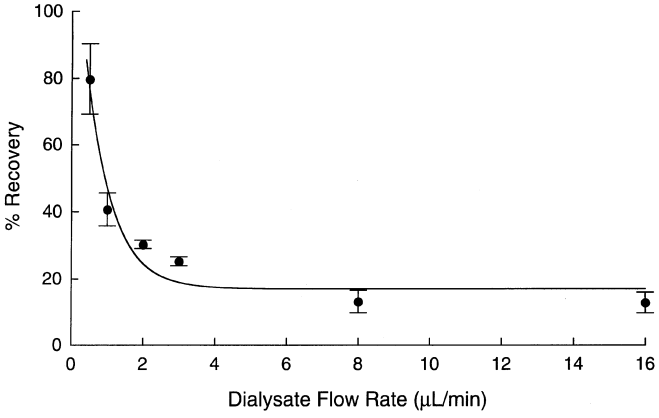

Fig. 2 Relative in vitro recovery of topotecan at a concentration of 10 ng/ml in Ringer's solution, USP. The probe was perfused at flow rates of 0.5, 1, 2, 3, 8, and 16 µl/min, and dialysate samples were obtained every 25 min for 5 samples

The in vivo recovery values as measured by the retrodialysis method for topotecan in the tumor ECF were 0.41 ± 0.10 and 0.36 ± 0.14 for NB-1691 and NB-1643 tumor xenografts, respectively ($P > 0.05$). In the dual-probe studies the ratio of in vivo recovery from probe A to probe B for NB-1691 tumor xenografts was 1.23 ± 0.05 ($P > 0.05$), whereas for NB-1643 tumor xenografts it was 0.85 ± 0.11 ($P > 0.05$). Topotecan was not detectable in the dialysate sample collected at the end of the washout period immediately prior to the beginning of the topotecan experiment. The position of the probe was established at the end of these studies, and studies in which the probe was broken or dislodged from the tumor were excluded (NB-1691, $n = 2$; NB-1643, $n = 3$).

Topotecan tumor ECF and systemic disposition

The results of our dual-probe studies showed that the average (±SD) ratios of probe A to probe B in NB-1691 tumor xenografts as recorded for AUC_{ECF} and tumor penetration were 1.2 ± 0.8 (range 0.45–2.4, $P > 0.05$) and 1.2 ± 0.9 (range 0.45–2.4, $P > 0.05$), respectively. The average (±SD) ratios of probe A to probe B in NB-1643 tumor xenografts as noted for AUC_{ECF} and tumor penetration were 0.69 ± 0.33 (range 0.43–1.1, $P > 0.05$) and 0.68 ± 0.32 (range 0.43–1.1, $P > 0.05$), respectively.

Representative topotecan plasma and tumor ECF concentration-time plots generated for mice bearing NB-1691 and NB-1643 tumors are presented in Fig. 3. Topotecan pharmacokinetic parameters recorded for NB-1691 and NB-1643 are summarized in Table 1. The

