Treatment of Neuroblastoma in Mice with Bromoacetylcholine and Bromoacetate

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Abstract □ Bromoacetylcholine (30 mg/kg intratumor one to three times per day) and bromoacetate (12 mg/kg intratumor two times per day) inhibited neuroblastoma in A/J mice efficiently and prolonged the lifespan of these animals at least 200%. Since the neuroblastoma-inoculated A/J mice are considered to be comparable to human neuroblastoma, the cytolytic action of bromoacetylcholine and bromoacetate on murine neuroblastoma warrants further studies on patients. The fact that these tumors were cured in adult mice is very important because older children and adults with neuroblastoma have the poorest prognosis.

Keyphrases □ Bromoacetylcholine—effect on neuroblastoma in mice □ Bromoacetate—effect on neuroblastoma in mice □ Antitumor activity—bromoacetylcholine and bromoacetate, effect on neuroblastoma in mice

Except for leukemia and brain tumors, neuroblastoma is the most common malignancy seen in children. Because metastasis has occurred in over two-thirds of the patients by the time of diagnosis, definitive surgical treatment is not possible in most cases. Similarly, radiation therapy has had very limited success in patients with disseminated disease and has most often been only palliative (1). Treatment, therefore, frequently emphasizes a chemotherapeutic approach, although all three modes of therapy are used in many cases.

A regimen consisting of vincristine, cyclophosphamide, and daunorubicin has shown the best results in clinical trials, but these drugs are far from satisfactory because of their short duration of action and low tumor remission rate (1–4). Newer, more effective drugs are urgently needed to treat this highly malignant disease, which is responsible for at least 8% of childhood deaths by cancer.

Recently, it was shown that bromoacetylcholine is an effective agent for killing murine C-1300 neuroblastoma cells in culture at concentrations as low as 1×10^{-5} *M* (5, 6). It was also noted that moderate doses (10–30 mg/kg) of this compound suppressed the growth of tumors resulting from subcutaneous implantation of the C-1300 cells in A/J mice (6). Since the LD_{50} of bromoacetylcholine in tumor-free A/J mice was found to be 250 mg/kg, the therapeutic index appears to be reasonably high. These results strongly suggest a possible role for this drug in the chemotherapy of neuroblastoma.

Since bromoacetate, the hydrolysis product of bromoacetylcholine, was found early in the present study to inhibit neuroblastoma in cell culture, its effects on subcutaneous neuroblastomas in A/J mice were also investigated.

Generally, the major problem hindering the use of chemotherapeutic agents in cancer treatment is intolerable toxicity and other side effects. To reduce the possible parasympathomimetic side effects of bromoacetylcholine, atropine was included in some regimens in the hope that the combination might provide potent antitumor activity with few undesirable side effects. This use presumed that the cytotoxic effects of bromoacetylcholine were not somehow mediated by its parasympathomimetic action.

Table I—Effects of Doses and Frequencies of Injection on Neuroblastoma Inhibition by Bromoacetylcholine *In Vivo*

Bromoacetylcholine	Frequency of Injection per Day	*n*	Range, days	Mean ± *SE*, days	CLS[a], %	Median Lifespan, days
Control	3	10	11–38	23.9 ± 3.1	—	25
Control	2	10	11–38	24.3 ± 2.9	—	25
Control	1	10	11–38	21.2 ± 3.3	—	18
1 mg/kg	3	7	28–40	33.9 ± 1.7	+42	35
10 mg/kg	3	7	14–150[b]	52.9 ± 16.9	+121	38
30 mg/kg	3	10	30–150[b]	73.4 ± 17.1	+207	53
30 mg/kg	2	8	14–150[b]	60.1 ± 20.2	+147	38
30 mg/kg	1	7	25–150[b]	67.1 ± 21.6	+217	35
60 mg/kg	3	11	4–150[b]	36.0 ± 11.9	+51	32
60 mg/kg	3	13	2–150[b]	35.5 ± 10.7	+49	28
for 1 week; then 30 mg/kg for 2 weeks; then 10 mg/kg for 3 weeks	3					

[a] Percent change of lifespan calculated from mean lifespan. [b] Many mice survived beyond 150 days after drug treatments began; 150 days was chosen arbitrarily as the cutoff date for the purpose of performing statistical analysis.

