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ORIGINAL ARTICLE

**Joyce Thompson · Sylvie M. Guichard
Pamela J. Cheshire · Lois B. Richmond
Catherine A. Poquette · Susan T. Ragsdale
Bruce Webber · Robert Lorsbach
Mary K. Danks · Peter J. Houghton**

Development, characterization and therapy of a disseminated model of childhood neuroblastoma in SCID mice

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Abstract *Purpose*: To develop a highly reproducible model of disseminated childhood neuroblastoma in mice to allow secondary evaluation of therapeutics against microscopic disseminated disease. *Methods*: CB17/Icr SCID were injected i.v. with 10^3 to 5×10^6 human NB-1691 neuroblastoma cells. NB-1691 cells were detected by PCR for synaptophysin and tyrosine hydroxylase in peripheral blood, and bone marrow. Therapeutic studies evaluated topotecan and vincristine as single agents or in combination. Topotecan was administered i.v. daily for 5 days on two consecutive weeks. Courses were repeated every 21 days for three cycles. Vincristine (1 mg/kg) was administered i.v. every 7 days for nine consecutive weeks. Treatment started 11–21 days after tumor cell inoculation. *Results*: Following injection of $\geq 1 \times 10^5$ cells 100% of mice developed disease. Mice inoculated with 10^7 cells survived a median of 42 days. Survival time was a linear function of the cell inoculum. At autopsy, gross tumor was routinely detected in many organs in particular liver, ovaries, kidneys and adrenals. NB-1691 cells were detected by PCR in peripheral blood, and bone marrow. Immunohistochemical staining showed that lesions were strongly positive for synaptophysin, chromogranin A and negative for leukocyte common antigen. Topotecan (0.6 mg/kg) alone extended median survival from 44 days (controls) to 95 days. When treatment was started 21 days after inoculation of NB-1691 cells, topotecan extended median survival from 39 days (controls) to 91 and 99 days at dose levels of 0.3 and 0.6 mg/kg, respectively. Vincristine (1 mg/kg) extended survival by a median of 9.5 days. In combination with vincristine (1 mg/kg), median survival was increased to 141 days (topotecan 0.6 mg/kg) and 159 days (topotecan 1.0 mg/kg). *Conclusion*: This model of disseminated neuroblastoma is highly reproducible. As this model may more closely simulate childhood disease it may be a valuable adjunct in developing new approaches to advanced stage, poor prognosis neuroblastoma.

Key words Neuroblastoma · SCID mice · Topotecan · Vincristine

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J. Thompson · S. M. Guichard[1] · P. J. Cheshire
L. B. Richmond · M. K. Danks · P. J. Houghton (✉)
Department of Molecular Pharmacology,
St Jude Children's Research Hospital, 332 N. Lauderdale,
Memphis, TN 38105-2794, USA
E-mail: peter.houghton@stjude.org
Tel.: +1-901-4953440; Fax: +1-901-5211668

C. A. Poquette
Department of Biostatistics and Epidemiology,
St Jude Children's Research Hospital, Lauderdale,
Memphis, TN 38105-2794, USA

S. T. Ragsdale
Department of Experimental Oncology,
St Jude Children's Research Hospital, Lauderdale,
Memphis, TN 38105-2794, USA

B. Webber
Department of Pathology, Baptist Memorial Hospital,
Memphis, TN, USA

R. Lorsbach
Department of Pathology, St Jude Children's Research Hospital,
Lauderdale, Memphis, TN 38105-2794, USA

Present address:
[1]Institute Claudius Regaud, 20-24,
rue du pont St. Pierre, Toulouse, Cedex France 31052

Introduction

Neuroblastoma is the most common extracranial solid malignancy of childhood. It is thought to arise from embryonic neural crest tissue and can occur anywhere along the sympathetic nervous system. The majority of patients present with metastatic disease at diagnosis [1]. Treatment modalities traditionally used in neuroblastoma include chemotherapy, surgery and radiotherapy. However, chemotherapy continues to be the cornerstone

of management for patients with unresectable localized tumors or disseminated disease. Prognosis for children over 1 year of age with advanced-stage disease remains poor and only 12–20% achieve long-term survival with conventional chemotherapy [1–7]. The increasing use of high-dose myeloablative therapy with autologous or allogeneic bone marrow transplantation [8–15] appears to improve survival rates, but definitive conclusions await the outcome of ongoing randomized prospective trials.

Neuroblastoma grafted directly, or after in vitro culture, into athymic nude mice has provided some insights into the biology and chemosensitivity of these tumors. In vivo models of neuroblastoma afford the opportunity to evaluate potentially useful therapies in both untreated and clinically drug-resistant forms of this tumor [16]. A major obstacle towards developing relevant in vivo models has been the poor success rate in establishing xenografts directly from patient specimens [17, 18]. In contrast, the use of established cultured human neuroblastoma cell lines inoculated into athymic nude mice has resulted in growth of tumors. Numerous models of neuroblastoma have been developed since the initial report of the C1300 murine model. Each model has its own value and limitations.

Current in vivo models represent syngeneic or transplantable tumors of murine or human origin. More recently, a transgenic model of MYCN overexpression has been reported [19], but as yet the use of this model for therapeutic studies has not been reported. Immunotherapy experiments using syngeneic models have relied almost exclusively on variants of the C-1300 neuroblastoma (reviewed in reference 20). Tsuchida et al. have extensively characterized the chemosensitivity of subcutaneously (s.c.) implanted human neuroblastomas in nude mice [21] and have also demonstrated fidelity of these models. Of note is that xenografts can be established only from tumors with unfavorable histology, as defined by Shimada classification criteria, and successful growth in mice is associated with a dismal patient outcome. These results are consistent with those from other groups who have reported establishing tumors as xenografts only from advanced-stage disease [22]. The use of athymic nude mice however is limited because of the environmental and breeding requirements and the cost to investigators.

As an alternative we have demonstrated that neuroblastomas can successfully be heterografted into immune-deprived mice [23–25] and used for therapeutic studies. As with other models that represent human tumors, it is essential that in mice they retain the chemosensitivity of tumors in patients. Our own studies, and those of others, have indicated that chemosensitivity profiles are retained when tumors are heterografted into mice. Despite the description of human neuroblastoma xenografts and their proposed value for developing therapy very few studies have been published. Extensive experience with pediatric solid tumor xenografts has already suggested these models may be of value in identifying novel or new chemotherapeutic agents [26, 27]. These experiments may be of particular importance to drug development in rare malignancies such as pediatric cancers. However, since s.c. transplanted human tumors rarely metastasize in mice, these models do not accurately recapitulate the biologic characteristics of neuroblastoma in children. The need for a suitable animal model of disseminated neuroblastoma to prospectively study and test new therapeutic strategies is important and has been emphasized by others [28, 29]. Such models should mimic, as far as possible, the biologic behavior of the human disease and be easily reproducible.

