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Chemotherapy of Neuroblastoma in Mice with Anticancer Agents

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Abstract □ Neuroblastoma-inoculated A/J mice were treated with various anticancer chemotherapeutic agents, including cyclophosphamide, daunorubicin, vincristine, α-bungarotoxin, dihydroxytryptamine, and diaminopropane. Cyclophosphamide and diaminopropane inhibited neuroblastoma as effectively as bromoacetylcholine and bromoacetate. The effectiveness of these drugs could be related to the inhibition of ornithine decarboxylase, a rate-limiting enzyme for the synthesis of polyamines.

Keyphrases □ Anticancer agents—chemotherapy of neuroblastoma in mice □ Ornithine decarboxylase—inhibition by anticancer agents, chemotherapy of neuroblastoma □ Cyclophosphamide—chemotherapy of neuroblastoma in mice □ Diaminopropane—chemotherapy of neuroblastoma in mice

Despite many efforts, the prognosis of the clinical treatment of neuroblastoma in patients over the age of 1 year is very poor (1). Since the A/J mouse inoculated with C-1300 neuroblastoma cells is considered to be a model of the neuroblastoma disease of humans (2), various anticancer drugs were tested on this animal model to determine an effective way to treat the neuroblastoma disease. Bromoacetylcholine and bromoacetate recently were reported to be effective in inhibiting the neuroblastoma growth *in vivo* (3–6). Therefore, a combination of bromoacetylcholine and other anticancer drugs also was tested in this neuroblastoma mouse model.

METHODS

Adult male A/J mice[1] were inoculated subcutaneously in the interscapular area with 1×10^6 cells of murine C-1300 neuroblastoma cells. Ten days was allowed for a measurable tumor to develop. Drug injections began on the 10th day after neuroblastoma inoculation, and this time was designated as Day 0 of drug treatment. Identical conditions were maintained for the control groups, except that saline was injected instead of drug solutions. Drug treatment was continued for 6 weeks unless otherwise specified. For all injections, the volume of the drug solutions was <50 μl.

Bromoacetylcholine perchlolate was synthesized by a previously described method (7). Sodium bromoacetate, cyclophosphamide, 1,3-diaminopropane, 5,6-dihydroxytryptamine, α-bungarotoxin, and vincristine were purchased commercially. All drug solutions were freshly prepared at the time of injection.

RESULTS

It has been established that bromoacetylcholine (30 mg/kg) injected intratumorly one to three times per day and bromoacetate (12 mg/kg) injected intratumorly twice per day inhibit neuroblastoma growth in A/J mice efficiently and prolong the lifespan of these animals by >200% (4, 5). The only anticancer agent that was able to produce an effectiveness equivalent to bromoacetylcholine and bromoacetate was cyclophosphamide (100 mg/kg) injected only once intraperitoneally (Table I). A higher dose (200 mg/kg) of cyclophosphamide reduced its effectiveness due to toxicity. Combination of cyclophosphamide with bromoacetylcholine or bromoacetate did not additionally prolong the lifespan. On the contrary, the lifespan was shortened somewhat as compared to the lifespan of mice receiving the optimal doses of bromoacetylcholine, bromoacetate, and cyclophosphamide administered individually (Table I).

Daunorubicin[2] at a dose of 2 mg/kg injected intravenously twice per week worsened the disease as compared to the control. Combined use of daunorubicin and bromoacetylcholine prolonged the lifespan by only 33%, which was much shorter than the result with bromoacetylcholine alone (Table I).

Vincristine (1 mg/kg) injected intraperitoneally three times daily did not improve the neuroblastoma disease and did not potentiate the effect of bromoacetylcholine (Table I). On the contrary, it shortened the mean lifespan of bromoacetylcholine-treated animals from 73.4 (207% change of lifespan) (5) to 52.1 (129% change of lifespan) days (Table I).

