

## Antitumour Effect of L-2,4 Diaminobutyric Acid on a Hepatoma Cell Line

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**Abstract.** Pharmacological treatment of malignant disease is often insufficient highlighting the need for more efficient treatment based on new principles. We have observed that the amino acid analogue diaminobutyric acid (DAB), accumulates in malignant cells apparently without saturation kinetics, leading to hyperosmosis and subsequently to cell lysis. In the first *in vitro* part of the present study hepatoma cells were incubated with DAB in miniwells in the presence or absence of physiological amino acids. In the *in vivo* part malignant cells were inoculated into rat liver after laparotomy. The tumour was treated by continuous infusion of DAB via a catheter, the tip of which was placed in the center of the tumour. DAB had a significant antitumour effect both *in vitro* and *in vivo*. The principle of action of DAB as an antitumour agent is unique and therefore would be the ideal partner to practically any other cytostatic drug for a combined treatment to achieve a synergistic effect.

Pharmacological treatment of malignant disease is often unsuccessful so the search for new drugs is of great interest. The growth of both benign and malignant cells depends on nutrient uptake activity (1, 2). Consequently, the growth of malignant cells depends, at least partly, on the transport capacity of amino acids (a.a.) into the cell.

System A is a universal transport system of a group of a.a. into cells. In Ehrlich cells System A has been shown to transport both physiological a.a. and some a.a.-analogues, e.g. L-2,4 diaminobutyric acid (DAB) and  $\alpha$ -aminoisobutyric acid (AIB) (3). The observation that accumulation of AIB in cultured malignant glioma cells is considerably higher than in cultured non-malignant glia cells may be true e.g. for most malignant cells (4). The accumulation of AIB in Balb/3T3 virus-transformed cell lines is much higher than in non-

transformed cells (5-8). Furthermore, DAB is transported into the cell against a much steeper concentration gradient than are monoamino acids (3). It may therefore be expected that the accumulation of DAB in the cell exceeds that of monoamino acids. The extensive accumulation of DAB results in increased osmotic strength eventually leading to lysis of the malignant cells. In *in vitro* experiments we explored whether incubation of a hepatoma cell line with DAB influenced cell proliferation, and whether presence of physiological a.a. interfered with the effect of DAB. Further, we investigated the possibility of DAB exerting its effects under *in vivo* conditions.

### Materials and Methods

The hepatoma cell line used for both the *in vitro* and *in vivo* experiments was a diaminobenzidine-induced hepatocellular cancer. The tumour was kept viable by preparation under sterile conditions of a suspension of tumour cells (9) and retransplantation into the thigh every 10<sup>th</sup> day in a new set of rats. Lister Hooded rats (B&K Universal AB, Sollentuna, Sweden) were used to keep the tumour cell line viable and for the *in vivo* experiments.

***In vitro* study.** A tumour cell suspension was prepared (9). The tumour cells were cultured in a multiwell plate (density of approximately 180,000 cells per well) for 21 hours in a standard incubator at 37° C. The composition of the culture medium (Nord Junter & Cell AB, Bromma, Sweden) is given in Table I. Culture medium with or without the presence of physiological amino acids was used. Different concentrations of DAB (2-20 mmol/L) were used in the various *in vitro* experiments. The experimental protocol is given in Table II.

Cell survival rate was determined by cell proliferation assay (4 hour incubation) based on the living cell capacity of reducing tetrazolium salts into formazan derivatives (EZ4U, Biomedica GmbH, Vienna, Austria).

***In vivo* study.** The protocol for the *in vivo* experiments is given in Table III. Via a laparotomy (day 0), 0.2 mL of tumour cell suspension containing 1x10<sup>6</sup> malignant cells per mL, were inoculated into the antero-caudal aspect of the median lobe of the liver (9). On day 7 a relaparotomy was performed to determine tumour size (9), and to allow continuous administration of 80 mmol/L DAB to the tumour via a cannula in the treatment groups of rats, or continuous administration of an identical volume of isotonic saline in the control groups. The infusion

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Table I. Composition of hepatoma cell culture medium.