Here we detail the development, characterization, and therapeutic application of the human neuroblastoma line NB-1691 grown as disseminated disease in the severe combined immune deficiency (SCID) mouse.

Materials and methods

Mice

Female CB-17/Icr SCID mice at 8–13 weeks of age were obtained from the SJCRH breeding colony. They were housed in a positive pressure HEPA filtered barrier room (25 °C) in autoclaved polycarbonate cages. All litter, food, and water were autoclaved. Mice were exposed to 12-h cycles of light and dark. All aspects of the animal work were performed in accordance with protocols approved by the animal care and use committee.

Tumor

The NB-1691 neuroblastoma was initially cultured in vitro. Cells were grown in RPMI-1640 with 10% fetal calf serum and 2 m*M* glutamine in a humidified atmosphere of 5% CO_2/95% air. When the monolayers became confluent, cells were harvested (0.5% trypsin, 0.53 n*M* EDTA solution) and triturated to produce a single-cell suspension. The cells used were from early passages of the original isolates (passage less than 20). The cell line was established from a patient with stage D, MYCN-amplified disease originating in the adrenal. Prior to biopsy the patient had been treated with cytosine arabinoside, daunorubicin, 6-thioguanine, etoposide and 5-azacytidine. We have reported previously the growth and chemosensitivity of NB-1691 s.c. xenografts maintained in immune-deprived CBA/CaJ mice [25].

Establishment of xenografts

To establish s.c. xenografts in SCID mice each received 2×10^7 NB-1691 cells (suspended in 0.1 ml serum-free tissue culture medium) injected s.c. into bilateral dorsal flanks. For therapeutic trials mice were implanted s.c. with a single tumor piece. All procedures used aseptic technique in a class 2 biologic safety cabinet. After tumors became palpable, serial growth was determined weekly by measurement of two perpendicular diameters using vernier calipers. Tumor volumes were calculated assuming tumors to be spherical using the formula $(\pi/6 \times d^3)$, where d is the mean diameter [25]. The growth rate of s.c. tumors was determined by plots of tumor volume versus time.

Development of disseminated neuroblastoma

Cells were grown in vitro, as described above. SCID mice were injected with 1×10^7 NB-1691 cells via the lateral tail vein using a

25-gauge needle attached to a 1-ml syringe. The mice were monitored daily for any signs of sickness manifested by slow or difficult movement, arched backs or enlargement of the abdomen. All mice were killed when any of the preceding symptoms were noted, but some mice died before this became necessary. The decision to kill animals was made by two independent observers.

Postmortem pathologic examination

Postmortem examinations were performed on all animals killed or found dead (if minimal tissue autolysis). Tissue specimens from sites of tumor growth were taken for histologic examination, and for fluorescence in situ hybridization (FISH) analysis to identify MYCN amplification. All tissues for histology were fixed in 10% buffered formalin, paraffin embedded, sectioned, and routinely stained with hematoxylin and eosin (H&E).

Immunohistochemistry

All tissues were fixed in 10% buffered formalin. Paraffin sections of thickness 5 μm were prepared and stained with H&E. Immunoperoxidase staining was performed on paraffin sections of formalin-fixed tissue following heat-induced antigen retrieval. The following antibodies were employed: antihuman chromogranin A (LK2H10(2); Boehringer Mannheim), antihuman synaptophysin (rabbit polyclonal; Zymed Laboratories), and antihuman leukocyte common antigen (RP2/18; Ventana). Antigen detection was performed using an automated stainer and a Basic DAB detection kit from Ventana.

Detection of bone marrow disease and NB cells in peripheral blood

Microscopic disease was detected by RT-PCR and Southern blotting of the PCR products, all by standard methods. RNA was extracted using the SNAP kit from Invitrogen (Carlsbad, Calif.). The white blood cell fraction was separated by a standard Ficoll procedure. Forward and reverse primers for tyrosine hydroxylase spanned two exons to eliminate PCR products that might result from contaminating DNA. Primers for β-actin were used as positive controls for the PCR reaction. A probe end-labeled with ^{33}P which did not overlap the primers for tyrosine hydroxylase and synaptophysin were used to verify the identity of the PCR products. Specific primer sequences, the sensitivity of the method, and detailed procedures will be described completely in a separate report.

Blood and marrow samples from which RNA was extracted were obtained from two mice at each time-point. Time-points included day 0 prior to injection of NB-1691 cells i.v. and at weekly intervals thereafter from day 7 through day 42. On day 42, only one sample was available, as the second mouse for that time-point had died.

Cytogenetic analysis

The karyotype of the parent cultured cell line and the corresponding xenograft and disseminated model were obtained by a modified trypsin-Wright technique [30]. Ten metaphases from each were karyotyped and chromosomes identified and numbered according to the International System of Human Cytogenetic Nomenclature (1995).

Fluorescence in situ hybridization

Cells in culture, fresh s.c. tumor, and metastatic tumor in the SCID model were all examined for the presence of MYCN copy number by FISH. MYCN gene amplification was investigated by simultaneous interphase and metaphase FISH [31]. A minimum of 25 nuclei were analyzed for each sample.

Therapeutic studies

Topotecan was kindly provided by SmithKline Beecham (King of Prussia, Pa.). Topotecan was dissolved in 0.9% sodium chloride USP, filter sterilized, and administered i.v. to SCID mice by direct tail vein injection (duration of infusion < 1 min). Commercially available vincristine (Eli Lily and Company, Indianapolis, Ind.) was also administered by a short i.v. infusion (< 1 min). Animals received topotecan alone, or vincristine alone, or topotecan in combination with vincristine.

Cohorts of mice ($n = 5$ or 6) were treated with topotecan i.v. at doses of 0.3, 0.6, or 1.0 mg/kg per day. Drug was administered daily for 5 days on two consecutive weeks followed by a 9-day rest period, referred to as one cycle of therapy. Mice received a maximum of three cycles, designated [(d × 5)2]3, over an 8-week period. Vincristine was administered at a fixed dose (1 mg/kg), and was injected every week for nine consecutive weeks. For combination studies, vincristine was administered on the first day of the initial topotecan course, and at 7-day intervals thereafter for nine consecutive weeks.