Table II shows the effects of various agents known to inhibit neuroblastoma growth in cell culture on the neuroblastoma growth in A/J mice. α-Bungarotoxin is an inhibitor of neuroblastoma in cell culture (8) but is too toxic to be used *in vivo*. The mice survival rate was shorter than that of the untreated controls (Table II).

5,6-Dihydroxytryptamine has degenerated serotonergic neurons (9, 10) and inhibited gliomas in cell culture (11). However, it did not prolong the lifespan of neuroblastoma-inoculated A/J mice and did not enhance the inhibition of neuroblastoma growth when used in combination with bromoacetylcholine (Table II).

1,3-Diaminopropane was shown to inhibit ornithine decarboxylase, a rate-limiting enzyme for the synthesis of polyamines, which in turn suppressed neuroblastoma growth in cell culture (6). It also produced a significant improvement in mice inoculated with neuroblastoma cells when injected intratumorly twice per day for 6 weeks at a dose of 110 mg/kg (Table II). The combination of diaminopropane and bromoacetylcholine was even more instrumental in prolonging the lifespan of neuroblastoma-inoculated mice.

Various routes of administration were used to find an effective alternative to the intratumor route. Bromoacetylcholine and bromoacetate were ineffective with intraperitoneal administration (Table III), mainly because bromoacetylcholine has a permanent positive charge at the

[1] Jackson Laboratory, Bar Harbor, Me.

[2] Obtained from the Drug Liaison and Distribution Section, Division of Cancer Treatment, National Cancer Institute.

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Table I—Inhibition of Neuroblastoma Growth in A/J Mice by Cyclophosphamide, Daunorubicin, and Vincristine

Drug	Dose (mg/kg) and Route	Frequency per Day	n	Range, days	Mean $\pm SE$, days	Change of Lifespan[a], %	Median, days
Saline	— it	2	10	13–63	33.0 ± 5.1	—	29
Cyclophosphamide	100 ip	Once only	10	34–150[b]	108.2 ± 17.2[c]	227	150
Cyclophosphamide	200 ip	Once only	9	9–150[b]	66.4 ± 29.0	101	26
Cyclophosphamide +	100 ip	Once only	10	11–150[b]	73.6 ± 17.2[c]	124	56
bromoacetylcholine	30 it	2					
Cyclophosphamide +	200 ip	Once only	10	5–86	21.7 ± 9.0	−34	10
bromoacetylcholine	30 it	3					
Cyclophosphamide +	100 ip	Once only	10	34–150[b]	78.5 ± 15.8[c]	138	56
bromoacetate	12 it	2					
Daunorubicin	2 iv	Twice per week	10	11–34	25.1 ± 2.4	−24	27
Daunorubicin +	2 iv	Twice per week	10	18–150[b]	52.7 ± 16.6	60	32
bromoacetylcholine	30 it	2					
Saline	— it	3	10	11–38	22.8 ± 3.4	—	18
Vincristine	1 ip	3	8	8–43	23.9 ± 4.4	5	25
Vincristine +	1 ip	3	8	29–150[b]	52.1 ± 14.6	129	34
bromoacetylcholine	30 it	3					

[a] Calculated from the mean lifespan of the control. [b] Many mice survived beyond 150 days after drug treatment began; 150 days was chosen arbitrarily as the cut-off date for the statistical analysis. [c] $p < 0.05$ as compared to the control.

