Activity of Intrathecal 4-Hydroperoxycyclophosphamide in a Nude Rat Model of Human Neoplastic Meningitis

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ABSTRACT

Neoplastic meningitis can result from leptomeningeal dissemination of a variety of cancers. We now report the development of animal models of human neoplastic meningitis and activity of Intrathecal 4-hydroperoxycyclophosphamide (4-HC) against the human rhabdomyosarcoma cell line TE-671 and the human glioma cell line D-54 MG grown in the subarachnoid space of athymic rats. The injection of $5 \times 10^6$ TE-671 or D-54 MG cells resulted in leptomeningeal tumor growth from the base of the brain to the cauda equina. Daily weights and neurological examinations revealed progressive neurological deficits and weight loss, with death occurring between Days 21 and 27 for TE-671 and Days 14 and 26 for D-54 MG. 4-HC toxicity in non-tumor-bearing rats was assessed at dose levels of 2.0, 10.0, 15.0, and 20.0 mg, with clinical and histological evidence of neurotoxicity observed at the 2 highest dose levels. Intrathecal treatment with 4-HC on Day 8 following injection of TE-671 resulted in an increase in median survival of 20% ($P = 0.04$) at 1.0 mm 4-HC and 41% ($P < 0.001$) at 2.5 mm 4-HC. Intrathecal treatment with 4-HC (2.5 mm) on Day 5 following injection of D-54 MG resulted in an increase in median survival of 23% ($P = 0.009$). These studies show the usefulness of the athymic rat model of human neoplastic meningitis and demonstrate the efficacy in vivo of intrathecally administered 4-HC against a human glioma and a human rhabdomyosarcoma cell line and the lack of toxicity at therapeutic levels of 4-HC in normal athymic rats.

INTRODUCTION

Neoplastic meningitis can result from leptomeningeal dissemination of a variety of cancers, either arising from the central nervous system or resulting from invasion by lymphoid neoplasms, carcinoma, sarcoma, or melanoma. The majority of patients with leptomeningeal disease due to metastatic carcinoma or melanoma have a dismal prognosis, with minimal response to i.t. chemotherapy and a mean survival of 2–3 months (1–4). Patients with primary neural tumors such as medulloblastoma and glioma which relapse in the CSF pathways demonstrate a similarly poor outcome with a mean survival of only 12–13 months (5, 6). Although many of the tumors metastasizing to the leptomeninges are radiosensitive, therapeutic dosages of radiotherapy are often limited by the risk of central nervous system toxicity, particularly radionecrosis. This is a particular problem when prior therapy has included neurexia irradiation (conventionally used for patients with medulloblastoma or pineoblastoma). Intrathecal administration into the subarachnoid space of a chemotherapeutic agent may achieve higher drug levels in the CSF than is possible by systemic administration with consequently greater antineoplastic activity (and potentially less systemic toxicity). Furthermore, the steep dose-response relationship demonstrated by alkylating agents suggests that efforts designed to maximize alkylator levels in the subarachnoid space of patients with leptomeningeal tumor dissemination may result in an improved therapeutic outcome (7).

Cyclophosphamide is a bifunctional alkylating agent active in a wide spectrum of human malignancies including lymphoma, leukemia, medulloblastoma, glioma, and breast cancer (8–10). However, the requirement for intrahepatic conversion of cyclophosphamide (which is a prodrug) into biologically active metabolites precludes its use as an agent for regional chemotherapy. 4-HC is a preactivated derivative of cyclophosphamide which spontaneously converts into 4-hydroxycyclophosphamide, the principal cytotoxic metabolite of cyclophosphamide, which itself subsequently converts to phosphoramide mustard, the ultimate alkylating moiety of this prodrug (8). 4-HC is active in vitro against a diverse group of human cell lines including medulloblastoma (11), L1210 murine leukemia (8), Burkitt’s lymphoma (12), and breast carcinoma (13).

Ardnt et al. (14, 15) have recently demonstrated that the i.t. administration of 4-HC may provide higher levels of the active metabolites than systemic administration of cyclophosphamide while avoiding systemic toxicity. We now report the development of an animal model of human NM using the human rhabdomyosarcoma cell line TE-671 and the human glioma cell line D-54 MG grown in the subarachnoid space of athymic nude rats, allowing evaluation of the activity and toxicity of i.t. 4-HC. The neurotoxicity of i.t. 4-HC was observed only at dose levels of 15.0 and 20.0 mm. Treatment of TE-671 or D-54 MG NM with 1.0 or 2.5 mm 4-HC resulted in statistically significant increases in median survival.

