

## **Antitumor Activity of L-2,4 Diaminobuturic Acid Against Mouse Fibrosarcoma Cells in Vitro and in Vivo**

G. Ronquist, R. Hugosson, and B. Westermarck

Departments of Clinical Chemistry and Neurosurgery,  
University Hospital, S-750 14 Uppsala 14, Sweden  
and Division of Cell Biology,  
Wallenberg Laboratory, University of Uppsala, S-751 22 Uppsala, Sweden

### **Zelldestruktive Wirkung von L-2,4 Diaminobuttersäure gegen Fibrosarkomzellen bei Mäusen in vitro und in vivo**

**Zusammenfassung.** Mäusefibrosarcom in Zellkultur wurde mit L-2,4 Diaminobuttersäure (DAB), einer nicht metabolisierbaren Aminosäure, inkubiert. Die Tumorzellen wurden völlig und irreversibel bei der Inkubation mit 10 mM DAB unter 20 h bei 37 °C zerstört. Die zelldestruktive Wirkung von DAB beruht wahrscheinlich auf einer osmotischen Lysis. Die schädliche Wirkung von DAB ließ sich durch gleichzeitige Inkubation mit L-Alanin oder L-Methionin eliminieren. Beide konkurrieren mit DAB, während D-Formen der gleichen Aminosäuren und Sarcosin schwache Wirkung hatten.

Die Fibrosarcomzellen wurden auch auf Mäuse transplantiert, welche danach mit i.p. Injektionen einer isotonen 0,1 M DAB-Lösung behandelt wurden. Die neoplastischen Zellen wurden insgesamt auf 90 Tiere transplantiert. Das Gewicht des Tumors von 42 behandelten Tieren war im Mittel 1,16 g ( $\pm 0,77$  g) im Vergleich zu 2,05 g ( $\pm 1,22$  g) bei 27 nicht behandelten Tieren. Dies kommt einer Reduktion von 43,4% gleich.

Siebzehn Mäuse starben infolge der Behandlung, und bei einigen Tieren führte sie zu schlechter Nahrungsaufnahme und zu neurologischen Symptomen. Diese Nebenwirkungen begrenzen die Anwendung von DAB allein als Mittel gegen Tumoren.

Allerdings kann DAB bei der Eigenart seiner Wirkung neue Möglichkeiten für Kombinationsbehandlung in der Tumorthherapie bieten.

**Schlüsselwörter:** L-2,4-DAB, Inkubationsexperimente und Behandlung mit – Zelldestruktive Wirkung, in vitro, in vivo – Mäusefibrosarkomzellen, Zellkultur, Transplantation

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*Offprint requests to:* Dr. G. Ronquist, Department of Clinical Chemistry, University Hospital, S-750 14, Uppsala 14, Sweden

*Abbreviations:* AIB =  $\alpha$ -aminoisobuturic acid; DAB = L-2,4 diaminobuturic acid; MEM = minimum essential medium; LD = lactic acid dehydrogenase

**Summary.** Mouse fibrosarcoma cells were grown in vitro and incubated with L-2,4 diaminobuturic acid, a non-metabolizable amino acid. The tumor cells were irreversibly and totally damaged by incubation with 10 mM DAB for 24 h at 37 °C. The cell-destructive effect by DAB was probably due to an osmotic lysis induced by the non-saturated intracellular accumulation of DAB. The harmful effect of DAB could be abolished by concomitant incubation with L-alanine and L-methionine, that compete with DAB for the same transport system, while the D-forms of the same amino acids as well as sarcosine had a weak effect.