Statistical methods

Event-free survival (EFS) was defined as the time interval (in weeks) from initiation of therapy to death from any cause (disease, killing). EFS was estimated using the method of Kaplan and Meier. Exact log-rank tests were used to examine differences in EFS among treatment groups; no adjustments were made for multiple comparisons. Two-sided *P*-values are presented. As there were no censored mice the median survival denotes the time (days) at which 50% of the mice were still alive and 50% had died or been killed.

Results

Tumor characteristics

The original patient NB-1691 tumor was composed of both well differentiated and poorly differentiated neuroblastoma. Histologically, there were stroma-poor areas containing closely packed cells with rounded nuclei and other areas which were stroma-rich. There was some rosette formation, and calcification was evident. In the SCID mouse models, however, only the poorly differentiated component was evident.

NB-1691 cell line in culture, as a s.c. xenograft, and in the disseminated model, demonstrated amplification of MYCN. The source of the amplified MYCN signal was almost exclusively in the form of expanded intrachromosomal homogeneously staining regions (HSRs). Representative FISH data demonstrating MYCN gene amplification from one of the SCID models are shown in Fig. 1. No difference was noted in the number of HSRs between the NB-1691 cell line in culture and tumors from the mice. In all cases the amplified MYCN remained in the form of HSRs. The karyotype of the NB-1691 neuroblastoma cell line was also compared with the corresponding xenograft and SCID model. Cytogenetics demonstrated cells to be hyperdiploid with an HSR on chromosome 3.

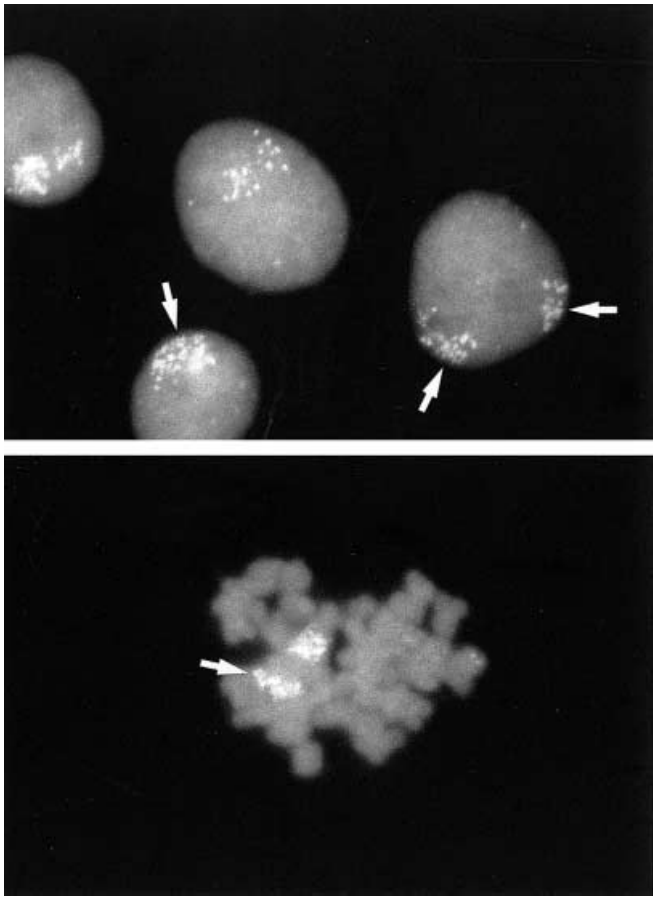

Fig. 1 **A** MYCN amplification (*arrows*) in homogeneously staining regions (HSRs) detected by FISH in interphase NB-1691 cells. **B** Metaphase FISH showing an HSR on a marker chromosome from NB-1691 tumor

Disseminated model of neuroblastoma

To determine whether injection of NB-1691 cells i.v. would result in disseminated disease in organ sites associated with clinical neuroblastoma, SCID mice were given with 1×10^7 cells by slow tail vein injection. Mice were observed daily, and were killed using criteria described in Materials and methods. The median survival was 45 days (range 44 to 50 days). Disseminated neuroblastoma was demonstrated at autopsy in all mice injected with NB-1691 cells (Fig. 2). The macroscopic anatomic sites of tumor growth included liver, kidneys, ovaries, adrenal glands, spleen, heart, lungs, lymph nodes and s.c. sites. Ascites, often hemorrhagic, was also present in the majority of mice. Microscopic examination of all tumors revealed similar histopathologic features. The tumors were comprised of spindle cells arranged in a cohesive, nested pattern; perivascular rosette-like structures were noted focally (Fig. 3A). The tumor cell nuclei contained fine chromatin with indistinct nucleoli. Immunohistochemical analysis of multiple tumors showed that they were uniformly, strongly reactive for the neural markers chromogranin A and synaptophysin, supporting the neuroblastic origin of the tumor (Fig. 3B, C). No tumor cells were positive for leukocyte common antigen (Fig. 3D).

To further characterize the model, we injected cohorts of mice ($n = 10$) with 1×10^3 to 1×10^7 NB-1691 cells. Mice were observed for development of disease and killed using criteria described above. After injection of $\geq 10^5$ cells 100% developed disease. Linear regression of survival of groups that had 100% incidence of disease (10^5 to 10^7 cells injected) indicated that a tenfold decrease in cell inoculum resulted in an increase of 12.8 days in lifespan.

Detection of NB-1691 cells in bone marrow and peripheral blood

Tyrosine hydroxylase mRNA, synaptophysin mRNA or both was detected in circulating cells coisolated with the white blood cell fraction at all time points following i.v. injection of NB-1691 cells (Fig. 4A). The single exception to this result was the sample from one of the mice killed on day 35 in which mRNA for neither synaptophysin nor tyrosine hydroxylase was detected. We conclude that the peripheral blood did not clear of tumor cells at any time following i.v. injection of NB-1691 cells. Tyrosine hydroxylase mRNA was not detected in bone marrow samples until the third week following i.v. injection of NB-1691 cells (Fig. 4B). The mRNA was detected from day 21 until the experiment was terminated. It is not known whether the second RNA sample obtained on day 28 contained intact RNA and was truly negative for expression of tyrosine hydroxylase, since the β-actin control was also negative. We conclude that the occurrence of bone marrow disease was a delayed event in this model of disseminated neuroblastoma.

