Biochemical Pharmacology, Vol. 22, pp. 1793–1799. Pergamon Press, 1973. Printed in Great Britain.

MECHANISM OF INHIBITION OF DNA SYNTHESIS IN EHRLICH ASCITES TUMOUR CELLS BY DIAZOACETYL GLYCINE-AMIDE

T. GIRALDI and L. BALDINI

Istituto di Farmacologia dell'Università di Trieste, Trieste, Italy

(*Received* 14 *December* 1972; *accepted* 19 *January* 1973)

Abstract—Treatment of Ehrlich ascites carcinoma bearing mice with DGA, the amide of diazoacetyl-glycine, leads to an inhibition of labelled thymidine incorporation into DNA of the tumour cells. This inhibition is not due to impairment of the nucleoside transport into the cell or to a modification in the activity of thymidine kinase. A possible explanation of the DGA effects resides in its partial inhibition of DNA polymerase and in its ability to alter the template activity of native DNA.

THE AMIDE of diazoacetyl-glycine (DGA)

$$N_2—CH_2—CO—NH—CH_2—CO—NH_2$$

is an interesting chemotherapeutic agent with a broad spectrum of biological activities, including remarkable immunodepressive[1,2] and antitumour effects. It displays in particular, a marked antitumour activity on the ascitic forms of Sarcoma 180 and Ehrlich ascites carcinoma* and on the Galliera rat sarcoma, and also a more limited tumour inhibiting activity on the solid forms of Sarcoma 180 and on Harding–Passey melanoma.[3]

The mechanism of action of this drug is under investigation in our laboratory. Preliminary data reported in a previous paper[4] have shown that DGA interferes with the incorporation of labelled formate in RNA and DNA, and of labelled thymidine and adenine in DNA.

The present communication reports some recent experiments, concerning the effects of DGA on the biochemical pathways which lead to DNA synthesis. The transport of labelled thymidine into the cell and the activity of the enzyme which catalyzes its phosphorylation to nucleotides have proved to be unaffected by the drug. On the contrary, DGA has shown the capacity of partially inhibiting DNA polymerase and of modifying the template properties of DNA in an *in vitro* polymerase system.

MATERIALS AND METHODS

Biochemicals, isotopes and drugs. ^{3}H-Methyl-thymidine and ^{3}H-methyl-thymidine triphosphate were obtained from the Radiochemical Center, Amersham, England. Deoxyribonucleotide triphosphates, adenine, calf thymus DNA were obtained from Sigma Chemical Co., St. Louis, U.S.A. DGA, synthesized in our laboratory, was

* Abbreviations used: DPBS, Dulbecco phosphate buffered saline;[20] TCA, trichloroacetic acid; EAC, Ehrlich ascites carcinoma.

dissolved in 0·1 N $NaHCO_3$, pH 8·4, and administered i.p. in volumes of 0·2 ml/10 g of body wt.

Tumour cells. Male Swiss albino mice weighing 20–25 g (Nossan, Milan, Italy), were inoculated i.p. with 5×10^6 EAC cells. Five days later, they were killed by cervical dislocation, and the ascitic fluid collected by Pasteur pipette after laparatomy. When indicated, DGA was injected i.p. a few hours before the sacrifice (see Results). Tumour cells were washed by two subsequent centrifugations at 500 *g* for 5 min and resuspended in chilled DPBS to twice the original volume. After counting in a haemocytometer chamber, the cell suspension was diluted with DPBS to the stated concentration. Ascitic fluids containing blood cells were discarded.

Thymidine incorporation in DNA of EAC cells. Two techniques were independently employed. When the specific radioactivity of DNA was determined, aliquots of 2 ml of cell suspension (5×10^7 cells/ml) were incubated at 37° for 30 min in a shaking water bath, after addition of 1 μCi of thymidine (0·1 ml aqueous solution, 5 Ci/mmole). The extraction of DNA and the determination of its specific radioactivity was carried out, with minor modifications, by the method of Schmidt and Tannhauser,[5] as described in a previous paper.[4]

When cold TCA-insoluble radioactivity was determined, 2 ml of cell suspension (1×10^6 cells/ml) were incubated as described above. Samples of 0·1 ml of the incubation mixture were pipetted onto Whatman 3 MM chromatographic paper disks (20 mm dia.), which were sequentially dipped in 10% TCA (w/v) at 4° for 30 min, in 5% TCA (w/v) at 4° for 5 min, in ethanol–ether 1:1(v/v) for 15 min, in ether for 5 min and finally allowed to dry. The disks were then placed on the bottom of scintillation vials containing 5 ml of a toluene scintillator and their radioactivity determined by liquid scintillation.

