

Unique Antitumour Effects of L-2,4 Diaminobutyric Acid on Cultured Hepatoma Cells

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Abstract. A single hepatoma cell line was grown *in vitro* and incubated with L-2,4 diaminobutyric acid (DAB), a non-metabolizable amino acid, under various conditions. The tumour cells were irreversibly damaged by incubation for 8 hours with 8 mmol/L of DAB. The tumour cell-destroying effect of DAB was dose- and time-dependent with no effect at a DAB concentration of 1.6 mmol/L. The presence of N-methyl α -aminoisobutyric acid (a specific substrate of amino acid transport system A) in the incubation medium abrogated the tumour cell destructive effect of DAB in a dose-dependent fashion. The presence of non-physiological amino acids in the incubation medium *per se* was not the cause of tumour cell destruction, since inclusion of α -amino-isobutyric acid and N-methyl α -aminoisobutyric acid in the incubation medium did not influence the viability of hepatoma cells. We conclude that the tumour cell destructive effect of DAB was the result of a huge and unlimited uptake of DAB energized by the Na⁺-gradient and that this uptake was not subjected to the law of saturation kinetics. This was combined with a tumour cell energy crisis in attempts to restore the Na⁺-gradient.

The pharmacological treatment of malignant disease is often unsuccessful. Consequently, the search for new drugs for cancer treatment is of great interest. In such a quest, we observed in a previous *in vitro* study that the amino acid analogue, L-2,4-diaminobutyric acid (DAB) is transported into hepatoma cells by System A, apparently without saturation kinetics, leading to hyperosmosis and subsequently to cell lysis (1). Moreover, we also noted an inhibitory effect of DAB on hepatoma volume growth when infused

continuously for one week using an osmotic pump system into the tumour in rats, all of which survived and did well during the study period (1). In these initial studies on DAB as an antitumour agents, several questions about its principle of action were raised. The aim of the present study was to further clarify the mechanism of DAB as an antitumour agent.

Materials and Methods

A single hepatoma cell line was used (2). The tumour cells were cultured in a multiwell plate (suspension of approximately 100,000 cells per well) in a standard incubator at 37°C. The composition of the culture medium (Nord Junter & Cell AB, Bromma, Sweden) is given in Table I. The culture medium was used with or without the presence of physiological amino acids. The cell survival rate was determined by a cell proliferation assay (2-to 5-hour incubation) based on the living cell capacity of reducing tetrazolium salts into formazan derivatives (EZ4U, Biomedica GmbH, Vienna, Austria). Incubations were done during various time-periods with different concentrations of DAB alone, or in combination with some other non-physiological amino acids, in the presence as well as in the absence of physiological amino acids. Light absorption values at 450 nm (dependent on cell suspension and type of culture medium) were determined in control wells on each multiwell plate used. The readings of control wells were subtracted from the readings of light absorbance after incubation with EZ4U. A standardization factor, making the reading in each of the wells in the column of controls 10, was calculated. All readings in each row were then multiplied by its standardization factor. Such standardization was considered appropriate as the experiments were performed over a time span of two years.

Firstly, we assessed whether tumour cells cultured in a multiwell plate could be kept viable for a sufficient time-period for the subsequent experiments. Secondly, we explored the dependency of cell viability on DAB concentration and incubation time. Thirdly, we investigated whether the presence of other amino acid analogues, *viz*, N-methyl- α -aminoisobutyric acid (N-methylAIB), a specific substrate amino acid for system A, and α -aminoisobutyric acid (AIB), also transported mainly by system A in culture medium, would cause cell death. These two compounds are examples of non-physiological amino acids sharing properties with DAB, although their cellular uptake is subjected to the law of saturation kinetics opposite to DAB. Fourthly, we elaborated on the combined actions in the culture medium of N-methylAIB and DAB, or N-methylAIB plus AIB, together with DAB on cell viability. Fifthly, all incubations were performed in both the presence and absence of