Therapeutic studies using the NB-1691 model

We have previously reported that NB-1691 s.c. xenograft in immune-deprived CBA/CaJ mice is relatively sensitive to the topoisomerase I poison, topotecan [25]. For dose levels that resulted in clinically relevant plasma systemic exposures of the lactone form (approximately 20–90 ng · h/ml) partial responses or stable disease were observed in that model. When grown as s.c. xenografts in SCID mice, NB-1691 tumors were slightly less sensitive to topotecan than in the CBA/CaJ host (Fig. 5). Topotecan caused significant growth retardation when administered at 0.61 mg/kg, and partial regressions at the higher dose level of 1 mg/kg administered using the [(d × 5)2]3 i.v. schedule. Of note was the rapid progression of tumor growth at the end of treatment (week 8). Responses of tumors in individual mice were highly consistent, as were the growth rates of tumors in untreated SCID mice.

We next evaluated the use of topotecan against systemic neuroblastoma. In the first study mice received topotecan i.v. at a dose of 0.6 mg/kg using the [(d × 5)2]3

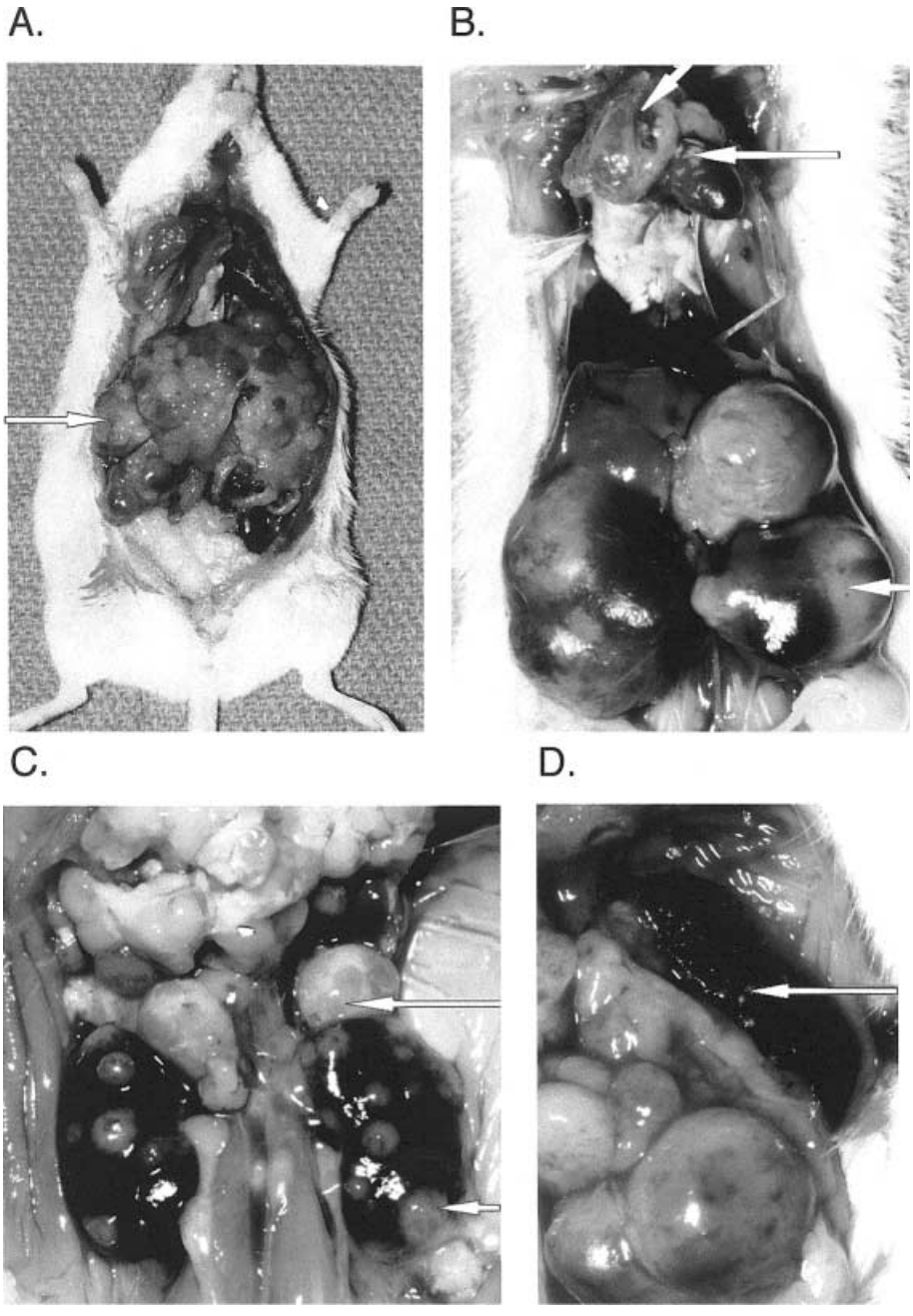

Fig. 2A–D Dissemination of i.v. injected NB-1691 cells in SCID mice (**A** Metastases to liver; **B** mediastinum, heart and ovary; **C** deposits in adrenal gland and kidney (arrows); **D** tumor deposits in the spleen). Tumor sites are indicated by *arrows*

schedule starting 11 days after cell inoculation. There was evidence of a statistically significant difference between the EFS for mice in the control group and mice in the 0.6 mg/kg topotecan group ($P = 0.001$). Mice in the control group survived a median of 44 days as compared to those receiving 0.6 mg/kg in which median survival was 95 days (Fig. 6A). At autopsy all mice had disseminated disease. Of note is the narrow range for survival times of mice in the control group.

We also evaluated topotecan against a more advanced stage of disease in which macroscopic tumor foci were already detectable by visual examination. Topotecan treatment was commenced on day 21 following injection of cells. Median survival for the control group was 39 days. Here again there was evidence of a statistically significant difference between the EFS for mice in the control group and mice in both the 0.6 mg/kg topotecan group (median survival 99 days) ($P = 0.008$) and the 0.3 mg/kg topotecan group (median survival 91 days) ($P = 0.008$). In addition, in this study there was also a significant difference between treatment with topotecan 0.6 mg/kg and 0.3 mg/kg ($P = 0.016$; Fig. 6B).