MATERIALS AND METHODS

Animal Model

Female athymic nude rats (200–250 g) maintained in the Duke University Animal Laboratory and Isolation Facility were anesthetized with ketamine/xylazine (55 mg/ml ketamine, 9 mg/ml xylazine) administered by i.p. injection at a volume of 1 ml/kg. Subarachnoid catheters were placed using a modification of the technique described by Kooistra et al. (16). Briefly, rats were placed with the neck flexed 90 degrees in a stereotactic frame with tilt adaptor (David Kopf Instruments, Tujunga, CA). A midline sagittal incision was made from the occiput to the lambdoid suture. The atlantooccipital membrane was exposed by sharp dissection. The tough, outer membrane and underlying cisterna magna dura were opened using the tip of a 20-gauge needle as a knife, under 5–10× magnification using an operating microscope (Zeiss OPMI 99). A PE-10 catheter (Intramedic, Clay Adams, Franklin Lakes, NJ) with a 5-0 stainless steel wire stylet was inserted into the subarachnoid space and passed along the posterior aspect of the spinal cord to the lumbar region (8.5 cm). A loose knot was tied in the catheter and fixed with dental epoxy (Lang Dental Manufacturing Co., Chicago, IL). The catheter was passed through the skin lateral to the incision using a 19-gauge needle. The wound was closed in 3 layers using 6-0...
Ethion (Ethicon Co., Somerville, NJ). After documenting spontaneous flow of CSF, the catheter was occluded using a short length of 2-0 stainless steel wire. The animals were allowed to recover for 3–5 days, and only animals showing normal motor and sensory function were used in experiments (>99% of animals undergoing catheter placement). Injections of tumor cells or drugs were performed through the catheter in volumes of 20–40 µl using a Hamilton syringe and injector equipped with a 30-gauge needle. All injections were followed by 20 µl of sterile saline to rinse the catheter.

Cell Lines

TE-671 is a well-characterized human cell line initially reported to be derived from a cerebellar medulloblastoma (17) but now known to be the subline of a rhabdomyosarcoma cell line, RD, established in the same laboratory 9 years earlier (18–21). D-54 MG is the Duke University subline of A-172 established by Giard et al. (22) from a human glioblastoma multiforme. Cell culture techniques for both lines were as described previously (23). Both cell lines grown as s.c. xenografts in athymic mice demonstrate sensitivity to cyclophosphamide (11, 24); additionally, the dose-response relationship of TE-671 in cell culture to 4-HC has been described previously (11).

Optimization of Tumor Injection Volume

For preliminary studies to determine the optimum volume of tumor injection, TE-671 cells were harvested in log phase into phosphate-buffered saline at 6.2 x 10⁶ or 1.25 x 10⁷ viable cells/ml (as assessed by trypan blue exclusion) and 5 x 10⁵ cells injected into subarachnoid catheters within 1 h of harvest. Groups of 5 animals each were given injections of 5 x 10⁵ cells in either 20 or 40 µl. Animals were followed until death with daily weights and neurological examinations, including the presence or absence of the stepping and placing reflex and the ability to negotiate a 60-degree incline ramp. These functions have been reported to correlate with subarachnoid tumor growth in other systems in contrast to sensory function tests (16). After death, the spinal column and skull were removed intact and processed for histology as described below.

Survival Studies

TE-671. Initial survival studies were performed in 25 animals given injections of 5 x 10⁵ TE-671 cells in 40 µl. One group of 10 animals was followed until death as described above. A separate group of animals was randomized (prior to tumor inoculation) for histological examination of tumor progression on Days 4, 8, 12, 16, and 20 posttumor inoculation (3 animals/day).

D-54 MG. Tumor inoculates for subarachnoid administration of D-54 MG were prepared from xenografts growing s.c. in athymic mice, due to the higher tumorigenicity of the disaggregated xenografts compared to D-54 MG grown in cell culture (data not shown). D-54 MG xenografts were grown s.c. in athymic mice, harvested as described previously (24), cut into fine pieces, and dissociated into a single cell suspension with 0.8% collagenase in a trypsinization flask for 2 h at 37°C. The cells were washed twice in phosphate-buffered saline, diluted to a final cell count of 1.25 x 10⁶ viable cells/ml (assessed by trypan blue exclusion), and 5 x 10⁵ cells in 40 µl were injected into 25 animals. One group of 10 animals was followed until death. A separate group of 15 animals was randomized (prior to tumor inoculation) for histological examination of tumor progression on Days 3, 6, 9, 12, and 15 posttumor inoculation (3 animals/day).