Uptake of thymidine by EAC cells. A procedure similar to that described by Peters *et al.*[6] was followed; 1×10^7 cells in 2 ml of DPBS were incubated at 37° for 30 min with 1 μCi of thymidine (0·1 ml aqueous solution, 5 Ci/mmole): the incubation was stopped by cooling at 0°. When the total cell radioactivity was determined, aliquots of 0·2 ml of the incubation mixture were filtered through a Millipore membrane (0·45 μm pore size). The filter was washed with 4 ml of ice-cold DPBS, dried and placed in toluene scintillator for the measurement of radioactivity. When the acid-soluble radioactivity had to be determined, 1 ml of cell suspension was added to 1 ml of ice-cold 10% TCA (w/v). After 30 min at 0°, the sample was centrifuged at 2000 *g* for 5 min, and the radioactivity of the supernatant measured, after neutralization with NaOH, in Bray's[7] scintillation fluid.

Thymidine kinase activity. Packed EAC cells were suspended in 4 vol. of 0·1 M phosphate buffer (pH 8·1), and disrupted with a MSE ultrasonic disintegrator at maximum power setting with a 30 sec burst. The test tubes containing the cells were immersed in an ice-bath during sonication. The sonicates were centrifuged at 105,000 *g* for 90 min, and the supernatant fraction carefully collected.

Thymidine kinase activity (ATP: thymidine 5′-phosphotransferase; EC 2.7.1.21) was determined according to Grav and Smellie.[8] The assay mixture (1 ml) contained: ^{3}H-methyl-thymidine (5 μCi, 100 nmoles), Tris–HCl buffer (pH 8) 100 μmoles, $MgCl_2$ 5 μmoles, ATP 5 μmoles, 2-mercaptoethanol 0·1 μmole, and 1 mg protein of the 105,000 *g* supernatant fraction. The mixture was incubated at 37° for 30 min: throughout this period the enzymatic reaction was linear. The reaction was stopped by heating

at 100° for 2 min. After removal of coagulated protein by centrifugation, unreacted thymidine was separated from thymine nucleotides by chromatography on 1 × 9 cm columns of Ecteola cellulose (exchange capacity approximately 0·5 m-eq./g). 0·5 ml Aliquots of the deproteinized reaction mixture were adsorbed on the column: thymidine was eluted with water (50 ml) and the thymine nucleotides with 0·5 N HCl (50 ml).[10] The concentration of thymidine and thymine nucleotides in these fractions was determined by measurement of the radioactivity of 0·5 ml samples of the eluates neutralized with NaOH, in Bray's sintillation fluid.[7]

DNA polymerase system. DNA polymerase (DNA nucleotidyltransferase: EC 2.7.7.7) was prepared from EAC cells by hypotonic shock according to Roychoud hury *et al.*:[10] the purification was limited to Fraction 1.

The enzymatic reaction was carried out as described by Shepherd *et al.*[11] 0·25 ml of the standard assay mixture contained: Tris–HCl buffer (pH 7·5) 5 μmoles, KCl 15 μmoles, EDTA 0·1 μmole, $MgCl_2$ 1 μmole, 2-mercaptoethanol 1·25 μmoles, 50 μg of native calf thymus DNA, dATP, dGTP, dCTP, dTTP, 100 nmoles each, labelled dTTP 5 μCi (29·3 Ci/mmole) and 100 μg protein of the enzymatic fraction. At the end of the reaction, the test tubes were chilled at 0°, aliquots of 0·1 ml of their content were pipetted onto Whatman 3 MM chromatographic paper disks, which were then treated as already described in a previous section. Suitable blanks were run.

Protein. Protein content was determined by the biuret method,[12] using cristalline bovine serum albumin for the standard.

RESULTS AND DISCUSSION

Data shown in Fig. 1 indicate that in cells derived from animals treated with DGA there is a substantial decrease in the incorporation of labelled thymidine in DNA. This inhibitory effect of DGA might depend on a number of biochemical modifications: decreased transport of the pyrimidine nucleoside through the cell membrane,

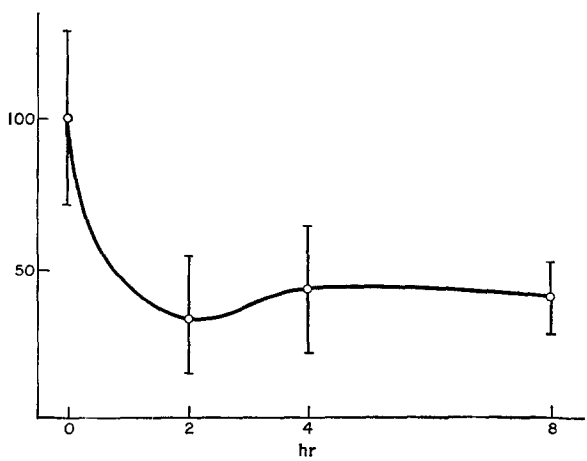

FIG. 1. Effects of DGA on thymidine incorporation in DNA of EAC cells. Tumour bearing mice were treated with 1·5 g/kg of DGA i.p., at the indicated time, they were sacrificed, and the tumour cells were incubated with the labelled precursor for 30 min. Each value represents the average of the specific radioactivity of DNA expressed as percentage of controls, with confidence limits at P = 0·05. Four groups of five mice were used.