Recently we have reported synergy between topotecan and vincristine in several s.c. models of pediatric solid tumors, including NB-1691 neuroblastoma [32]. It was of interest, therefore, to determine whether similar activity could be demonstrated using this disseminated model. Vincristine was administered at a dose of 1 mg/

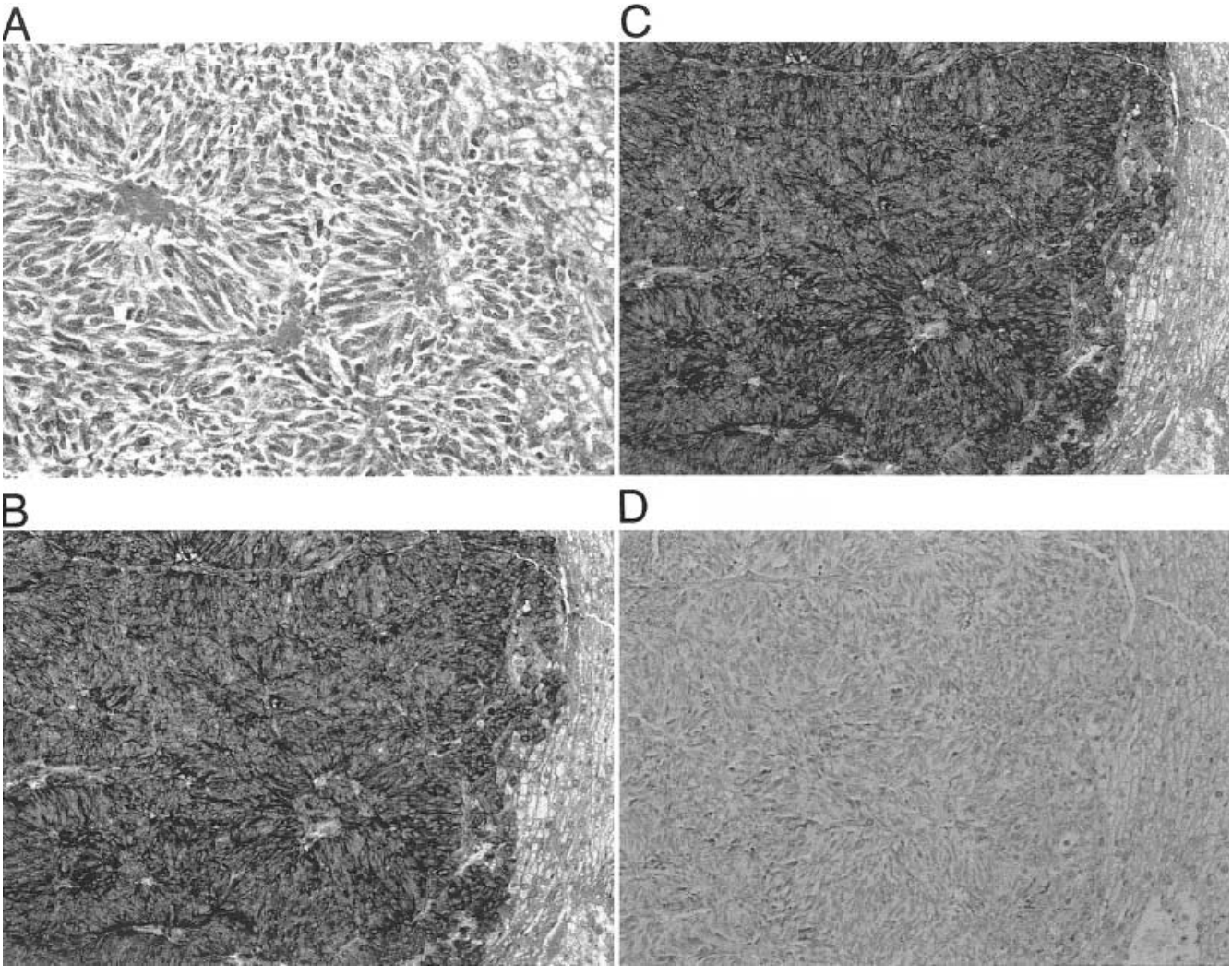

Fig. 3A–D Histopathology of neuroblastoma xenografts in SCID mice. **A** Metastatic tumor deposit in liver (H&E). **B–D** Immunohistochemical analysis of metastatic neuroblastoma for expression of synaptophysin (**B**), chromogranin A (**C**), and leukocyte common antigen (**D**)

kg every 7 days for nine consecutive weeks by i.v. injection. Topotecan was administered at doses of 0.6 mg/kg or 1.0 mg/kg as a single agent or in combination with vincristine (1.0 mg/kg). Distributions of EFSs were significantly different for all treatment groups compared to the control group ($P = 0.002$ for all tests). Median survival for the control group was 49.5 days, and 58.5 days for the vincristine-treated group. Topotecan at doses of 0.6 mg/kg (median survival 116.5 days) and 1.0 mg/kg (median survival 110 days) was superior to vincristine ($P = 0.002$), but there was no significant difference between the two doses of topotecan ($P = 0.68$) in this study (Fig. 7).

There was evidence of a significant difference in EFS distributions between mice that received topotecan 0.6 mg/kg and those that received the combination of topotecan 0.6 mg/vincristine 1.0 mg/kg (median survival 141 days; $P = 0.004$). However, we did not see a statistically significant difference in EFS among the mice which received 1.0 mg/kg topotecan compared to those that had the combination of topotecan 1.0 mg/vincristine 1.0 mg/kg (median survival 159 days; $P = 0.115$), although animals receiving the combination appeared to survive considerably longer (Fig. 7B).

Discussion

Children diagnosed with stage D, MYCN-amplified neuroblastoma, have a dismal prognosis. Tumors recur due to intrinsic or acquired resistance to current chemotherapeutic agents, or may recur as a consequence of reinoculation of tumor cells from inadequately purged bone marrow at autologous transplant. Preclinical models that closely simulate disseminated neuroblastoma may be valuable in identifying new agents, and evaluating new strategies, for clinical application.

We report a highly reproducible model of disseminated neuroblastoma in SCID mice, derived from a stage D, MYCN-amplified tumor. Reproducible patterns of dissemination and survival indicate that it may be of