Substance	Concentration (mg/L)	Substance	Concentration (mg/L)
NaCl	6400	Choline chloride	4
KCl	400	Folic acid	4
CaCl <sub>2</sub> (anhydride)	200	i-Inositol	7.2
MgSO <sub>4</sub> 7H <sub>2</sub> O	200	Nicotineamide	4
NaH <sub>2</sub> PO <sub>4</sub> H <sub>2</sub> O	140	D-Ca-Pantothenate	4
Glucose	4500	Pyridoxal HCl	4
Fe(NO <sub>3</sub> ) <sub>3</sub> 9H <sub>2</sub> O	0.1	Riboflavin	0.4
Sodium puruvate	110	Thiamine HCl	4
Phenol red	15		
NaHCO <sub>3</sub>	3700		

was performed using an osmotic pump (ALZA Corporation, Palo Alto, California, USA) in the abdominal cavity and connected to an infusion cannula by a catheter tube. The tip of the infusion cannula was placed at a depth of 5 mm in the tumour and fixed using a 6.0 polyglycolic suture to adjacent normal liver parenchyma. The animals were sacrificed on day 14. The 0.2 mL pump emptied at an infusion rate of 0.5 µL per hour (Alzet pump Model 2001), and the 2.0 mL pump at an infusion rate of 10.0 µL per hour (Model 2ML1) during one week. All reservoirs of the osmotic pumps were opened and verified as being empty at time of sacrifice. In order to examine the consistency of the results, experimental series 1 and 2 were performed under identical conditions. Peri- and intraoperative handling of the animals was as described (10).

The study was approved by the local Ethical Committee for animal experiments at Umeå University, Sweden.

**Statistics.** The Mann-Whitney U test (two-tailed) was used for analysis of data for comparison between groups. Calculations were made with the aid of the program Statistica for Windows, version 4.5, StatSoft, Tulsa, OK, USA, 1993.

## Results

Results of the *in vitro* study are given in Table II. In culture medium lacking physiological a.a., presence of 80 mM DAB resulted in significantly lower cell survival than in control cells. Even in culture medium containing physiological a.a. together with DAB a lower survival was noted.

Table III summarizes the results of the *in vivo* study. The relative increase in tumour size was significantly smaller in rats receiving DAB than those receiving saline both in experimental series 1 and 2 (0.2 mL, osmotic pumps), emphasising the consistency of the experiments. Also in rats with 2 mL pumps (experimental series 3), those given DAB had numerically smaller tumour size than those given saline.

Table II. Survival rate of a hepatoma cell line after 21 hours incubation with or without L-2,4 diaminobutyric acid (DAB), in medium with or without physiological amino acids (a.a.). Survival rate was determined by differences in absorbance following subsequent 4 hours incubation with tetrazolium salts.

No. of observations	a.a. included	DAB included	Absorbance Median (range)	
6	No	No	2.409 (2.271 - 2.514)	p=0.01
6	No	Yes	2.154 (0.733 - 2.317)	
6	Yes	No	1.961 (1.195 - 2.034)	p=0.02
6	Yes	Yes	1.932 (1.687 - 1.991)	

Intratumoural odema, as determined macroscopically, in experimental series 3 was, however, considerably more pronounced than in series 1 and 2 in which edema appeared equally. The volume of intratumoural oedema in experimental series 3 made estimation of the tumour size less reliable than for the other two series.

## Discussion

We observed, in the *in vitro* study, that cell survival rate was reduced in the presence of DAB in the culture medium. Also, the finding that the effect of DAB on cell survival rate was more pronounced in the absence of physiological a.a. supported the assumption that the DAB-inhibitory action on tumour cell growth was exerted after DAB had been transported into the cells. It should be noted that DAB is not further metabolized inside the cells. The DAB transport system is shared by several of the physiological a.a. and therefore, in their presence, the DAB uptake process was compromised by competitive inhibition (11). DAB in its extracellular environment was not harmful to the cells. It is therefore reasonable to conclude that, after DAB uptake against an unusually steep concentration gradient (3), it becomes osmotically active which may lead to cellular lysis.