The fibrosarcoma cells were also transplanted s.c. into mice that were subsequently treated with i.p. injections of an isotonic 0.1 M DAB solution. The neoplastic cells were transplanted into totally 90 animals. The mean tumor weight of 42 treated animals was 1.16 g ( $\pm 0.77$  g) compared with the corresponding figures of the 27 untreated mice, that were 2.05 g ( $\pm 1.22$  g), i.e., a 43.4% reduction of tumor growth. There were, however, 17 drug-related deaths. Treatment with DAB generally resulted in weight reduction, at least partly due to loss of appetite, in the animals. In addition, neurological symptoms of a specific character could develop among several of the treated animals. The side effects apparently restrict the usefulness of DAB alone as an anti-tumor agent, but since the principle of action of DAB is unique and not shared by other known chemotherapeutics it might offer new possibilities in the combined treatment of neoplastic growth.

**Key words:** L-2,4 diaminobuturic acid, incubation experiments and treatment with – Antitumor activity, in vitro, in vivo – Mouse fibrosarcoma cells, cell culture, transplantation

Alterations in nutrient uptake activity that accompany changes in cellular proliferation rate have been implicated in models of cell cycle control (Holley 1972; Pardee 1974; Cecchini et al. 1976). However, studies of the regulation of transport rate of nutrients such as certain amino acids into intact cells have been difficult to interpret due to the complexity of the system. Hence, it has been difficult to distinguish unambiguously between effects of intracellular metabolic changes, changes associated with the plasma membrane and effects of surface area on transport rates. Attempts have been made to separate uptake from subsequent metabolism by the use of nonmetabolizable analogues, such as  $\alpha$ -aminoisobuturic acid. This is the preferred substrate by System A, that is  $\text{Na}^+$ -dependent and energy-requiring, as defined for the Ehrlich cell by Oxender and Christensen (1963).

In a study of cultured human glia and glioma cells Ronquist et al. (1976) observed a manyfold increase in uptake and  $V_{\max}$  for AIB by the malignant human cells. This difference between normal and neoplastic cells in the ability to accumulate amino acids might be of a more general significance, since others have found a similar difference between Balb/3 T3 cells and their derived virus-transformed cell lines (Foster and Pardee 1969; Isselbacher 1972; Quinlan et al. 1976; Hamilton and Nilsen-Hamilton 1976).

The preferred substrates for System A are monoaminoacids with short carbon chains, but also diamino acids, such as DAB, can share this transport system as

has been shown for Ehrlich cells (Christensen 1975). What is more, the diaminoacids are generally transported against a much steeper concentration gradient into the Ehrlich cells than the monoamino acids (Christensen et al. 1973). Therefore, the kinetics of the uptake of DAB in Ehrlich cells were apparently of the non-saturated type and resulted in damage to these cells (Christensen 1977), most probably due to osmotic lysis possibly combined with a lowered adenylate charge potential within the cells as a consequence of the unlimited uptake of the diamino acid (Ronquist and Christensen 1973).

The present investigation describes the DAB induced, complete damage to mouse fibrosarcoma cells, when rapidly growing *in vitro*. In addition, the reduction in size of the solid tumor is reported when the fibrosarcoma cells had been transplanted into and grown in mice, that were subsequently treated with intraperitoneal injections of DAB daily.

## Material and Methods

### *Chemicals*

All chemicals were of analytical grade. L-2,4 diaminobuturic acid (dihydrochlorid form MW 191.1) was from Serva Fine Biochemicals, Federal Republic of Germany. It was dissolved in water and maintained as a 0.1 M solution after adjustment of pH to 7.4 with aqueous sodium hydroxide. The osmolarity of the amino acid solution was 300–310 mOsm. N-methylglycine (sarcosine), alanine and methionine, L- and D-forms, respectively, were all obtained from Sigma, USA, and maintained as isotonic 0.1 M solutions with a neutral pH.

### *In Vitro Experiments*

**Cell Culture.** The fibrosarcoma cell line was derived from spontaneously transformed cells of normal embryonic mouse lung cultures (Pontén 1971). The cells were routinely grown in Eagle's MEM supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 U/ml and streptomycin, 50 µg/ml). The cultures were grown at 37 °C in humidified air with 5% CO<sub>2</sub>. Subcultivation was performed 2 or 3 times a week at a 1:4 split ratio.

