Biochemical Pharmacology, Vol. 23, pp. 289–293. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF DIAZOACETYL-GLYCINE AMIDE ON PURINE NUCLEOTIDE METABOLISM IN EHRLICH ASCITES TUMOUR CELLS

T. GIRALDI and L. BALDINI
Istituto di Farmacologia, Università di Trieste, Trieste, Italy

(*Received* 16 *May* 1973; *accepted* 25 *June* 1973)

Abstract—On the basis of the known inhibitory activity of diazoacetyl-glycine amide (DGA) on DNA and purine nucleotide synthesis, a series of experiments was performed to study, in greater detail, the effects of the drug on purine nucleotide metabolism. DGA produces a marked inhibition of *de novo* utilization of glycine and formate and a less pronounced inhibition in the "salvage" utilization of hypoxanthine for IMP synthesis. The synthesis of adenine and guanine nucleotides from hypoxanthine is also depressed by DGA, and the drug also causes unbalance in the ratio of the concentration of adenine and guanine nucleotides and a drop in the energy status of nucleotides. These findings and those already reported are related to a rather unspecific interaction of the drug with cellular components.

A SERIES of diazoacetyl derivatives of glycine and glycyl–glycine were synthesized and tested for their antitumour properties by Baldini *et al.*[1] Of these, diazoacetyl-glycine amide (DGA) proved to be one of the most active against rodent tumours.

Subsequent investigations intended to detect the possible immunodepressive properties of DGA, showed that in some transplantation systems it has a remarkable activity, comparable to, or better than, that of azathioprine and methotrexate.[2]

The interesting pharmacological activities of DGA have stimulated investigations into its possible mode of action. The data obtained so far indicates that treatment of tumour-bearing animals with DGA causes a pronounced depression in the utilization of labelled thymidine and adenine for DNA synthesis in the tumour cells. There is a concomitant inhibition of purine nucleotide synthesis, indicated by a reduction in the amount of labelled formate incorporated into both DNA and RNA.[3] Further experiments have shown that there is no evident correlation between the inhibition of purine nucleotide and DNA synthesis and that the effects of the drug do not seem to be selective as far as the target (at a biochemical level) is concerned.[4]

In this communication the results of experiments carried out to study the detailed effects of DGA on purine nucleotide metabolism are reported.

MATERIALS AND METHODS

Tumour cells and treatment with DGA

Male Swiss albino mice, weighing about 25 g, purchased from Nossan, Milan,

Italy, were injected i.p. with 5×10^6 EAC* cells 5 days before use. When animals were treated with DGA the drug was administered i.p., dissolved in 0·2 ml of 0·1 N $NaHCO_3$ (pH 8·4) solution per 10 g of animal weight (1·5 g/kg). After 2 hr the animals were sacrificed by cervical dislocation, and the ascitic fluid was collected after laparotomy using a Pasteur pipette. Tumour cells were washed by centrifugation for 5 min, at 500 *g* and resuspended in DPBS. Having determined the packed cell volume with a microhematocrit, the cell suspension was diluted with DPBS to the stated concentration.

Incubation with labelled precursors

Aliquots of 2% (v/v) cell suspension in DPBS containing 5·5 mM glucose were pre-incubated at 37° for 20 min;[5,6] 1-[^{14}C]glycine (10 μCi, 0·4 mM final concentration), [^{14}C]formate (10 μCi, 0·35 mM) or 8-[^{14}C]hypoxanthine (2·5 μCi, 0·1 mM) were then added, and the incubation continued for 60 min. The metabolic utilization was stopped by chilling at 4°, and the cells were washed by centrifugation at 500 *g* and resuspension in DPBS.

Nucleotide extraction

Two techniques were employed independently of each other: hot ethanol,[7] and cold TCA extraction.[5] When ethanol extraction was performed, the packed cells were suspended in 1 ml of 70% ethanol–water solution (v/v), and maintained at 70° for 10 min. When TCA extraction was used, the packed cells were suspended in 1 ml of cold 5% (w/v) TCA, and maintained at 4° for 10 min. In both cases the clear supernatant was collected after centrifugation at 2000 *g*, and the nucleotides were separated by chromatography. As TCA seriously interferes with chromatographic procedures, it was first removed by five extractions with 3 ml of ether.