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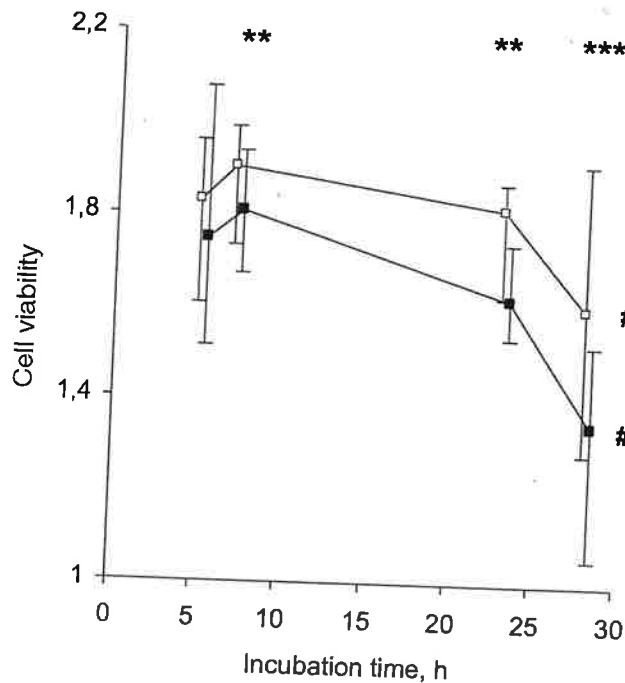


Figure 1. Viability (median, minimum and maximum values) of a single hepatoma cell line as a function of time in presence (■) and absence (□) of physiological amino acids. ** denotes $p < 0.01$ and *** $p < 0.001$. # denotes $p > 0.001$ at 27 hours vs. baseline. $N = 16$.

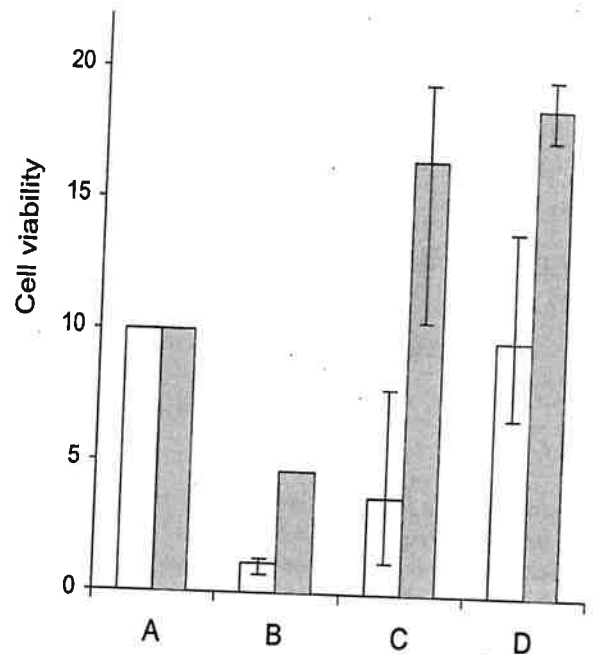


Figure 2. Dose-dependent inhibitory effects of N-methylAIB on DAB-induced lysis of a single hepatoma cell line in the absence (empty columns) and presence (filled columns) of physiological amino acids. Incubation time 19.5 hours. $N = 6-9$ except in B (empty column, $N = 4$ and filled column, $N = 1$). A. Control, B. DAB, 8 mmol/L, C. DAB, 8 mmol/L + N-methyl AIB, 4 mmol/L, D. DAB, 8 mmol/L + N-methylAIB, 10 mmol/L. ** denotes $p < 0.01$.

physiological amino acids in incubation medium for determination of their assumed modifying role of the DAB - induced lysis of hepatoma cells.