For experiments, cells were trypsinized (2.5 mg of trypsin per ml in phosphate buffered saline) and suspended in culture medium. The cell number was adjusted to  $2 \times 10^5$  cells per ml and an appropriate number of 35 mm Nunc plastic culture dishes were inoculated with 2 ml of cell suspension. The cells were permitted to grow in Eagle's MEM with 10% fetal calf serum for 24 h before incubation experiments.

**Incubation with DAB and Selected Amino Acids.** DAB in different concentrations (1–20 mM, usually 10 mM) was added to the fibrosarcoma cells, when still growing in a simplified culture medium for incubation in the plates, either alone or together with one (sarcosine) or two other amino acids (alanine and methionine, D- and L-forms, respectively) in 10 mM each. The simplified culture medium consisted basically of Eagle's MEM lacking serum and it was adjusted osmotically to compensate for the amino acids added. Controls were always run concomitantly, i.e., cells maintained in the simplified culture medium. Incubation of cells proceeded for 4–48 h, generally for 24 h, at 37 °C in humidified CO<sub>2</sub>/air atmosphere.

The cells were examined as regards viability at the end of the incubation by light microscopy. The incubation media from control cells (with no additional amino acid) as well as from those with DAB and possibly other additional amino acids were separated from the cells and saved for lactic acid dehydrogenase (L-lactate: NAD oxido reductase, E.C. 1.1.1.27) analyses.

**Determination of LD.** For comparison, an equal amount of fibrosarcoma cells to that used for incubation experiments was subjected to a hypotonic lysis combined with freezing and thawing by suspending the cells in 2 ml of distilled water, i.e. the corresponding volume to that of the simplified culture medium. LD activity in cell-free media from incubated cells as well as in the cell-free water extracts was

**Table 1.** Weight of solid tumor in treated and untreated animals among altogether 90 mice comprising six series of experiments performed on different occasions. The results are given in mean value  $\pm 1$  standard deviation

	N	Tumor weight (g)	Per cent reduction	Casualties during treatment
DAB-treated mice (N=42) <sup>a</sup>	61	1.16 $\pm 0.77$ (N=42) <sup>a</sup>	43.4	17
Controls not treated	29	2.05 $\pm 1.22$ (N=27) <sup>a</sup>	—	—

<sup>a</sup> Two animals are not included in the calculation since no tumor growth developed in them

**Table 2.** Effect of different treatment regimens on tumor growth in mice. The number of untreated animals was five in each of the series except in series 3, where it was four. Calculations in series six are based on five treated animals and three untreated ones due to no tumor growth development in two of the treated and two of the untreated mice

Series	Number of treated mice	Total amount given of 0.1 M DAB solution in ml/animal, mean value, range given in brackets	Time period of treatment (days)	Casualties during treatment	Tumor weight, g Mean value, range given in brackets	
					Treated animals	Control animals
1	10	9.5 (9.5)	19	1	1.7 (1.0–2.6)	3.1 (2.0–4.5)
2	10	6.6 (3.0–9.3)	18	6	1.3 (0.6–1.8)	2.5 (0.9–5.0)
3	11	4.6 (4.6)	8	2	1.1 (0.5–1.8)	1.2 (0.4–2.0)
4	10	7.4 (5.75–9.25)	15	5	0.5 (0.1–1.3)	1.3 (0.3–2.3)
5	10	8.0 (5.95–9.45)	15	0	0.9 (0.2–2.0)	1.6 (1.0–2.2)
6	10	12.2 (8.1–14.7)	22	3	1.4 (0.5–3.9)	2.6 (0.8–3.6)

determined in accordance with the method employed by The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974). The LD activity in the water extracts from the osmotically destructed cells ( $49\text{--}51 \text{ nkat}/4 \times 10^5$  fibrosarcoma cells) was regarded as total and set to 100%, and the LD activity in the medium of incubated cells was expressed in per cent of the total after correction had been made for endogenous LD activity in the medium (control cells).