Histology

The entire spine and skull were removed en bloc, fixed in 10% formalin for 7–10 days, and then decalcified using RDO solution (Apex Engineering Products Co., Plainfield, IL). The brain and spinal cord were cut into 6 sections. Coronal sections of the brain were taken at the level of the coronal suture and the pituitary gland. Axial sections of the spinal cord were taken from the cervical, thoracic, and lumbar cord and the cauda equina. Sections were embedded in paraffin, and 6-µm sections were stained with hematoxylin and eosin and examined by light microscopy.

4-HC Toxicity Studies

For toxicity studies, non-tumor-bearing rats (groups of 10) were given injections of 40 µl of a 2.0, 10.0, 15.0, or 20.0 mM solution of 4-HC in 0.9% saline synthesized by the method of Hohorst et al. (25). Immediately following injection, the remaining dose solution was snap frozen in dry ice-ethanol and stored at −135°C until analysis by the method of Arndt et al. (14). The animals were sacrificed 6 weeks posttreatment and submitted for histological analysis as above. These injections were chosen to provide estimated final CSF 4-HC concentrations of 200, 1000, 1500, and 2000 µM based on the rat CSF volume of 400 µl (26). Tumor Inoculation Volume Response

D-54 MG. 4-HC diluted in 0.9% saline was administered via subarachnoid catheter on Day 8 following injection of TE-671 (5 x 10⁶ cells/animal). In the first experiment, one group of 7 animals was treated with 40 µl of a 1.0 mM solution of 4-HC, with a control group of 10 animals receiving 40 µl of 0.9% saline. In the second experiment, 1 group of 9 animals was treated with 40 µl of a 2.5 mM solution of 4-HC, with a control group of 9 animals receiving 40 µl of 0.9% saline. Animals were followed as described above, with histopathology obtained on all animals after death. Statistical analysis was performed using the Wilcoxon rank sum test. P < 0.05 was considered significant.

RESULTS

Tumor Inoculation Volume Response

The effect of altering the volume of inoculated tumor cells was assessed using a constant dose of 5 x 10⁵ TE-671 cells. At a 20-µl inoculation volume, animals developed progressive neurological deficits and died at Days 14–18. Histological analysis documented leptomeningeal tumor extension from the cauda equina to the low cervical cord. With a 40-µl injection volume, animals died by Days 21–27, with leptomeningeal tumor extending from the cauda equina to the base of the brain (Fig. 1). For all further studies, a 40-µl injection volume was used.

Survival Studies

TE-671. Animals given injections of 5 x 10⁵ TE-671 cells in 40 µl developed a progressive quadriparesis, with loss of ramp-
climbing ability and stepping and placing reflexes by Days 18–24, with death occurring by Days 21–27.

D-54 MG. Animals given injections of $5 \times 10^5$ D-54 MG cells in 40 $\mu l$ developed a progressive quadriparesis, with loss of ramp-climbing ability and stepping and placing reflexes by Days 12–23, with death occurring by Days 14–26.

Histology

TE-671. On Day 4 no tumor could be demonstrated. By Day 8 sections from most animals showed small, focal nests of tumor cells in the subarachnoid space (Fig. 2, A, B). By Day 20 sections from most animals showed confluent filling of the subarachnoid space by tumor cells, which extended from the base of the brain to the cauda equina (Fig. 2, C, D).

D-54 MG. On Day 6 all animals showed focal aggregates of tumor cells in the subarachnoid space. By Day 15 sections from all animals demonstrated that the subarachnoid space was filled with tumor cells from the base of the brain to the cauda equina.

4-HC Toxicity Studies

Actual dose concentrations of 4-HC, as measured by high pressure liquid chromatography, were 10.73 mM for 10.0 mM, 15.49 mM for 15.0 mM, and 21.56 mM for 20.0 mM. There was no neurological dysfunction noted in the animals treated with 40 $\mu l$ of 2.0 or 10.0 mM 4-HC dose solutions. One animal treated with 15.0 mM 4-HC showed a transient loss of incline ramp function; of animals treated with 20.0 mM 4-HC, 3 exhibited loss of ramp function and 5 showed a loss of the stepping and placing reflex (Table 1). Histological examination of the brains and spinal cords from animals treated with 2.0 and 10.0 mM revealed no abnormalities, while animals treated

with 15.0 or 20.0 mM showed myelin degeneration with lipid-laden macrophage accumulation in the peripheral white matter of the spinal cord (Table 2). The cervical and thoracic levels were most frequently affected; lumbar involvement was seen only in the 20.0 mM dosage group. The lateral white column was most often involved, followed by the posterior and anterior columns. The 20.0 mM dosage group showed more frequent involvement of multiple spinal cord levels and multiple white matter columns. In all cases, myelin degeneration was located at the periphery of the white matter columns (Fig. 3). Only one animal in the toxicity studies died. This animal was in the group treated with a 4-HC dose of 2.0 mM and did not exhibit any neurological dysfunction prior to death or histological evidence of neurotoxicity.