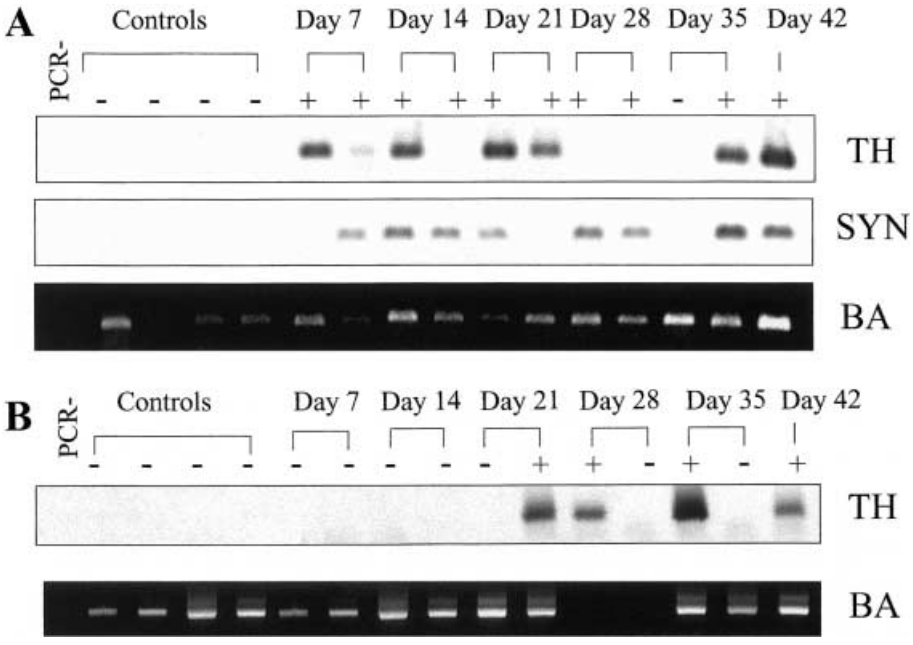

Fig. 4A, B Detection of NB-1691 cells in (**A**) peripheral blood and (**B**) bone marrow by polymerase chain reaction. Mice were injected with 5×10^6 cells, and tissues were sampled at weekly intervals from day 7. NB-1691 cells were detected by expression of synaptophysin (*SN*) and tyrosine hydroxylase (*TH*). β-actin (*BA*) was used as an internal control, and tissues from nontumored mice were used as negative controls for PCR reactions

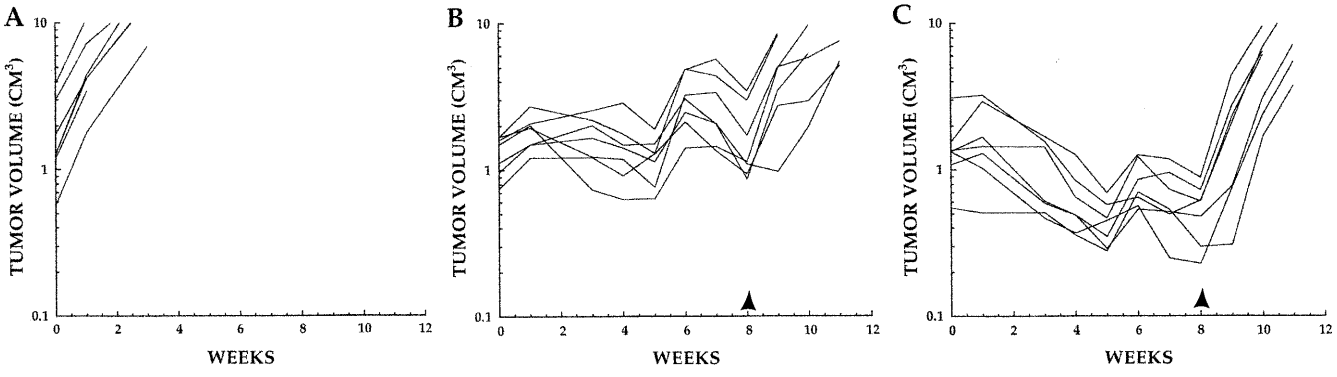

Fig. 5A–C Responses of s.c. NB-1691 neuroblastoma xenografts in SCID mice treated with topotecan. Mice received topotecan 0.61 or 1.0 mg/kg by i.v. administration using the (d × 5)2 schedule every 21 days for three cycles (**A** control, **B** 0.61 mg/kg, **C** 1.0 mg/kg). Each curve shows the growth of an individual tumor. *Arrowheads* mark the end of treatment (day 56)

value in therapeutic studies designed to identify or optimize new therapeutic agents. In the SCID model, reported here, tumor was evident macroscopically in multiple organs. The liver was involved in all cases and was often largely replaced by tumor. The kidneys were very commonly involved, and disseminated disease could also be seen macroscopically in the spleen, heart and lungs. Metastatic lesions were strongly positive for chromogranin A and synaptophysin, but negative for leukocyte common antigen. Bone marrow infiltration, often a feature of advanced disease in children, was not detected microscopically or by immunostaining in this study. Immunocytology techniques have shown that as many as 70% of patients have bone marrow tumor at diagnosis and 50% have circulating tumor cells [33]. Involvement of bone marrow and peripheral blood was, however, detectable by PCR. Circulating neuroblastoma cells were detected in peripheral blood from 7 days after cell injection, suggesting that injected neuroblastoma cells were not completely cleared from the blood. Bone marrow involvement was detected from day 21. Involvement of the ovaries (usually gross) was apparent in virtually all cases, in contrast to the clinical setting where this would be an exceedingly rare metastatic site. The pattern of dissemination in the mice may reflect, to some extent, the site of injection into a peripheral vein, and distribution into the abdominal and portal venous system. Interestingly, no liver metastases were identified in the athymic nude mouse, when cells were injected intravenously into a tail vein [29], or in the athymic nude rat when tumor cells were injected intra-arterially [34]. In our model, the incidence of disseminated disease was related to the number of cells injected. With inoculae of $\geq 10^5$ cells the incidence of disseminated tumor was 100%. Survival was highly correlated with inoculum size.

Failure to metastasize from the s.c. site appears not to be a consequence of loss of potential to form disseminated disease. Gilbert et al. [29] first reported the 'metastatic' capacity of human neuroblastoma in a murine system by demonstrating disseminated disease in Balb/c