#### *In Vivo Experiments*

*Transplantation of Fibrosarcoma Cells into Mice.* Adult white male mice (N.M.R.I., Anticimex, Stockholm, Sweden) with a weight of about 35 g and fed with an ordinary diet (from Astra-Ewos AB, Sweden, Type R 3, containing a protein concentrate from fish and soybean, fodder yeast, wheat-germs, animal fat, soybean oil, vitamins, tracer elements and minerals) were used for the experiments. The cells to be transplanted ( $5 \times 10^7$ ) were brought via a blunt-ended cannula into a few mm wide incision, that was made in the right flank of the anesthetized animals. The neoplastic cells were in this way deposited

s.c. and the surgical incision was closed by two sutures. Fifteen mice were transplanted within each series of which five mice usually served as controls and 10 were subjected to treatment with DAB: Totally 90 animals were transplanted comprising six series of experiments performed on different occasions.

*Treatment with DAB.* Treatment began on day 1–4 after transplantation and the mice were given 0.50–0.90 ml (50–90  $\mu$ mol of DAB) of the 0.1 *M* DAB solution i.p. generally as a single dose once daily and the dosages and intervals between them were somewhat different for the six series (Table 2). The weight and clinical condition of the treated mice were followed every day. A weight loss of more than 2 g per 24 h resulted in withdrawal of treatment until the animals regained in weight. A weight reduction of the animals in the range of 0.5–2.0 g per 24 h resulted as a rule in a reduced dosage. The animals were treated for 8–23 days and were killed 2–12 days after the treatment had discontinued, when the tumor weight of the untreated mice had reached a value corresponding to about 10% of the total body weight. The solid tumors were removed and weighed. In some cases the tumors were extirpated from the mice (all together 7 animals) in anesthesia and the course and clinical condition of the 7 DAB treated and operated animals were followed.

## Results

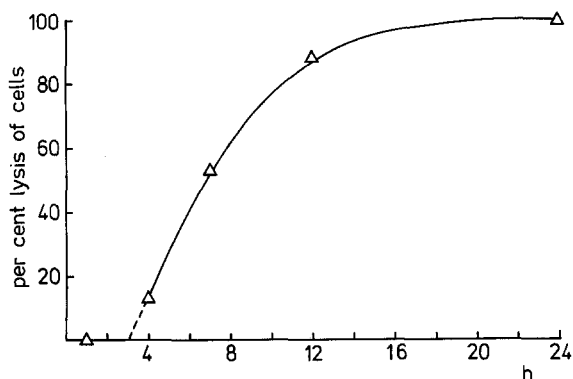
### *In Vitro Studies*

Figure 1 clearly demonstrates that 100% of the fibrosarcoma cells were lysed at 24 h of incubation in the presence of 10 mM DAB. The irreversible nature of the damage of the cells was confirmed in the light microscope where the cells appeared as empty craters. Fifty per cent lysis had occurred at 7–8 h of incubation. Ten millimolar DAB concentration or just below that seemed to be critical since 5 mM DAB in the medium had only a limited lytic effect on the malignant cells (Fig. 2). The effect of 5 mM DAB was only moderately increased by extending the incubation period to 48 h (Fig. 2). A further extension of time was not possible out of experimental reasons, since it was difficult to maintain the viability of the control cells for any longer periods in the simplified culture medium used for incubation.