EXPERIMENTAL

Materials—Bromoacetylcholine perchlorate was synthesized according to a previously published method (7). Bromoacetic acid, atropine sulfate, choline iodide, acetylcholinesterase[1], and butyrylcholinesterase[1] were obtained commercially. Murine C-1300 neuroblastoma cells were obtained from a collection institute[2] and had been maintained in culture in this laboratory for 12 months.

Cell Culture Experiments—Conditions and culture media were described previously in detail (5, 6). To determine whether the hydrolysis products of bromoacetylcholine are capable of inhibiting neuroblastoma, choline and bromoacetate were added to murine C-1300 neuroblastoma cells in culture and their cell growth inhibitory activity was compared with that of the parent compound.

Inhibition of Cholinesterases by Bromoacetate—The enzyme activities of acetylcholinesterase and butyrylcholinesterase were determined spectrophotometrically[3] using the method of Ellman *et al.* (8). The reaction mixture contained 3.0 ml of pH 8 phosphate buffer, 0.017 U of butyrylcholinesterase/ml or 0.083 U of acetylcholinesterase/ml, and 1.32 mg of 5,5-dithiobis(2-nitrobenzoate)/ml in a final volume of 3.135 ml. Various concentrations of bromoacetate were added to the incubation mixture to determine the I_{50} for each enzyme. The cell was shaken and allowed to stand at 25° for 15 min, followed by addition of acetylthiocholine (5.9×10^{-4} *M*).

The yellow anion, 5-thio-2-nitrobenzoate, formed in the reaction was measured at 412 nm. The reversibility of the enzyme inhibition was determined with various concentrations (noted in the *Results* section) of the enzymes and bromoacetate. The initial acetylthiocholine hydrolysis rate was expressed in terms of change in absorbance per minute.

[1] ICN Life Sciences Group, Cleveland, Ohio.
[2] American Type Culture Collection, Rockville, Md.
[3] Gilford automatic recording spectrophotometer, model 2400, Gilford Instrument Laboratories, Oberlin, Ohio.

Table II—Effects of Doses and Frequencies of Injection on Neuroblastoma Inhibition by Bromoacetate *In Vivo*

Bromoacetate	Frequency of Injection per Day	*n*	Range, days	Mean ± *SE*, days	CLS[a], %	Median Lifespan, days
Control	1	10	11–38	21.1 ± 3.3	—	18
Control	2	10	11–38	24.3 ± 2.9	—	25
Control	3	10	11–38	23.9 ± 3.1	—	25
12 mg/kg	1	10	25–150[a]	63.2 ± 14.7	+200	45
12 mg/kg	2	7	28–150[a]	62.4 ± 15.2	+157	57
12 mg/kg	3	8	8–150[a]	45.1 ± 15.9	+89	41
30 mg/kg	3	10	18–150[a]	54.4 ± 16.0	+128	32

[a] See Table I.

***In Vivo* Experiments**—Adult, male A/J mice, 16–18 g, were obtained commercially[4]. A suspension of 1×10^6 murine C-1300 neuroblastoma cells was injected subcutaneously in the interscapular area of these mice and allowed 10 days to develop a measurable tumor. Animals with tumors were then divided into control and treatment groups. Saline (0.9% NaCl) injections were given directly into the tumors of the control mice on a daily schedule, except weekends, for 6 weeks. Treatment animals were injected according to the dosage schedules shown in Tables I–IV. All solutions injected were adjusted to a volume of 30 μl.