Statistics. The data were presented as median and range. The Mann-Whitney U-test was used for analysis of data for comparison between groups and the Sign-test for comparison of data within groups. Calculations were made with the aid of Statistica for Windows, version 4.5, StatSoft, Tulsa, OK, USA, 1993.

Results

Hepatoma cells incubated in a multiwell plate could be kept viable, both in the presence and absence of physiological amino acids, for at least 28 hours for subsequent experiments with DAB (Figure 1). In culture medium with and without physiological amino acids, 88% and 77% cell viability, respectively, remained after 28 hours of incubation.

The effect of DAB was dose- and time-dependent (Table IIA and IIB). For example, after incubation in medium without physiological amino acids, for 8 hours with DAB ≥ 8 mmol/L, less than 33% cellular viability remained ($p < 0.01$). Physiological amino acids in the incubation medium mitigated the DAB destroying effect on tumour cells, which in turn was invalidated by an extension of the incubation time (Table IIA and IIB). Hence, after 28 hours of incubation in the presence of 4 mmol/L of DAB, cell viability was lower than in the controls ($p < 0.01$), but higher than in the presence of 12

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 ₂ (anhydride)	200	i-Inositol	7.2
MgSO ₄ ·7H ₂ O	200	Nicotinamide	4
NaH ₂ PO ₄ ·H ₂ O	140	D-Ca-Pantothenate	4
Glucose	4500	Pyridoxal HCl	4
Fe(NO ₃) ₃ ·9H ₂ O	0.1	Riboflavin	0.4
Sodium pyruvate	110	Thiamine HCl	4
Phenol red	15		
NaHCO ₃	3700		

Table II. Survival of a single hepatoma cell line over time exposed to different concentrations of DAB in absence (A) and presence (B) of physiological amino acids.

A				
DAB(mmol/L)	Incubation time, hours			
	0	8	25	28
0	10	10	10	10
1.6	10		10.02 (5.09-15.09), n=16	
4	10		5.79 (3.72-6.69), n=8	6.94 (5.00-8.39), n=8
8	10	3.20 (1.17-6.38), n=8	2.52 (0.54-10.43), n=7	0.71 (0.37-3.35), n=24
12	10	2.67 (2.14-3.16), n=8	1.50 (0.82-2.00), n=8	0.51 (0.26-0.72), n=16
16	10	2.28 (1.95-3.05), n=8	3.66 (0.19-10.85), n=7	0.47 (0.30-0.69), n=16
B				
DAB (mmol/L)	Incubation time, hours			
	0	8	25	28
0	10	10	10	10
1.6	10		9.59 (8.39-10.33), n=15	
4	10		7.67 (5.62-8.98), n=8	6.84 (4.75-8.24), n=8
8	10	9.31 (8.40-9.90), n=8	6.57 (0-23.6), n=8	1.84 (0-9.48), n=24
12	10	7.74 (4.68-8.84), n=8	2.13 (1.44-2.58), n=7	1.06 (0.67-2.50), n=16
16	10	6.87 (4.73-8.23), n=8	3.57 (2.54-18.91), n=8	0.40 (0.23-0.77), n=16

Note, significant differences ($p < 0.01$) between baseline values in all cases except at 1.6 mmol/L of DAB (A, B) and 4.0 mmol/L DAB (B) at 25 hours.

mmol/L or more of DAB ($p < 0.01$) (Table IIA and IIB). Cell viability was totally unaffected by 1.6 mmol/L DAB in the culture medium.

The presence of N-methylAIB (4 mmol/L) in the incubation medium abrogated the cell destructive effect of DAB (8 mmol/L) in a dose-dependent fashion (Figure 2). This effect of N-methylAIB was somewhat modified by physiological amino acids. Inclusion of either AIB or N-methylAIB in the absence of DAB in the incubation medium did not influence the viability of hepatoma cells (Figure 3). The inhibitory effect of AIB on DAB was significantly higher than that of N-methylAIB (Figure 3).