TABLE 1. EFFECTS OF DGA ON THYMIDINE UPTAKE BY EAC CELLS

DGA	Total cellular radioactivity (counts/min)	Acid soluble radioactivity (counts/min)
−	7091 ± 641 (100%)	1658 ± 293 (100%)
+	6524 ± 734 (92%)	2194 ± 220 (132%)

Tumour bearing mice were treated i.p. with 1·5 g/kg of DGA. Two hr later they were sacrificed, and the tumour cells incubated *in vitro* with labelled thymidine for 30 min. The above values are the mean ± confidence limits at P = 0·05 (values in parentheses are the per cent of controls): four groups of six mice were used.

impaired phosphorylation of thymidine to the corresponding nucleotides, unbalance of the cell nucleotide pool, modified template activity of DNA or decreased DNA polymerase activity.

TABLE 2. EFFECTS OF DGA ON THYMIDINE KINASE ACTIVITY IN EAC CELLS

	Adenine mg/kg DGA	0 −	0 +	30 +	60 +
Thymidine kinase activity*	3 hr after DGA 1·5 g/kg	16·6 (100%)	13·5 (81%)	14·0 (84%)	19·2 (116%)
	13 hr after DGA 1·5 g/kg	19·2 (100%)	14·3 (74%)	22·0 (114%)	19·8 (103%)
	25 hr after DGA 2 × 0·5 g/kg	15·9 (100%)	14·2 (89%)	15·1 (95%)	18·1 (114%

* Nanomoles of phosphorylated thymidine/milligram of protein/hour.
DGA was administered i.p. in a single dose of 1·5 g/kg or in two subsequent daily doses of 0·5 g/kg. Treatment with adenine was performed 1 hr before sacrifice. Each value represents the mean of three determinations, carried out on the ascites pooled from three mice. (Values in parentheses are per cent of controls.)

The effects of DGA on some of these processes or enzyme activities have been investigated, and the results are hereby reported.

Data shown in Table 1 allow to exclude an inhibitory effect of DGA on thymidine

TABLE 3. EFFECTS OF DGA ON THYMIDINE KINASE ACTIVITY OF EAC CELL SONICATES *in vitro*

	DGA	0	10^{-4} M	10^{-3} M	10^{-2} M
Thymidine kinase activity*		15·1 (100%)	14·9 (98%)	14·4 (95%)	15·2 (100%

* Nanomoles of phosphorylated thymidine/milligram of protein/hour.
Each value represents the mean of two determinations carried out on the ascites pooled from three mice. (Values in parentheses are per cent of controls.)

uptake by EAC cells. The higher concentration of thymidine in TCA soluble material of cells coming from treated animals is consistent with the lower thymidine incorporation in DNA of these cells.

An inhibition of thymidine kinase by DGA can also be ruled out, since the activity of this enzyme is not modified by the drug either when using whole cells (Table 2) or a crude enzymatic preparation (Table 3).

A slight effect on the template activity of DNA due to DGA is observed when the nucleic acid is incubated with DGA for 4 hr (Fig. 2). This treatment, unlike that with alkylating agents such as nitrogen mustards,[13] does not reduce the template activity of heat inactivated DNA (unpublished results), but, on the contrary, enhances that of native DNA. This enhancement suggests that the interaction of DGA with DNA leads to a labilization of hydrogen bonds between the two strands of the polymer. The

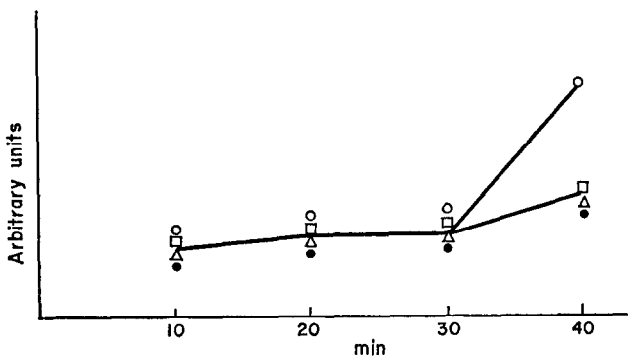

FIG. 2. Effects of DGA on the template activity of native calf thymus DNA in a polymerase system *in vitro*. 0·5 mg of native calf thymus DNA were incubated for 1 hr (□), 2 hr (△) or for 4 hr (○) in 1 ml of 10 mM Tris–Cl buffer (pH 7·5) containing 10 μmoles of DGA (10 mM), and used as the template in the polymerase system described in the experimental section (controls ●). The results are represented in arbitrary units as labelled dTTP incorporated in cold TCA insoluble material. Experiments were carried out in triplicate.