Solutions of bromoacetylcholine and bromoacetate were prepared fresh each day and stored on ice to prevent hydrolysis and decomposition. Solutions containing bromoacetic acid were adjusted to approximately pH 7.4 with 5 *M* NaOH to prevent irritation of tissues at the injection sites. Since bromoacetylcholine is a salt, its solutions needed no such adjustment. These solutions also were injected directly into the tumors, except in experiments testing the effects of intraperitoneal administration. Atropine was given intramuscularly. Thirty milligrams of bromoacetylcholine/kg is equimolar with 12 mg of bromoacetate/kg.

RESULTS

Inhibition of Neuroblastoma Cells in Culture by Hydrolysis Products of Bromoacetylcholine—Since bromoacetylcholine is hydrolyzed both enzymatically and chemically to choline and bromoacetate (7), the effect of these compounds was first examined on neuroblastoma growing in culture (Fig. 1). Although cell growth was not significantly inhibited by choline concentrations as high as 1×10^{-4} *M*, bromoacetate at 1×10^{-5} and 1×10^{-4} *M* did decrease the number of viable cells compared to the control. The cytotoxic potency of bromoacetate is thus similar to that of bromoacetylcholine on an equimolar basis.

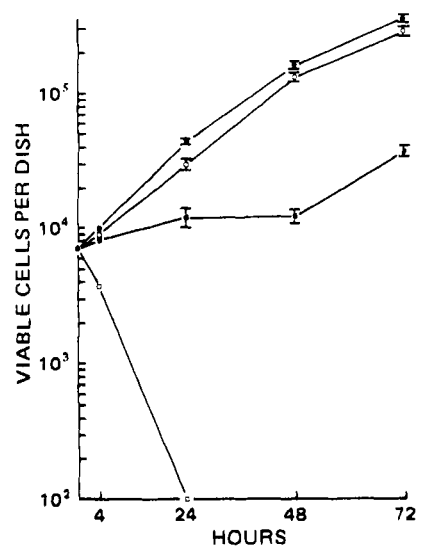

Figure 1—*Effects of bromoacetate and choline, hydrolysis products of bromoacetylcholine, on the cell growth of neuroblastoma* in vitro. *Key:* ●, *control;* ○, *1* × 10^{-4} M *choline;* ■, *1* × 10^{-5} M *bromoacetate; and* □, *1* × 10^{-4} M *bromoacetate. Each point represents the mean of four values, and bars represent the standard error of the mean.*

[4] Jackson Laboratories, Bar Harbor, Me.

Table III—Effect of Atropine on the Neuroblastoma Inhibition by Bromoacetylcholine (I) *In Vivo*

Drugs and Doses	Frequency of Injection per Day	*n*	Range, days	Mean ± *SE*, days	CLS[a], %	Median Lifespan, days
Control	3	5	9–30	22.2 ± 3.7	—	35
Atropine, 1 mg/kg	1	9	18–51	31.8 ± 3.7	+43	28
I, 10 mg/kg	3	7	14–150[a]	52.9 ± 16.9	+121[b]	38
Atropine, 1 mg/kg, plus I, 10 mg/kg	1 3	6	9–32	26.2 ± 3.5	+18	30
I, 30 mg/kg	3	10	30–150[a]	73.4 ± 17.1	+207[b]	53
Atropine, 1 mg/kg, plus I, 30 mg/kg	1 3	7	16–56	28.7 ± 5.3	+29	28

[a] See Table I. [b] These data were transferred here from Table I for comparison.

Inhibition of Cholinesterases by Bromoacetate—Although the cholinesterases are inhibited irreversibly by bromoacetylcholine (9), they were inhibited reversibly by bromoacetate (Fig. 2). Fifty percent inhibition of acetylcholinesterase and of butyrylcholinesterase occurred with $2.96 \pm 0.16 \times 10^{-3}$ *M* and $4.18 \pm 0.38 \times 10^{-3}$ *M* bromoacetate, respectively. These concentrations are about 100-fold higher than those required for inhibition of neuroblastoma in culture. It is thus unlikely that cholinesterase inhibition plays a significant role in the control of neuroblastoma growth.