Discussion

By showing that hepatoma cells could be cultured for at least 28 hours, a set-up allowing further studies on the effects of various amino acid analogues on hepatoma cell survival was available.

It was apparent that DAB, a specific non-physiological amino acid, could kill tumour cells *in vitro* in a dose- and time-dependent fashion. That this was a specific effect of DAB was proven by the failure of inducing cell death induction by N-methylAIB or AIB, both being non-physiological amino acids as well, but differing from DAB in that their cellular uptake was subjected to the law of saturation kinetics. We also have reason to believe that the unique effect of DAB can be attributed to the fourth carbon atom with its amino group of the molecule rendering DAB unique amino acid substrate properties of transport system A (3). That system A is involved in the cell lysis was proven by the total abolition of the DAB effect by adding N-methylAIB to the culture medium, since it is a specific amino acid substrate for system A (4).

The DAB uptake by system A was against an unusually high concentration gradient leading to intracellular accumulation of DAB, to the extent that it became osmotically active, in turn causing an influx of water and

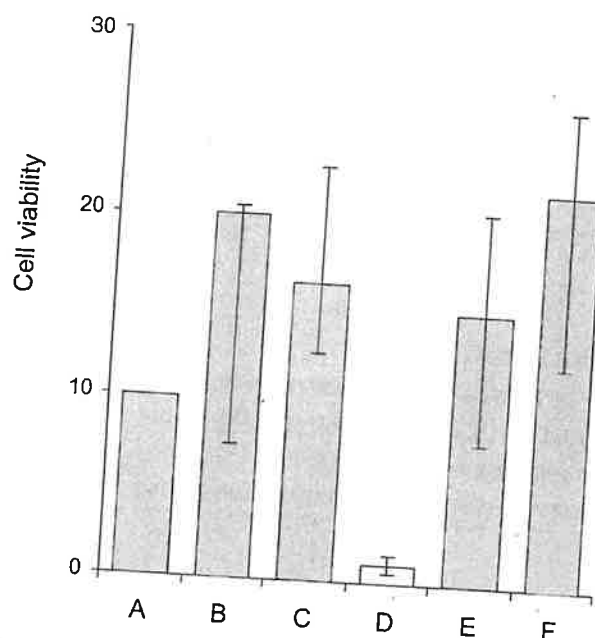


Figure 3. Effect of different amino acid analogues, alone or in combinations, on survival of a single hepatoma cell line. Incubation time: 19.5 hours
A. Control
B. N-methyl AIB, 8 mmol/L
C. α -Aminoisobutyric acid (AIB), 8 mmol/L
D. DAB, 8 mmol/L
E. DAB, 8 mmol/L + N-methylAIB, 8 mmol/L
F. DAB, 8 mmol/L + AIB, 8 mmol/L
D. vs. E, $p=0.02$, D vs. F, $p=0.02$, E vs. F, $p<0.05$.
N=6, except B (N=4) and C (N=3).

ultimately lysis of the hepatoma cells. Furthermore, system A amino acid transport is known to be sodium-dependent (4), implying that DAB uptake occurred at the expense of the sodium gradient. Restoration of the dissipated sodium gradient took place at the expense of ATP, possibly leading to

an energy crisis in the cell, due to the huge uptake of DAB. Consequently, we may conclude that cell death was the result of the combined effect of hyperosmosis and intracellular energy crisis.

The action of DAB on tumour cells, thus, may be modulated by the actual concentration, time of exposure and presence of other amino acids (both physiological and non-physiological), which may be competitors for the same transport system (System A). Our study demonstrates the unique principle of action of DAB; non-saturation kinetics of uptake, thereby ensuing intracellular hyperosmosis, energy crisis due to the huge Na^+ -dependent uptake and, ultimately, lysis of tumour cells. The unique properties of DAB could be favourable in the clinical setting e.g., in combination with other anticancer drugs with other principles of action.

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