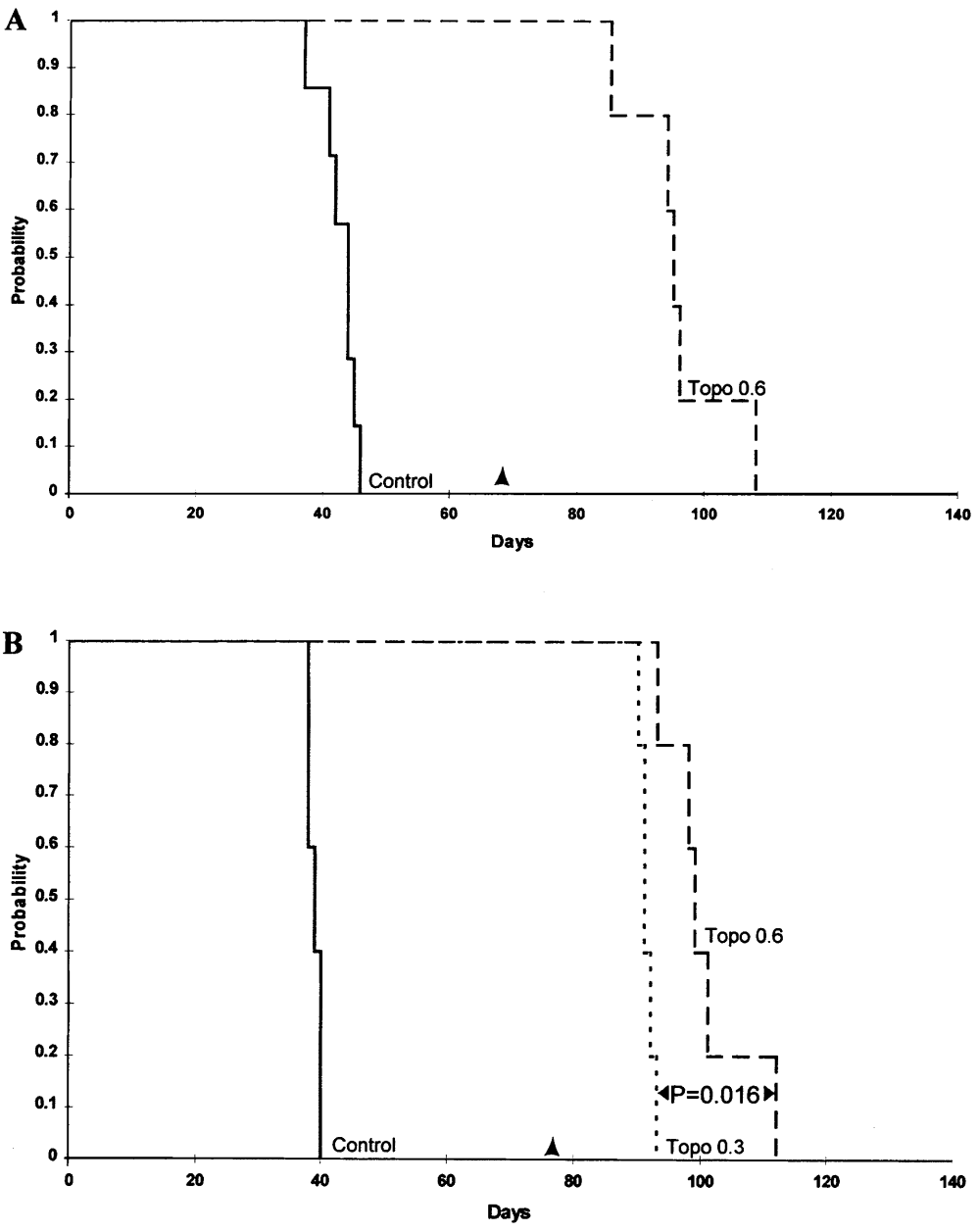

Fig. 6A, B Event-free survival of SCID mice bearing disseminated NB-1691 neuroblastoma. **A** Treatment with topotecan (0.6 mg/kg [(d × 5)2]3 i.v.) was started day 11 after inoculation of 1×10^7 cells. **B** Treatment with topotecan (*Topo*) 0.3 and 0.6 mg/kg [(d × 5)2]3 i.v. was started day 21 after inoculation of 1×10^7 NB-1691 cells. *Arrowheads* mark the end of treatment (**A** day 67, **B** day 77)

nude mice (aged 6–8 weeks) after intravenous tail vein injection of 5×10^6 IMR-5 cells. The resultant tumor metastases were evident in kidneys, spleen and multiple abdominal lymph nodes. A more comprehensive study has been reported in which growth of CHP-100 neuroblastoma cells was studied after s.c, intraperitoneal and i.v. injection in athymic nude (T-cell deficient) and beige-nude mice (T-cell and NK-cell deficient). Growth at the s.c. site was equivalent in both strains, but following i.v. injection tumor growth was more extensive in beige-nude mice in which gross metastases were observed more frequently [35]. Tumor was found in the lungs, bone marrow, ovaries, adrenals, kidneys, abdomen, and in one case pancreas.

A nude rat model of metastatic neuroblastoma has also been reported [35]. An advantage of the rat model over more conventional mouse models is that it offers access to intra-arterial pathways of tumor cell dissemination that may be important in the metastatic process. Intracardiac inoculation results in tumor growth in 95% of rats, but in 29% tumor is evident only at the site of injection (pericardial). The anatomical sites of disseminated tumor growth have been found to be similar to the sites of neuroblastoma in children. These include the adrenal gland and paravertebral regions. Bone and marrow involvement have been also noted. Interestingly, the host age determines tumor growth. Of 30 nude rats injected at 5–8 weeks of age, 25 (83%) developed disseminated tumor whereas none of the rats aged 9–13 weeks developed disseminated disease. This would suggest caution in the use of nude rats, particularly for therapeutic studies, because tumor regrowth may take