Inhibition of Neuroblastoma in Mice—Tables I–IV summarize the effects of various therapy regimens on survival rates of A/J mice with implanted neuroblastoma tumors. For bromoacetylcholine, 30 mg/kg injected into the tumors one to three times per day gave the highest success rate, with an average prolongation of life of 147–217% (Table I). Comparatively, bromoacetate (12 mg/kg) given once or twice a day also increased the average survival time 200 and 157%, respectively (Table II).

Atropine (1 mg/kg) given with 30 mg of bromoacetylcholine/kg reduced the survival time to that of control mice (no drug) (Table III). This result is in contrast to its effect upon the cells in culture where it had no effect on bromoacetylcholine inhibition.

Experiments designed to test the effectiveness of bromoacetylcholine and bromoacetate by the intraperitoneal route showed no significant enhancement of survival time (Table IV). Poor absorption, because of the permanent charge in the molecule, and dilution of drug at the tumor site may have contributed to these results.

Mice given bromoacetylcholine or bromoacetate directly into the tumor showed significant reductions in tumor growth (Figs. 3 and 4). Those surviving more than 150 days were free of tumors and had no recurrence of neuroblastoma during the remainder of their natural lives.

DISCUSSION

With the advent of chemotherapy, a marked improvement was made in the treatment of Wilm's tumor, a less common solid tumor in children. No such advances have been made in the chemotherapy of neuroblastoma (1). Several agents, including vincristine, cyclophosphamide, and daunorubicin, have been tried without significant improvement in the overall survival rate of treated patients. Following an initial slowing of tumor growth, there appears to be an escape from the effects of these noncell-specific antineoplastic agents. Remission rates remain low, and the duration of drug effectiveness is relatively short (1–4).

The present work demonstrated the remarkable effectiveness of bromoacetylcholine and bromoacetate in reducing neuroblastoma growth

Table IV—Effects of Intraperitoneal Injection of Bromoacetate (II) and Bromoacetylcholine (I) on Neuroblastoma Inhibition

Drugs	Frequency of Injection per Day	*n*	Range, days	Mean ± *SE*, days	CLS[a], %	Median Lifespan, days
Control	3	9	8–49	30.3 ± 4.0	—	35
I, 30 mg/kg	3	8	31–45	34.3 ± 1.7	+13	35
II, 12 mg/kg	3	8	31–47	38.0 ± 1.9	+25	38

[a] See Table I.

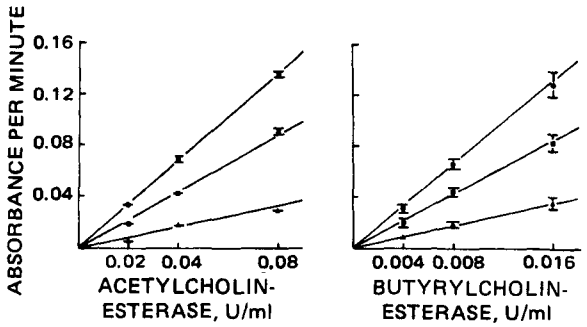

Figure 2—*Inhibition of acetylcholinesterase and butyrylcholinesterase by bromoacetate. Key: ●, control; ■, 3 × 10⁻³ M bromoacetate; and ▲, 1 × 10⁻² M bromoacetate. Each point represents the mean of four values, and bars represent the standard error of the mean. All plotted lines passed through the origin, and the lines obtained with bromoacetate treatments had slopes that were smaller than those of uninhibited ones. These results indicate a reversible inhibition of cholinesterases by bromoacetate.*

both *in vitro* and *in vivo*. Since the murine C-1300 neuroblastoma A/J mouse model has been noted to be comparable to the human disease (10), the present findings open the possibility of an important advance in chemotherapy. They are even more significant in light of the fact that these tumors have been cured in adult mice. Older children and adults with neuroblastoma have a much poorer prognosis than do babies less than 1 year old.