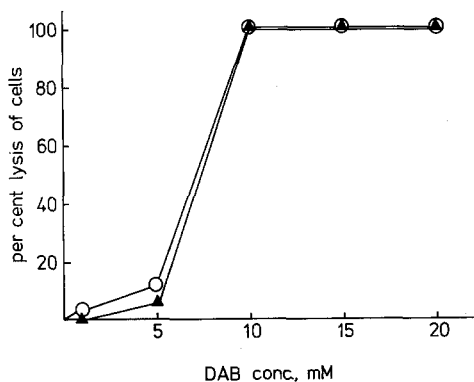
The 100% lysis of the neoplastic cells obtained by the presence of 10 mM DAB in the medium for 24 h never occurred if L-alanine and L-methionine were concomitantly added with DAB (Fig. 3). Instead a complete survival of the cells was observed and a result well comparable to that of the control cells was obtained (Fig. 3). L-Alanine and L-methionine are both physiological amino acids that are transported to a great extent by System A and they act as competitive inhibitors to DAB for the receptor-moiety of the carrier complex. The receptor is stereospecific in its choice of substrates (Christensen 1975), and it was therefore not unexpected that the D-analogues of alanine and methionine were less active as inhibitors in the fibrosarcoma cells (Fig. 3). Sarcosine that was found to have weak interacting properties for System A in human glia and glioma cells (Ronquist et al. 1976) also displayed weak inhibitory activity in the fibrosarcoma cells (Fig. 3).

### *In Vivo Studies*

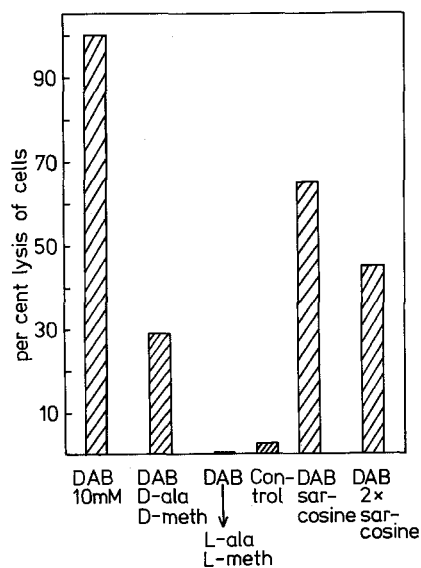
Totally six series of in vivo experiments were carried out at different occasions comprising in all 90 transplanted animals of which 27 served as controls. A survey is given in Table 1 of all series taken together and the average reduction in tumor size on a weight basis was 43.4% among 42 treated animals. Two of the treated animals and two of the controls did not develop any tumor growth and were not in-



**Fig. 1.** Time course of cytolytic activity by 10 mM DAB on fibrosarcoma cells. The tumor cells were incubated for various times with DAB in the simplified culture medium at 37 °C



**Fig. 2.** Effect of different concentrations of DAB to induce cytolysis in fibrosarcoma cells. The tumor cells were incubated with various amounts of DAB for 24 h (filled symbols) as well as for 48 h (open symbols) at 37 °C



**Fig. 3.** Cytolytic activity of DAB in fibrosarcoma cells in the absence and presence of other amino acids. The tumor cells were incubated with 10 mM of DAB either alone or together with 10 and 20 mM sarcosine or 10 mM each of alanine and methionine, D- and L-forms, respectively, for 24 h at 37 °C

cluded in the calculations. Seventeen died during treatment. It should be emphasized, however, that several treatment regimens were tried (see below) of which some lead to more death casualties than others due to individual tolerances for this diamino acid.

Generally, regardless of dosage and interval between doses, the animals displayed a weight reduction on treatment with DAB. The tumor growth did not contribute to any weight reduction since the untreated control animals with tumors did not lose in weight. In addition, neurological symptoms of a specific character could develop among several of the treated animals, the most prominent features of which being a more or less pronounced rigo-spasm combined with stiffness in the tail and extremities. Furthermore, the body movements became jerky and incoordinate and a typical characteristic was the tactile sensitivity. Indolence and loss of appetite were often concomitant peculiarities. As a rule these symptoms were reversible on withdrawal of DAB therapy for 1–3 days. The general finding on extirpation of the solid tumor was a local expansion with growth through the abdominal wall although the tumor never penetrated the peritoneum. A certain infiltration of the tumor through the surface-located thigh muscles was generally the rule but not to the extent that it was a real hindrance for the total extirpation of the tumor. Autopsy was performed in most of the animals but there were no evidences that the tumor had metastasized into the visceral organs, which showed a normal appearance.