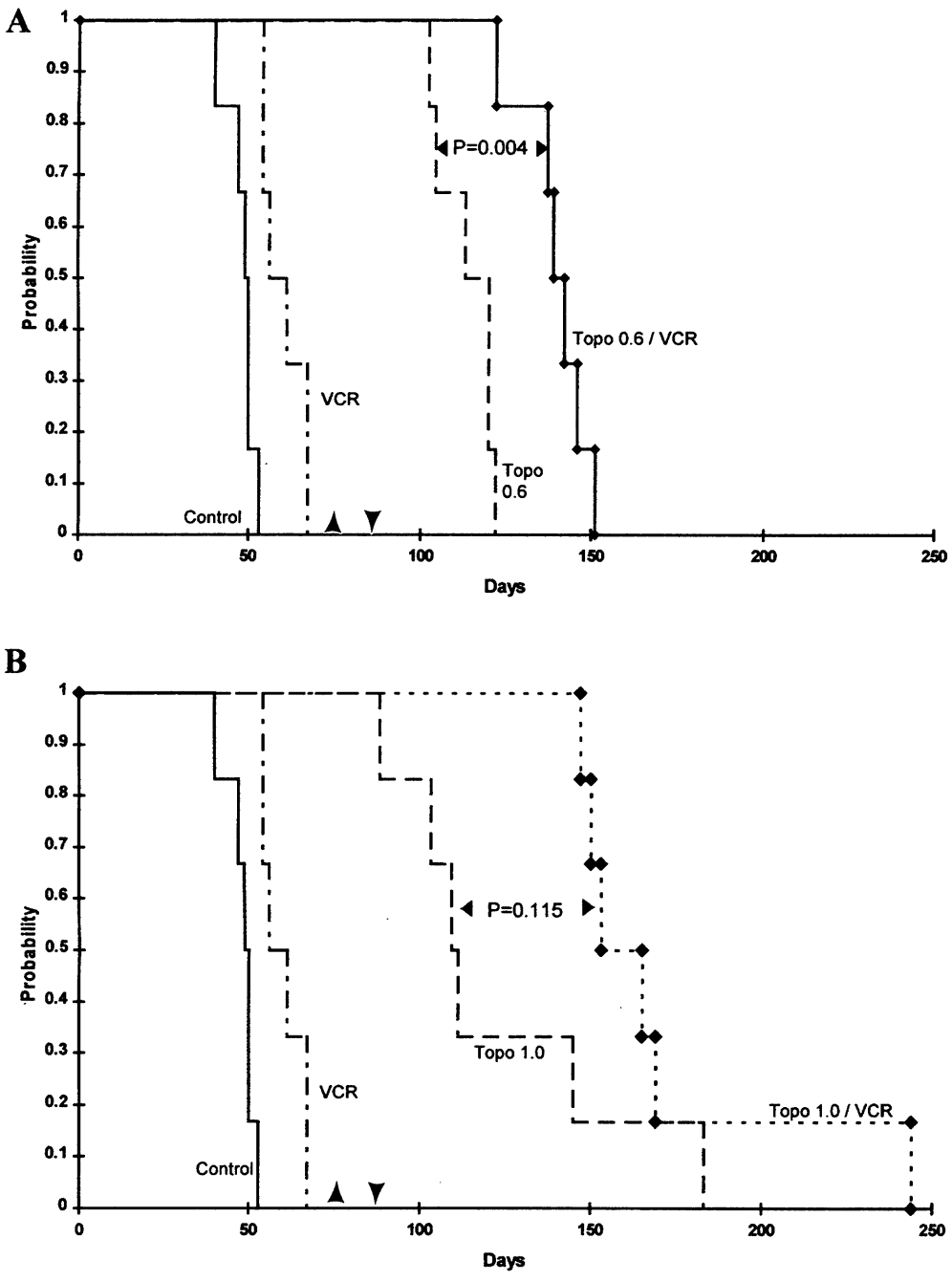

Fig. 7A, B Efficacy of vincristine and topotecan alone or in combination in the NB-1691 disseminated model. **A** Event-free survival of mice treated with vincristine (*VCR*) 1 mg/kg every 7 days for 9 weeks, topotecan (*Topo*) 0.6 mg/kg (d × 5)2 for three cycles, or the combination. All drugs were administered i.v. starting 21 days after inoculation of NB-1691 cells. **B** Survival of mice receiving vincristine 1 mg/kg, topotecan 1 mg/kg, alone or in combination as described in **A**. *Arrowheads* mark the end of treatment with vincristine on day 84 (▼), and topotecan (▲) on day 77

several months following complete regression, and regrowth may be inhibited due to immunological changes as the host animals mature. Consequently, time to regrowth or survival as experimental end points may overestimate the effectiveness of therapy

Although several models of disseminated neuroblastoma have been reported, few if any therapeutic studies have been published to support their value in developmental therapeutics. Bergman et al. have reported developing spinal epidural xenografts in rats and using this model to evaluate systemic anti-GD2 monoclonal antibody therapy [36]. Our major objective in developing this model was to use it for developing therapeutic strategies for treatment of disseminated, MYCN-amplified disease. For the model to be of value it is essential that patterns of metastasis and survival are reproducible. In particular, it is important that survival of animals is consistent. In separate experiments, median survival of groups of mice that received 1×10^7 cells was 39, 44, 44, and 49 days. In the experiment in which cell number was varied, a tenfold reduction in inoculum resulted in an increase in mean survival of approximately 13 days.

Recently, we have reported synergy between topotecan and the antimitotic agent vincristine in vivo against a panel of pediatric tumor xenografts [32]. It was

of interest, therefore, to examine the activity of these agents against the disseminated NB-1691 model, and to compare this with activity against s.c. tumors in the SCID mice. Topotecan was administered over 8 weeks on a schedule found previously to be optimal [25]. At the highest dose level used (1.0 mg/kg) there was no tumor progression and five of seven tumors demonstrated a volume regression of at least 50% during the period of treatment. The lower dose of topotecan (0.61 mg/kg) significantly inhibited growth relative to control tumors, but did not cause regressions. Tumor response in SCID mice was therefore slightly less than previously reported for the same s.c. tumors in CBA/CaJ immune-deprived mice [25]. Of note was the rapid progression of tumor at the end of treatment.

In the disseminated model longer survival was observed in groups of mice receiving topotecan. In three experiments topotecan (0.6 mg/kg) extended median lifespan over controls by 51, 60, and 69 days. At the higher dose (1.0 mg/kg) topotecan extended the median lifespan to 110 days compared to 49.5 days for control mice. No mice were cured of disease at either dose level. This delay in death is very similar to tumor growth delay observed in s.c. tumors, being essentially equivalent to the duration of topotecan treatment (8 weeks). Vincristine was less effective than topotecan, and extended the lifespan from 49.5 to 58.5 days. Mice receiving topotecan plus vincristine survived longer than would be anticipated from the activity of either drug administered alone. For example, median lifespan for mice treated with topotecan (0.6 mg/kg) plus vincristine was 141 days, whereas additive activity is 128 days. Median survival for mice treated with topotecan (1.0 mg/kg) plus vincristine was 159 days, whereas additive activity is calculated to be 121 days. Thus, against the disseminated model this combination demonstrated greater than additive activity, and is in agreement with data previously reported derived from evaluation of the combination against s.c. xenografts.

This model has certain strengths compared to the s.c. model, in that it more readily simulates disseminated disease. However, although highly reproducible, effective treatments such as topotecan necessitate very long observation times. Syngeneic models tend to have short survival times (for example P388 or L1210 is 6–8 days, B16 melanoma or Lewis lung carcinoma less than 25 days). Historically, such models have not proven particularly accurate for developing therapy against specific cancer types in humans. Although other investigators have reported the development of metastatic/disseminated models of neuroblastoma, this is the first report, to our knowledge, of the use such a model for therapeutic drug studies.

Identifying less toxic highly effective therapy for children with advanced neuroblastoma remains a challenge. Development of a suitable animal model of disseminated neuroblastoma in which new and innovative therapies can be tested will serve as a useful tool in meeting the challenge.

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