Nucleotide separation

Nucleotides were separated by thin-layer ion exchange chromatography on PEI-cellulose plates (E. Merck, Darmstadt, Germany), with minor modifications of the method of Randerath and Randerath.[8,9] Cell extract (90 μl) was added together with 10 μl of aqueous solution of carrier nucleotides (10 nmoles each), and spotted in 5 μl aliquots on the plate without intermediate drying. The plate was developed with 1 M NaCl up to 7 cm from the starting line and with 4 M sodium formate buffer pH 3·4, to the top. Spots were detected by a u.v. lamp, scraped off into vials containing 10 ml of a toluene scintillator, and their radioactivity determined by liquid scintillation counting. The purine nucleotides considered were: GTP, ATP, GDP, ADP, GMP + XMP, IMP, AMP and NAD.

Cellular nucleotide pool

The cellular nucleotide pools of the TCA extracts were determined. The ATP pool was determined according to the microtechnique of Randerath *et al.*[10] The ATP + ITP + GTP + UTP levels were determined by the phosphoglycerokinase-glyceraldehyde phosphate dehydrogenase system [ATP: D-3-phosphoglycerate 1-phosphotransferase (EC 2.7.2.3) and D-glyceraldehyde-3-phosphate: NAD oxidoreductase (EC 1.2.1.12)], using a Sigma kit (366-UV).[11]

*Abbreviations used: EAC, Ehrlich ascites carcinoma; TCA, Trichloro acetic acid; DPBS, Dulbecco phosphate buffered saline.[17]

Cell respiration

The respiration of tumour cells was determined by a polarographic technique, using a Clark electrode.[12]

RESULTS AND DISCUSSION

The data reported in Table 1 show that treatment of tumour-bearing mice with DGA causes an inhibition of the utilization of various labelled precursors for the

TABLE 1. EFFECTS OF DGA ON THE INCORPORATION OF VARIOUS PRECURSORS INTO IMP IN EAC CELLS

DGA	Labelled precursor	IMP radioactivity (counts/min × 10)	%
−	Glycine-1[^{14}C]	3602 ± 582	100
+		1104 ± 496*	30·6
−	Sodium formate[^{14}C]	1565 ± 83	100
+		947 ± 96*	60·5
−	Hypoxanthine-8[^{14}C]	5506 ± 1250	100
+		4918 ± 882	89·3

* Significantly different (Student's *t*-test, $P < 0{\cdot}005$). Each value is the mean (±S.E.) obtained from groups of five mice. Values obtained in two subsequent experiments did not differ significantly.

synthesis of IMP in the tumour cells. In particular, glycine and formate utilization for "de novo" synthesis of IMP and that of hypoxanthine for "salvage" IMP synthesis are affected, the effect increasing with the number of steps from precursor to end product. Later stages of purine nucleotide synthesis are also affected, as shown by the

TABLE 2. EFFECTS OF DGA ON PURINE NUCLEOTIDE METABOLISM IN EAC CELLS

	Treated (counts/min × 5)	Controls	T/C	Inhibition (%)
Hypoxanthine phosphoribosyl transferase	91,744	161,545	0·57	—
AMPS synthetase + lyase	76,413	127,619	0·60	−5·4
AMP kinase	73,573	124,347	0·59	1·2
ADP kinase	63,751	113,498	0·56	5·1
IMP dehydrogenase + GMP synthetase	15,331	33,926	0·45	20·5
GMP kinase	8594	14,329	0·60	−32·8
GDP kinase	2325	5131	0·45	24·4
Adenine nucleotide energy status	4·870	7·858		
Adenine/guanine nucleotides	4·98	3·76		