Table II—Effects of Various Agents, Administered Intratumorly, that Suppressed Neuroblastoma in Tissue Culture on Neuroblastoma Growth in A/J Mice

Drug	Dose, mg/kg	Frequency per Day	n	Range, days	Mean $\pm SE$, days	Change of Lifespan[a], %	Median, days
Saline	—	3	8	27–55	40.3 ± 3.4	—	43
α-Bungarotoxin	0.03	1	10	20–48	31.8 ± 3.1	−21	31
α-Bungarotoxin	0.1	1	9	16–16	16.0 ± 0	−60	16
α-Bungarotoxin	0.3	1	7	14–14	14.0 ± 0	−65	14
Saline	—	2	6	33–43	36.8 ± 1.6	—	36
Dihydroxytryptamine	50	2	8	29–68	43.1 ± 4.7	17	47
Dihydroxytryptamine +	50	2	8	3–63	32.6 ± 7.8	−11	40
bromoacetylcholine	30	2					
Dihydroxytryptamine +	50	1	7	22–43	30.4 ± 3.1	−17	29
bromoacetylcholine	30	1					
Dihydroxytryptamine +	50	1	6	15–150[b]	50.1 ± 17.1	36	38
bromoacetate	12	1					
Saline	—	2	6	33–43	36.8 ± 1.6	—	36
Diaminopropane	110	2	8	31–150[b]	68.8 ± 24.3[c]	87	50
Diaminopropane	220	2	8	29–58	44.3 ± 4.1	20	50
Diaminopropane +	110	2	8	26–150[b]	81.1 ± 20.4[c]	120	50
bromoacetylcholine	30	2					

[a,b,c] See Table I.

Table III—Inhibition of Neuroblastoma Growth in A/J Mice by Bromoacetylcholine and Bromoacetate with Various Routes of Administration

Drug	Dose (mg/kg) and Route[a]	n	Range, days	Mean $\pm SE$, days	Change of Life-span[b], %	Median, days
Saline	— ip	10	9–42	28.5 ± 2.8	—	32
Bromoacetyl-choline	30 ip	8	26–38	29.1 ± 1.4	2	30
Bromoacetate	12 ip	8	26–40	32.3 ± 1.6	13	32
Bromoacetate	6 im	10	9–32	21.3 ± 2.1	−25	21
Bromoacetate	12 im	10	11–35	22.9 ± 2.7	−20	25
Bromoacetate	24 im	10	4–35	17.3 ± 2.9	−39	18
Bromoacetate	12 sc	10	11–35	24.1 ± 2.5	−14	28
Bromoacetate	24 sc	10	4–37	18.3 ± 3.3	−36	18

[a] The drug was administered three times daily. [b] See footnote a, Table I.

quaternary nitrogen group of the molecule and bromoacetate has a negative charge in the molecule, which hinders absorption from the GI system. Intramuscular and subcutaneous injections of bromoacetate also were ineffective in suppressing neuroblastoma growth (Table III). Intratumor injection is the only viable route at this time. Intravenous administration was not tried because the mouse tail vein cannot withstand multiple daily injections for 6 weeks. Larger animal models are sought for experimentation to solve this problem.

DISCUSSION

Neuroblastoma is a cancer that cannot be treated well with current medical knowledge and methodology (1). After unsuccessful attempts to treat neuroblastoma surgically, with immunotherapy, and by radiation therapy, partial success was achieved with chemotherapy (1). Chemotherapy should be explored further.

Among the drugs tested, bromoacetylcholine, bromoacetate, cyclophosphamide, and 1,3-diaminopropane effectively inhibited neuroblastoma growth and prolonged the lifespan of A/J mice inoculated with neuroblastoma cells. Bromoacetylcholine, bromoacetate, and 1,3-diaminopropane are potent inhibitors of ornithine decarboxylase, a rate-limiting enzyme for the synthesis of polyamines. Since cellular levels of polyamines are closely related to cell proliferation and growth (12–17), it is highly possible that neuroblastoma growth is suppressed *via* the inhibition of ornithine decarboxylase (6). The only agent that is not clearly known to inhibit ornithine decarboxylase is cyclophosphamide, because cyclophosphamide must be activated *in vivo* while ornithine decarboxylase activity can only be determined *in vitro* with tissue culture experiments. The effect of cyclophosphamide also may possibly be manifested through the ornithine decarboxylase inhibition *in vivo*. Daunorubicin is an antibiotic that binds tightly with DNA (18) but has no inhibiting action on neuroblastoma growth.