Following the observations of cytotoxicity of these two bromo compounds, preliminary investigations of possible mechanisms of action were made. The results have been presented here and in an earlier paper (6). One proposed mechanism involves the binding of bromoacetylcholine irreversibly to cholinergic receptors and/or cholinesterases. It was speculated that such binding might be responsible for the specific cytotoxicity of this compound. That cholinesterase inhibition is not part of the mechanism can be derived from the fact that 100-fold higher concentrations of bromoacetylcholine and bromoacetate are needed to inhibit these enzymes than to inhibit neuroblastoma cell growth. The irreversible binding of these bromo compounds to the cholinergic receptor on neuroblastoma cell membrane seems to be responsible for their cytolytic effect[5].

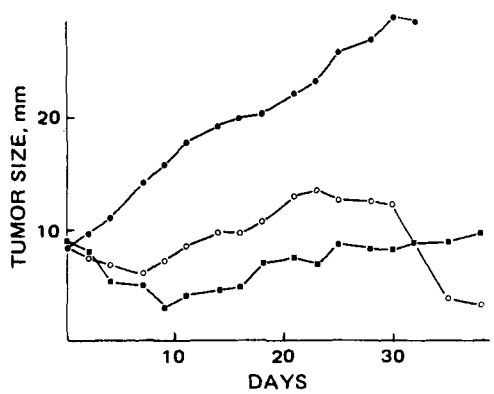

Figure 3—*Effects of bromoacetate and bromoacetylcholine on the tumor size of A/J mice inoculated with 1 × 10⁶ cells of neuroblastoma. Key: ●, control; ■, treated with bromoacetate, 12 mg/kg, twice a day; and ○, treated with bromoacetylcholine, 30 mg/kg, once a day. Note a significant depression of tumor growth in treated* versus *control animals.*

[5] Based on recent findings to be published shortly.

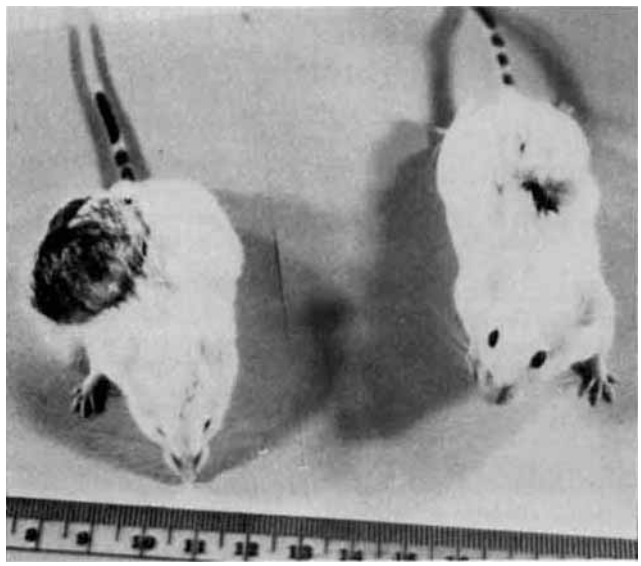

Figure 4—*Treatment of neuroblastoma-inoculated A/J mice with bromoacetylcholine. Left: Mouse inoculated with 1 × 10⁶ cells of neuroblastoma and injected with saline three times daily, except weekends, for 5 weeks as control. Right: Mouse treated with 30 mg of bromoacetylcholine/kg three times daily, except for weekends, for 5 weeks.*

In conclusion, the simple organic compounds bromoacetylcholine and bromoacetate are potent drugs in the inhibition of neuroblastoma growth. The optimal regimen was 30 mg of bromoacetylcholine/kg injected one to three times daily or 12 mg of bromoacetate/kg injected once daily, except weekends, for 6 weeks. Compared to untreated controls, the tumors were significantly reduced in size and the average lifespan was increased 200%. These drugs should be given by intravenous injection to patients with metastatic neuroblastoma since GI absorption is poor, as shown by the experiments utilizing intraperitoneal injections.

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