4-HC Activity Studies

TE-671. Treatment with i.t. 4-HC (40 $\mu l$ of a 1.0 mM solution) resulted in a delay in the loss of antigravity strength ($P = 0.035$) and an increase in median survival of 20% ($P = 0.04$) (Fig. 4). Increase in the dose of 4-HC (40 $\mu l$ of 2.5 mM solution) resulted in a further delay in the loss of antigravity strength ($P < 0.001$) and an increase in median survival of 41% ($P < 0.001$) (Fig. 5). There was no clinical evidence of toxicity with either dose. In both treatment studies, loss of stepping and placing reflexes and ramp-climbing ability preceded death by approximately 3 days. Sections from all animals in both the 4-HC treatment and saline-treated control groups showed a diffuse subarachnoid infiltrate of tumor cells extending from the base of the brain to the cauda equina.

D-54 MG. Treatment with i.t. 4-HC (40 $\mu l$ of a 2.5 mM solution) resulted in a delay in the loss of antigravity strength...
Table 1  Summary of neurological findings

<table>
<thead>
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<th>Neurological criterion</th>
<th>Concentration of 4-HC dose solution (mM)</th>
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<td></td>
<td>2.0</td>
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<tr>
<td>No. of animals that died</td>
<td>1/10*</td>
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<tr>
<td>No. of animals with a transient loss of stepping and placing reflex</td>
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</tr>
<tr>
<td>No. of animals with a transient loss of incline ramp function</td>
<td>0/10</td>
</tr>
<tr>
<td>Total no. of animals demonstrating a loss of either stepping and placing, or ramp function</td>
<td>0/10</td>
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* This animal survived for 43 days and showed no histological evidence of toxicity.
* Loss of stepping and placing reflex or ramp function was defined as a plus/minus or minus result on 3 consecutive days.
* Some animals exhibited a loss of both neurological parameters.

Table 2  Histological findings

<table>
<thead>
<tr>
<th>Histological criterion</th>
<th>Concentration of 4-HC dose solution (mM)</th>
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<tbody>
<tr>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>No. of animals with myelin degeneration/macrophage accumulation*</td>
<td>0/10</td>
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<tr>
<td>No. of animals having neuronal loss with gliosis</td>
<td>0/10</td>
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<tr>
<td>Total no. of animals exhibiting any of the above pathological changes*</td>
<td>0/10</td>
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* Assessed on sections stained with hematoxylin and eosin.
* Some animals showed more than one pathological change. In addition to the pathological findings listed, all animals exhibited a catheter reaction consisting of fibrosis and occasionally acute and/or chronic inflammation. Additionally, 6 of 10 animals in the 2000 μM dosage group showed focal periventricular and/or basilar pontine mineralization and vascular changes in the anterior spinal artery consisting of inflammation or hyaline thickening of the media.

(P = 0.004) and an increase in median survival of 23% (P = 0.009) (Fig. 6). Sections in both the 4-HC treatment and saline-treated control groups showed a diffuse subarachnoid infiltrate of tumor cells similar to that seen with TE-671, with extension from the base of the brain to the cauda equina. In some regions there was infarction of the underlying brain or spinal cord. Sections from some animals also showed invasion and replacement of portions of the brain and spinal cord with masses of tumor cells.

DISCUSSION

Diagnosis of neoplastic meningitis portends a dismal clinical outcome, with the majority of patients surviving less than 1 year from initiation of treatment (1–4). Although many of the malignancies that metastasize to the subarachnoid space are radiosensitive, limitations in the radiotolerance of the neuraxis preclude treatment at potentially curative dosages. Those chemotherapeutic agents that have been administered into the CSF (such as 1-β-D-arabinofuranosycytosine and methotrexate) are limited either by marginal activity against the tumors that are most frequently metastatic to the leptomeninges or by a propensity to produce a leukoencephalopathy that can be clinically devastating (27). Identification of drugs with a higher therapeutic index after i.t. administration would be a major therapeutic advance, particularly in the treatment of solid tumors metastatic to the leptomeninges.