The tumors were surgically removed from 7 of the DAB-treated mice and the clinical course of these animals was followed during 3 weeks after operation. They displayed in all respects a normal behavior but five of them had local recurrences of the tumors.

Some details of the different series are given in Table 2. Treatment of 10 mice began on day 3 after transplantation in Series 1. Each animal received 0.5 ml of the 0.1 *M* DAB solution once daily during 19 days. One mouse lost 6 g in weight during the first week of treatment and died. The remaining nine mice were killed 22 days after transplantation together with the five untreated control animals. The average tumor weight of the treated animals was 1.7 g (range 1.0–2.6 g) compared to that of the untreated animals being 3.1 g (range 2.0–4.5 g), i.e., an average weight reduction of 45.2%.

Treatment started on day 3 also in series 2 with 0.5 ml of DAB twice daily to 10 of the mice. A weight reduction was seen already after 2 days of treatment and since most of the mice also developed apathy the dosage was reduced. In spite of this, six animals died within the first week of treatment. The continued treatment of the remaining four mice during altogether 18 days was according to a modified schedule depending on the clinical condition of the animal, at the most 0.5 ml once daily to each animal. The four mice were killed 21 days after transplantation and the average weight of the tumors from the treated animals was 1.3 g (range 0.6–1.8 g) compared to that of the five controls being 2.5 g (range 0.9–5.0 g) constituting a weight reduction of 48.0%.

Treatment of the 11 animals in series 3 started 2 days after transplantation with 0.2 ml three times daily. Two animals died suddenly during the first days of treatment probably due to shock in connection with the injection. The treatment three times daily discontinued 10 days after transplantation for the remaining nine ani-

imals and they were killed 20 days after transplantation together with the four control animals. The average tumor weight of the treated animals was 1.1 g (range 0.5–1.8 g) compared to that of the controls being 1.2 g (range 0.4–2.0 g) which means that the effect was slight and only 8.3%.

Treatment with 0.75 ml of the 0.1 *M* DAB solution began on day 1 after transplantation for the 10 animals in series 4. The daily dosage was reduced to 0.50 ml in case of weight loss of 0.5–1.5 g/day in the animals. A one day pause was made in treatment 8 days after transplantation, since all animals had lost in weight 1–3 g. Treatment with 0.5–0.75 ml of DAB depending on the clinical condition of the animal continued until 15 days after transplantation. Five animals died, however, and the remaining five animals were killed 19 days after transplantation. They had an average weight of their tumors of 0.5 g (range 0.1–1.3 g) and the corresponding figure for the five control animals was 1.3 g (range 0.3–2.6 g). This means that the average weight reduction of the tumors was 61.5% in the treated animals.

The treatment with DAB of the 10 animals of series 5 started on day 4 after transplantation with 0.75 ml once daily to each animal. The daily dosage was then individualized to 0.50–0.75 ml depending upon the clinical condition of each mouse and possible weight reduction. An obligatory pause of treatment for 1 day for all animals took place 6 days after beginning of treatment. All mice were in relatively good condition during the period of treatment for 15 days and the animals were killed 19 days after transplantation. The average weight of the 10 tumors from the treated animals was 0.8 g (range 0.2–2.0 g). This implied an average weight reduction of 50% since the corresponding figures for the 5 tumors of the control animals were 1.6 g with a range of 1.0–2.2 g. No death casualties occurred during the treatment period in this series.