Each value is the mean obtained from groups of five mice. Values obtained in two subsequent experiments did not differ more than 20 per cent. The computation of apparent enzymatic activities and other parameters was done according to the method of Snyder *et al.*[5] The labelled precursor employed in these experiments is hypoxanthine-8[^{14}C].

experiments reported in Table 2, in which the incorporation of radioactivity from "salvage" synthesis of hypoxanthine was followed in adenine and guanine nucleotides. The treatment with DGA reduces the synthetic activity (mainly phosphorylative) of all the intermediates being considered to about half that of the controls. The apparent enhancement of the GMP synthetase activity should be regarded as an accumulation of GDP due to the inhibition of GDP kinase activity, since the figures of percent inhibition obtained by the method of Snyder *et al.*[5] reflect the pools of the nucleotides rather than the kinetic constants of the enzymatic activities. Furthermore, the treatment with the drug causes an unbalance in the ratio between the concentration of adenine and guanine nucleotides and a decrease in the energy status of adenine nucleotides (Table 2). The depression of the phosphorylative activity and of the energy status of adenine nucleotides is concomitant with a reduction in the pool of ATP, UTP, ITP, GTP (Table 3). These findings cannot however be ascribed solely to the

TABLE 3. EFFECTS OF DGA ON THE NUCLEOTIDE TRIPHOSPHATE POOL IN EAC CELLS

DGA	ATP + ITP + UTP + GTP pool*	ATP pool*	ATP in pool of ATP + ITP + UTP + GTP (%)
−	1289 ± 73 (100)	805 ± 27 (100)	62·4
+	975 ± 57 (75·6)†	629 ± 127 (78·1)†	64·5

* nmoles/10^8 cells.

† Significantly different (Students *t*-test, $P < 0{\cdot}05$). Each value is the mean (± S.E.) obtained from groups of five mice. Values obtained in two subsequent experiments did not differ significantly.

effect of DGA on the cellular pool of ATP, since the treatment with the drug reduces the ATP pool only to about 78 per cent (Table 3). This reduction should be attributed however to an inhibition of oxidative phosphorylation rather than to an uncoupling activity of DGA, as the drug reduces the respiration of the tumour cells to about 88 per cent (Table 4), while it shows no uncoupling activity on purified preparations of mitochondria (unpublished results).

TABLE 4. EFFECTS OF DGA ON THE RESPIRATION OF EAC CELLS

DGA	Cell respiration*	(%)
−	130·5 (± 4·67)	100
+	115·2 (± 37·9)†	88·3

* O_2 n atoms/min./10^7 cells.

† Significantly different (Students *t*-test, $P < 0{\cdot}05$). Each value is the mean (± S.E.) obtained from groups of ten mice. Values obtained in one subsequent experiment did not differ significantly.

These data and those already reported[3,4] seem to support the hypothesis that DGA acts in an unselective manner, inhibiting more than one enzymatic activity, unlike the action of azaserine, the best known diazoacetyl derivative of an amino acid.[13] The toxic and pharmacologically active doses of DGA are 1000 times greater than those of azaserine[14,15] and DGA requires time (at least hours when only a single dose is administered, and in the case of a therapeutic regimen involving repeated administrations it may require days) to exert its effects.[3] These facts cannot be explained in terms of a poor uptake of the drug by the cells, as *in vitro* incubation of both cells and cell sonicates with DGA had a similar effect on DNA synthesis (unpublished results). On the basis of the generally weak alkylative activity of azaserine at concentrations related to pharmacologically active doses (micromolar range) unless very particular conditions are satisfied,[16] it therefore seems conceivable to attribute the pharmacological activities of DGA to a rather unspecific reaction (alkylation?) with biological substrates which could occur because of the high concentration of the drug (millimolar range) in the ascitic fluid, and presumably within the cells.

Acknowledgements—The authors are delighted to thank Professor D. Romeo for encouragement and discussion, and Mr. G. Fonzari and Mrs. L. Ravalico for skilful technical assistance.

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