Cyclophosphamide is a bifunctional alkylating agent active against a very broad spectrum of malignancies, including lymphoma, leukemia, medulloblastoma, glioma, and breast cancer (8, 9). The use of the preactivated derivative of cyclophosphamide, 4-hydroperoxycyclophosphamide, bypasses the need for intrahepatic conversion of cyclophosphamide into biologically active metabolites and provides the rationale for regional chemotherapy. Furthermore, the steep dose-response relationships demonstrated by alkylating agents such as cyclophosphamide suggest that efforts designed to maximize drug levels may result in improvement of the therapeutic outcome (7). Arndt et al. (14) have recently demonstrated the CSF pharmacokinetics of 4-HC following intraventricular administration to rhesus monkeys and suggested that direct injection of this agent into the CSF will provide higher peak drug levels in the subarachnoid space than is seen following systemic therapy (thus maximizing therapeutic activity without an increase in systemic toxicity). They demonstrated peak CSF drug levels of 100 μM following intraventricular administration of 4-HC (0.4 mg), a level that is not achievable with the i.v. administration of cyclophosphamide at a dose (1 g/m2) associated with marked systemic toxicity (15). Blood levels of 4-HC following i.v. administration of this agent were undetectable. No acute or chronic neurological or systemic toxicity was seen following the intraventricular treatment with 4-HC.

Our studies were designed to develop an animal model of leptomeningeal dissemination of human neoplasms and evaluate the therapeutic benefit seen following i.t. administration of
4-HC. The human rhabdomyosarcoma cell line TE-671 and the human glioma cell line D-54 MG were implanted into the subarachnoid space of athymic nude rats via a chronic indwelling subarachnoid catheter. The growth of TE-671 and D-54 MG in the subarachnoid space of athymic nude rats was very reproducible, with a well-defined progression of neurological dysfunction leading to death. Histological analysis demonstrated a progression from nests of tumor cells through the neuraxis to virtual tumor replacement of the subarachnoid space. Toxicity studies in non-tumor-bearing animals showed neither clinical nor histological evidence of neurotoxicity with doses of 2.0 or 10.0 mM 4-HC. Animals treated with 15.0 or 20.0 mM demonstrated a dose-dependent loss of neurological function, and histological analysis demonstrated myelin degeneration of the spinal cord. (The one death in the 2.0 mM 4-HC dose group cannot be attributed to 4-HC toxicity since there was no evidence of neurological dysfunction prior to death and no histological evidence of neurotoxicity.) Doses of 4-HC (1.0 and 2.5 mM) designed to produce peak CSF drug levels of 100 or 250 µM were chosen for our initial therapeutic studies, reflecting previous measurement of the in vitro 90% inhibitory dose (against TE-671) of 12.8 mM (11). This therapy produced an increase in median survival of 20% with 1.0 mM 4-HC and 41% with 2.5 mM 4-HC. Similarly, treatment of rats bearing leptomeningeal D-54 MG with 2.5 mM 4-HC produced an increase in median survival of 23%.

These studies demonstrate the activity of 4-HC in the treatment of human neoplasms metastatic to the subarachnoid space and justify further efforts to define an optimal dose schedule regimen. Kooistra et al. (16, 28) have developed experimental models for meningeal neoplasia using i.t. injections of 9L gliosarcoma or Walker 256 carcinosarcoma into Sprague-Dawley rats, allowing a precise evaluation of the effect of i.t. chemotherapy dose and schedule modulations on therapeutic outcome. Multiple dose regimens of i.t. diaziquone or mitomycin C produced significant increases in median survival compared to those produced by single-dose regimens of these agents, suggesting that similar studies with 4-HC in the treatment of leptomeningeal TE-671 or D-54 MG will enable definition of a regimen maximizing therapeutic activity while minimizing toxicity.
These studies, demonstrating the antineoplastic activity of i.t. 4-HC against human rhabdomyosarcoma and glioblastoma cell lines growing in the subarachnoid space of athymic rats and the lack of toxicity of i.t. 4-HC (2.0–10.0 mm) in non-tumor-bearing athymic rats, provide the basis for evaluating the role of the i.t. administration of 4-HC in patients with cyclophosphamide-sensitive malignancies metastatic to the subarachnoid space and primary central nervous system tumors that recur or present with NM. Studies in progress will define the maximal tolerated dose and optimal therapeutic regimen for i.t. 4-HC as a prelude to initiating a Phase I trial for patients with recurrent NM from metastatic systemic extraneural neoplasms and from primary brain tumors that seed the neuraxis.

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