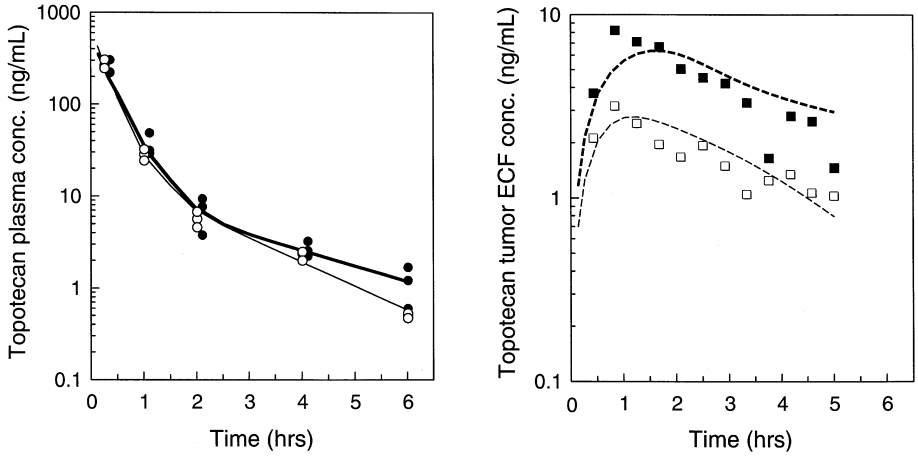

Fig. 3 Representative topotecan plasma (*left*) and tumor ECF (*right*) concentration-time plots generated for mice bearing NB-1691 and NB-1643 tumors. Individual data points and the best-fit line of the data are represented for (*left*) topotecan lactone plasma concentrations measured in mice bearing NB-1643 (—, ●) and NB-1691 (—, ○) human neuroblastoma tumor xenografts, and (*right*) topotecan lactone tumor ECF concentrations measured in mice bearing NB-1643 (- - ■ - -) and NB-1691 (- - - □ - - -) human neuroblastoma tumor xenografts. The topotecan lactone tumor ECF $AUC_{0\rightarrow5}$ values recorded for the representative NB-1643 and NB-1691 tumor xenografts were 22.3 and 9.1 ng ml^{-1} h, respectively

percentage of topotecan AUC_{ECF} that was extrapolated was greater than 30% in 6 of 10 tumors in mice bearing NB-1691 xenografts and in 5 of 11 tumors in mice bearing NB-1643 xenografts. Thus, we chose to calculate the topotecan AUC_{PL} and AUC_{ECF} from time zero to the last measured time point (i.e., 5 h).

Topotecan tumor ECF exposure and antitumor sensitivity

Previously we have reported a 6-fold difference in the dose and systemic exposure associated with a complete response in mice bearing NB-1691 (2.0 mg/kg, 290 ng ml^{-1} h) as compared with NB-1643 (0.36 mg/kg, 52 ng ml^{-1} h) xenografts [6]. The topotecan AUC_{PL} values from zero to 5 h as recorded in the present study after a dose of 1.25 mg/kg for NB-1691 and NB-1643 tumors were 173.6 and 171.9 ng ml^{-1} h, respectively ($P > 0.05$). The average (±SD) V_m/K_m ratios noted for NB-1691 and NB-1643 were 0.001 ± 0.001 and 0.010 ± 0.014, respectively ($P < 0.05$). The average (±SD) AUC_{ECF} values recorded for NB-1691 and NB-1643 tumors were 7.26 ± 6.09 and 25.6 ± 19.5 ng ml^{-1} h, respectively ($P < 0.05$). The average (±SD) tumor penetration calculated for NB-1691 and NB-1643 xenografts was 0.04 ± 0.04 and 0.15 ± 0.11, respectively ($P < 0.05$).

Growth-inhibition assays

Growth-inhibition curves for NB-1691 and NB-1643 human neuroblastoma cell lines were constructed after a single 6-h period of topotecan exposure and after 6 h of daily exposure to topotecan for 5 consecutive days. The topotecan IC_{50} values recorded for NB-1691 and NB-1643 neuroblastoma cell lines after a single 6-h period of exposure and following daily exposure for 6 h on 5

Table 1 Summary of topotecan lactone plasma and tumor ECF pharmacokinetic parameters in mice bearing NB-1691 ($n = 7$ and 3 for single- and dual-probe studies, respectively) and NB-1643 ($n = 8$ and 3 for single- and dual-probe studies, respectively) human neuroblastoma tumor xenografts. Parameters are presented as mean values (±SD) and median values (range)

Parameters	NB-1691			NB-1643		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range
K_{10} (h^{-1})	3.1 ± 0.29	3.2	2.4–3.5	2.3 ± 0.62	2.4	0.85–3.2
K_{12} (h^{-1})	0.42 ± 0.09	0.45	0.20–0.52	0.55 ± 0.57	0.37	0.14–2.3
K_{21} (h^{-1})	0.68 ± 0.10	0.72	0.43–0.76	0.54 ± 0.42	0.44	0.08–1.7
V_{c1} (l/m^2)	6.4 ± 0.84	6.4	5.3–8.7	8.0 ± 2.0	8.2	3.6–10.7
CL_{sys} ($l\ h^{-1}\ m^{-2}$)	19.8 ± 0.09	20.2	18.2–21.0	17.7 ± 4.3	19.9	9.1–22.4
V_m (ng/h)	0.002 ± 0.002	0.002	0.0004–0.007	0.03 ± 0.04	0.014	0.0014–0.098
K_m (ng/ml)	4.6 ± 4.5	2.3	0.39–14.0	33.0 ± 91.6	3.7	0.05–322.5
K_{31} (h^{-1})	1.1 ± 0.69	1.2	0.06–2.8	1.9 ± 1.8	1.1	0.41–6.1
CL_{ECF} ($ml^{-1}\ h^{-1}\ m^{-2}$)	5.6 ± 3.3	6.0	0.3–14.0	9.5 ± 8.9	5.5	2.1–30.5
AUC_{ECF} (ng ml^{-1} h)	7.3 ± 6.1	5.6	1.3–23.7	25.6 ± 19.5*	18.0	9.6–67.8
ECF penetration	0.04 ± 0.04**	0.03	0.01–0.14	0.15 ± 0.11	0.10	0.06–0.39