The *in vivo* experiment, also using a hepatoma cell line, comprised a study where DAB was not given systemically but administered directly into the tumour mass. That means that while the *in vitro* experiments concerned solitary tumour cells growing in a monolayer on a plate the *in vivo* experiments had to deal with a tumour parenchyma. However, a diffusional area of DAB along the interstitial space of the tumour parenchyma could be anticipated and by continuous administration of DAB into the tumour the maintenance of DAB concentration in the extracellular space could be satisfactorily guaranteed. We were also well aware that the composition of the extracellular fluid could not be adjusted in the same way as was the case for the culture medium. Hence,

Table III. Experimental protocol and results of infusion of L-2,4 diaminobutyric acid (DAB) or isotonic saline by an osmotic pump system into hepatoma tumour from day 7 to day 14. There were no statistically significant differences in tumour volume within each experimental series (1,2 and 3) in rats before administration of DAB or saline.

Experimental series	Infusate	No. of rats	Osmotic pump volume (ml/24 hours)	Tumour volume day 7 Median (range)	Tumour volume day 14/ tumour volume day 7	
1	DAB	4 <sup>a</sup>	0.2	394 (352 - 446)	24.5 (20.2 - 34.1)	p=0.027
1	Saline	5 <sup>b</sup>	0.2	320 (220 - 405)	38.9 (31.9 - 78.4)	
2	DAB	5 <sup>c</sup>	0.2	2678 (1688 - 3402)	4.2 (3.4 - 6.3)	p=0.01
2	Saline	6	0.2	1726 (550 - 3240)	9.0 (5.7 - 20.3)	
3	DAB	8	2.0	400 (319 - 936)	17.0 (7.5 - 26.5)	p=0.12
3	Saline	6 <sup>b</sup>	2.0	408 (288 - 750)	25.0 (13.1 - 40.9)	

<sup>a,b</sup>: Two and one rats in respective groups of 6 rats died before day 7

<sup>c</sup>: In one (excluded) out of six rats the tip of the infusion catheter was outside the tumour.

the interstitial fluid contained the physiological a.a, which could modify the response of DAB by competing for the same amino acid transport system. We tried to overcome this inconvenience by administering a higher concentration of DAB.

An attempt to validate a dose - response effect of intratumoural infusion of DAB was made by using osmotic pumps allowing two different infusion volumes and rates. Numerically diminished tumour growth, as compared to controls, was also observed in rats receiving DAB by the higher infusion rate. It seemed, however, that tumour volume measurement was less reliable in rats with the 2 mL pump because of intra- and extra-tumoural oedema, which was difficult to estimate quantitatively.

As DAB was administered intratumourally, leakage of the drug into the systemic circulation was anticipated and, therefore, general systemic effects of DAB could not be excluded a priori. As no rats died and as no side-effects of the drug, as judged from the general conditions of the rats, were noted it seemed that intratumoural infusion of DAB could be a useful principle for treatment of malignant tumours.

We conclude that DAB hampers hepatoma cell proliferation both in vitro and in vivo. The principle of action of DAB as an antitumour agent is unique and not shared by any other cytostatic compound encouraging further experimental work. Also, since DAB does not intrude on the principles of action of any other anticancer drug, it would be the ideal partner to practically any other cytostatic drug for a combined treatment to achieve a synergistic effect. Finally it could be pointed out that normal pharmacologic treatment failure is i.a. due to the induction of specific proteins extruding the

anticancer agent. Such a principle is invalid in the case of DAB since the DAB uptake mechanism is identical to the native transport of physiological amino acids which is essential for tumour cell proliferation.

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