The mice of series 6 were treated from day 4 after transplantation with 0.90 ml of the 0.1 *M* DAB solution once daily. This was a higher dosage than before and therapeutic pauses were instituted for all animals on the 6, 9, 13, 15, and 21 days, respectively, after the beginning of treatment. The dosages were also individualized in the range of 0.50–0.90 ml depending on the clinical condition of each animal and possible weight reduction. Three mice died during the treatment period which was prolonged due to the retarded growth of the tumors both in the treated and control animals. When the mice were killed 27 days after transplantation, it was revealed that two of the treated animals and two of the control animals had not developed any tumor growth. These four animals were excluded from the calculations. The remaining five DAB-treated mice displayed an average weight of 1.4 g (range 0.5–3.9 g) contrary to that of the three controls which was 2.6 g (range 0.8–3.6 g) i.e., a 46.2% reduction.

## Discussion

Fibrosarcoma cells were totally damaged by 10 mM DAB *in vitro*. This was most probably due to a lytic effect on the cells caused by the high osmotic pressure possibly combined with a lowered adenylate charge potential, both phenomena being the result of the energy-dependent, intracellular accumulation of DAB. The effect was controllable, since no lysis at all was induced by DAB in the presence of



L-alanine and L-methionine. It means that as long as DAB was in the extracellular space, it was harmless to the neoplastic cells.

By this induced, directed cytolytic effect on the fibrosarcoma cells DAB appears as a unique agent on these cells with a principle of action that concerns the plasma membrane as the only subcellular organelle primarily involved. A sharp "transition" apparently took place in the concentration interval between 5 and 10 mM DAB in the extracellular (incubation) medium. The damage of the neoplastic cells was slight by the lower concentration both at 24 and 48 h while the higher concentration of DAB resulted in a sharp and complete response with total cell death within 24 h. The explanation for such a distinct "transition" is not clear at present, but the possibility might exist that the "transition point" is determined by the  $V_{\max}$  parameter, i.e., the higher  $V_{\max}$  the lower is the "transition point" in a certain cell. This aspect has not been explored in the present investigation, but the  $V_{\max}$  for AIB was several times increased in human glioma cells as compared to glia cells (Ronquist et al. 1976). Such a difference between normal and malignant cells might have a more general significance and this would lead to also studies on malignant growth in vivo with the aim of arresting the growth of the tumor cells while the normal cells should be minimally damaged.

DAB did cause a consistent reduction in tumor mass, although an unequivocal effect, i.e., a complete reversion of the tumor growth with minimal side effects on the organism of the host, was not achieved in this study on tumor-bearing mice.

The neurological symptoms combined with weight loss were serious when intensifying the treatment with DAB. The neurotoxic symptoms may result from ammonia toxicity on deamination of DAB (Chen and Koeppel 1970; O'Neal et al. 1968). No examinations were performed in this study to confirm a possible ammonia presence in the blood of the animals, and even if it were possible to give effective treatment in reducing peripheral ammonia toxicity, the central ammonia toxicity would be very difficult to control (Fenster 1972). Some of the neurological symptoms might also be explained by increased GABA levels in the brain (Sutton and Simmonds 1974) and DAB was recently found to be a more effective inhibitor of GABA transaminase in vivo than in vitro (Beart and Bilal 1977).

Because of the serious side-effects of DAB when administered in vivo, it is not possible at present to intensify the treatment with DAB of the tumor-bearing mice. A possible way to evade this difficulty would be to selectively block the System A receptors of normal cells, provided that there are also qualitative differences in the receptors between normal and malignant cells. A different behavior of human glia and glioma cells versus sulfhydryl reactants was indeed observed indicating an active involvement of membrane sulfhydryls in the maintenance of System A transport in the human glia cells while such an involvement was apparently lacking in the glioma cells (Ronquist et al. 1976). A similar involvement was also observed in chick embryo fibroblasts (Smith-Johannsen et al. 1977).

Another possibility that might offer advantages would be the combination of DAB treatment, the active principle of which being cell-physiological phenomena related to the plasma membrane, with other agents with known antitumor activities working on other subcellular levels thereby accomplishing a synergistic effect on tumor growth.

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