*$P < 0.05$ versus NB-1691 xenografts; **$P < 0.05$ versus NB-1643 xenografts

Table 2 IC_{50} values recorded for topotecan in NB-1643 and NB-1691 human neuroblastoma cell lines after a single 6-h exposure period and following daily 6-h periods of exposure for 5 consecutive days. IC_{50} data are presented as average values ± SD

	6-h Exposure IC_{50} (ng/ml)	6-h Exposure daily for 5 days IC_{50} (ng/ml)
NB-1643	10.1 ± 6.74*	0.53 ± 0.19
NB-1691	32.1 ± 22.1*	2.70 ± 1.14

*$P < 0.05$ versus NB-1691

consecutive days were derived from these curves and are presented in Table 2. Consistent with our previous studies of neuroblastoma xenografts, we noted a steep response relationship between topotecan lactone exposure and inhibition of tumor cell growth (data not presented) [6, 28].

Discussion

Although the clinical response of children with neuroblastoma to topotecan has been significant, factors associated with a favorable antitumor response are incompletely understood. In the xenograft model of childhood neuroblastoma we have shown that the antitumor activity is directly related to the topotecan dose and systemic exposure [6]. Results of these studies have identified two neuroblastoma tumor xenograft lines that respond differently to topotecan, one being relatively resistant (NB-1691) and one, relatively sensitive (NB-1643). Using these two lines, we showed in the present study that NB-1643 had a 3.5-fold higher tumor ECF exposure to topotecan than did NB-1691. Application of pharmacokinetic modeling to these data provides a mathematical model that describes the differences in topotecan disposition and penetration into tumor ECF between these two neuroblastoma xenograft lines. Our results suggest that topotecan tumor penetration may be one factor associated with neuroblastoma antitumor response.

Plasma systemic exposure is another factor that may account for the difference in antitumor effect observed with topotecan. However, some tumors fail to respond even on exposure to putative cytotoxic plasma concentrations. This is not unexpected, since barriers to drug delivery, penetration, and distribution exist in solid tumors [7, 8]. At best, plasma systemic exposure serves only as a surrogate measure of drug exposure in the tumor. Thus, exposure to anticancer drugs in tumor ECF represents a measure of drug exposure closer to the site of drug action.

Microdialysis has recently been used to evaluate tumor ECF disposition of anticancer agents in tumor xenografts and in patients with solid tumors [10, 11]. Microdialysis provides several advantages over autoradiography studies of tumor biopsies in the measurement of anticancer drug concentrations in tumor tissue. With microdialysis techniques it is possible to obtain serial sampling of anticancer drugs from the ECF of a single tumor with minimal tissue damage or alteration of fluid balance. Furthermore, the drug concentration can be measured specifically as opposed to quantitation of radioactivity, which may be nonspecific. Thus, the data obtained with microdialysis techniques may more closely reflect drug disposition within the tumor ECF [10, 11].

Even though microdialysis technology has several advantages, its use in the evaluation of tumor extracellular disposition of anticancer agents has several possible limitations. Although the use of the microdialysis technique results in less tissue damage as compared with other sampling methods (e.g., biopsy), insertion of the microdialysis probe into the tumor does induce some tissue damage and immune reactivity. Thus, samples collected immediately after probe insertion may not reflect basal tumor conditions due to acute tissue damage and changes in blood flow associated with probe insertion [10]. Therefore, we allowed 45 min for probe and tumor ECF to equilibrate prior to the start of the probe calibration studies. Microdialysis is a dynamic process in which substances are continuously removed from the tumor ECF by diffusion into the probe. Consequently, the concentration of drug in the perfusate does not reach equilibrium with the tumor ECF. However, under constant conditions (i.e., perfusate flow rate) a steady-state percentage of recovery that represents a constant fraction of the ECF concentration will be reached. In the present study we used the retrodialysis calibration method to estimate the steady-state percentage of recovery [10, 11, 23, 24]. This approach assumes that diffusion properties are equal on each side of the dialysis membrane. This method is performed in each tumor with each probe and, thus, is tissue-specific. This provides the advantage of accounting for processes that affect recovery in tissues and tumors. The in vivo recovery values as measured by retrodialysis were similar within and between NB-1691 and NB-1643 xenograft lines.