Vincristine inhibits cell mitosis at the metaphase and dissolves the microtubule structures (18). However, it has little effect on ornithine decarboxylase activity, and this fact could be why it is ineffective in the inhibition of neuroblastoma growth.

Although bromoacetylcholine binds to the nicotinic receptor at the neuromuscular junction irreversibly (19), as does α-bungarotoxin (20), the latter compound produced little effect on neuroblastoma growth, indicating that bromoacetylcholine inhibits neuroblastoma growth through an action mechanism that differs from α-bungarotoxin, similar to action at cholinergic receptors. Neuroblastoma cells possess adrenergic, cholinergic, and nonspecific receptors but very few serotonergic receptors. It thus is understandable that neuroblastoma cell growth was not inhibited by a serotonergic neuron degenerator, 5,6-dihydroxytryptamine.

Among the routes of administration studied, intratumor administration for bromoacetylcholine, bromoacetate, and 1,3-diaminopropane and intraperitoneal administration for cyclophosphamide were the best.

In summary, it seems that drugs capable of inhibiting ornithine decarboxylase can suppress the cell growth of neuroblastoma. A more potent ornithine decarboxylase inhibitor that produces few side effects may be developed as an effective weapon to treat neuroblastoma.

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Effect of pH, Chlorobutanol, Cysteine Hydrochloride, Ethylenediaminetetraacetic Acid, Propylene Glycol, Sodium Metabisulfite, and Sodium Sulfite on Furosemide Stability in Aqueous Solutions

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Abstract □ A study was conducted to determine the effects of pH, two antioxidants, a chelating agent, a preservative, and propylene glycol on furosemide stability. Aqueous solutions of furosemide containing 10% alcohol (v/v) were prepared in phosphate buffers with various pH values (5, 6, and 9) whose ionic strength was adjusted to 0.1 *M* with potassium chloride. Some solutions contained chlorobutanol, ethylenediaminetetraacetic acid, or sodium metabisulfite. Another set of aqueous solutions contained phosphate buffer (0.1 *M*), alcohol (10% v/v), and propylene glycol (40% v/v) with or without cysteine hydrochloride, ethylenediaminetetraacetic acid, and sodium sulfite. The solutions were divided into two parts, stored at 24 and 50°, and assayed frequently using a previously developed high-pressure liquid chromatographic procedure. At the lowest pH value (pH 5), furosemide appeared to be very unstable. Cysteine hydrochloride, ethylenediaminetetraacetic acid, and sodium sulfite failed to improve the stability of furosemide. Chlorobutanol and sodium metabisulfite had an adverse effect on the stability, probably due to the fact that they decreased the pH of the solution. The pH value appears to be the only critical factor for the stability of furosemide. Buffered solutions containing propylene glycol were very stable at both temperatures for 170 days, and they tasted good.

Keyphrases □ Furosemide—stability in aqueous solutions, effect of formulation factors □ Diuretics—furosemide, stability in aqueous solutions, effect of formulation factors □ Stability—furosemide in aqueous solutions, effect of formulation factors

Furosemide (I) is a widely used diuretic, but little information is available concerning the stability of this drug in dosage forms. Rowbotham *et al.* (1) reported that aqueous furosemide solutions undergo hydrolysis and photochemical degradation. Quantification of photochemical degradation products of furosemide by the USP XIX (2) UV assay procedures was not successful. A stability-indicating assay for furosemide using high-pressure liquid chromatography (HPLC) was developed by Ghanekar *et al.* (3). It also was reported that an aqueous furosemide solution containing sorbitol and 10% alcohol (v/v) had limited stability. The pH of the solution was adjusted to ~8.5. However, it was difficult to maintain the pH value of the solution, which caused rapid decomposition.

The objectives of the present investigation were to study

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