Given the heterogeneity of tumor tissue, we were concerned as to whether sampling of tumor ECF from a single microdialysis probe would be representative of the topotecan exposure throughout the tumor. For evaluation of the variability within a tumor, dual-probe studies were performed in which sampling of tumor ECF was performed simultaneously from two parallel sites within a single tumor. Since we found that topotecan disposition was relatively similar within a single tumor (i.e., the maximal difference between the probe A and B AUC_{ECF} was 2.4- and 2.3-fold for NB1691 and NB1643, respectively) as compared with the difference seen between the two xenograft lines, we conducted the remainder of our studies on these tumor lines using a single probe. However, we did observe sufficient variability that for the study of other tumors we would recommend that dual-probe studies be performed to characterize the intratumor variability. In addition, we found no relationship between the tumor size and topotecan disposition and intratumor variability. This may be

attributable to the relatively similar size of the tumors and to the placement of the probes near the tumor surface.

The differences in penetration and exposure observed between the neuroblastoma xenograft lines may be a result of a higher degree of vascularity or capillary permeability in NB-1643 xenograft tumors as compared with NB-1691 xenograft tumors. A study of children with primary untreated neuroblastoma found that a high degree of tumor neovascularization strongly correlated with widely disseminated disease ($P = 0.006$) and poor survival ($P < 0.0001$) [31]. The results of this study suggest that angiogenesis may play an important role in determining the biologic behavior of neuroblastoma. In addition, the greater interstitial pressure in NB-1691 tumor xenografts may decrease the diffusion of topotecan from the capillaries into the tumor ECF. Consistent with these possibilities is the higher delivery of topotecan into the tumor ECF, as estimated by the V_m/K_m ratio, in NB-1643 xenograft tumors as compared with NB-1691 xenograft tumors.

The results of the in vitro growth-inhibition studies on NB-1643 cell lines were consistent with the increased tumor ECF exposure observed in the sensitive NB-1643 line as compared with the resistant NB-1691 xenograft line. However, the difference (i.e., approximately 6-fold) observed in the dose and associated topotecan systemic exposure between the resistant and the sensitive xenograft line was not a product of the 4-fold difference in intrinsic sensitivity, as measured by the in vitro assay, and the 3.5-fold difference in tumor exposure. This suggests there may be a different basis for the difference in sensitivity seen between the in vitro and in vivo models. Moreover, this raises a novel aspect in that although in vivo sensitivity correlates with in vitro results, the basis for this may differ between the two growth conditions.

The development of effective chemotherapeutic agents for the treatment of solid tumors depends in part on the ability to achieve cytotoxic drug exposure within the tumor. Previously, we reported on the relationship between topotecan plasma exposure and response in mice bearing NB-1643 (sensitive) and NB-1691 (resistant) tumor xenografts. However, due to barriers to drug penetration and distribution in solid tumors, plasma concentrations are not direct measures of drug exposure at the tumor site. Thus, we used microdialysis methodology to evaluate tumor ECF exposure to topotecan in mice bearing NB-1643 and NB-1691 human neuroblastoma xenografts. Consistent with the difference in plasma exposure associated with response, NB-1643 xenografts had a 3.5-fold higher tumor penetration and tumor ECF exposure to topotecan as compared with the resistant NB-1691 xenografts. Moreover, these data suggest inherent differences in tumor vascularity, capillary permeability, and/or tumor interstitial pressure between NB-1643 and NB-1691 human neuroblastoma tumor xenografts, resulting in altered penetration of topotecan in the tumor ECF. The clinical importance of this study is underscored by the need to develop treatment strategies for adults and children with solid tumors, to select anticancer agents with characteristics associated with high degrees of tumor penetration, and to optimize dosing and administration schedules to increase tumor penetration.

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