alteration of the DNA structure, however, is unlikely to be very large, since the u.v. melting curve and the c.d. spectrum of DGA treated-DNA are virtually the same as that of untreated DNA (unpublished results).

A decreased incorporation of thymidine in DNA might also be caused by an altered activity of DNA polymerase. As shown in Fig. 3, the treatment of a partially purified DNA polymerase with DGA, results in a decreased activity of the enzyme. Because of the high K_m of the incubated polymerase for the nucleoside triphosphates, it is difficult to assess whether this change in activity is produced by a decreased affinity of the enzyme for the substrates or simply by a decrease of its V_{max}.

In conclusion, from the data so far obtained, it appears that DGA is able to alter, at least partially, the DNA template properties and the activity of DNA polymerase.

Under acidic conditions aliphatic diazocompounds easily originate very reactive carbocations.[14] It is therefore conceivable that in intact tumour cells, whose cytoplasm has a lower pH than that of the corresponding normal cells,[15] the effects produced might be greater than those exerted *in vitro* on purified cell components.

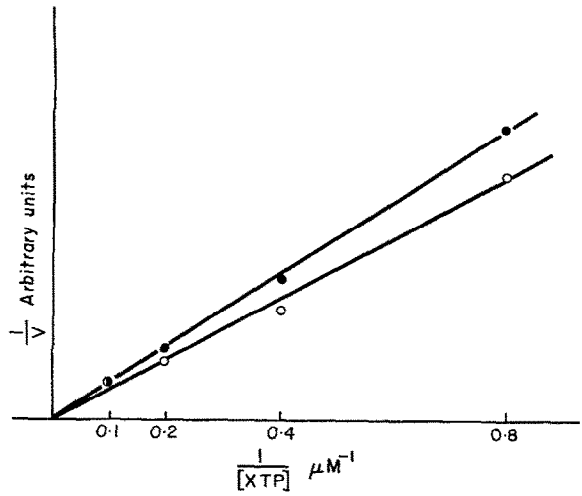

FIG. 3. Effects of DGA on DNA polymerase. Lineweaver–Burk plot[21] of a DNA polymerase reaction: V is expressed as arbitrary units of labelled dTTP incorporated in cold TCA insoluble material from the enzymatic reaction mixture. Details are described in the experimental section: enzymatic fractions were incubated for 2 hr at 37° with 10 mM DGA in 10 mM Tris–HCl buffer (pH 7·5). Experiments were carried out in triplicate. (○)−DGA; (●)+DGA.

Finally, it is interesting to compare the effects of DGA on the metabolism of tumour cells with those of azaserine, a drug which has many similarities with DGA in structure and in biological activity.[16] Azaserine inhibits thymidine incorporation into DNA, but not that of labelled adenine. Moreover, if one supplies the cells with an excess of adenine, the inhibition of thymidine incorporation caused by azaserine is removed.[17] These effects have been explained as being due to a pronounced depression in the activity of thymidine kinase and to a drop of adenine nucleotides in the cellular pool: treatment with preformed adenine brings back both biochemical parameters to normality.[18] The effects obtained with adenine are also consistent with the fact that azaserine exerts at an early stage a very selective inhibition of purine biosynthesis.[19] From this point of view, DGA behaves quite differently from azaserine. Firstly, it also inhibits the incorporation of labelled adenine in DNA,[4] and secondly, as shown in Table 4, adenine does not modify the inhibition of thymidine incorporation caused

TABLE 4. EFFECTS OF ADENINE ON THE DGA-INDUCED INHIBITION OF LABELLED THYMIDINE INCORPORATION

Adenine	0	10^{-4} M	3×10^{-3} M	10^{-2} M
DGA −	4399 ± 753 (100%)	4317 ± 1081 (98%)	4413 ± 754 (100%)	2799 ± 514 (64%)
DGA +	351 ± 45 (7·9)	269 ± 54 (6·1)	286 ± 37 (6·5)	311 ± 70 (7·1)

Tumour bearing mice were treated i.p. with 1·5 g/kg of DGA. Two hr later they were sacrificed and the tumour cells incubated *in vitro* with adenine: 45 min later, 1 μCi of thymidine was added and the incubation stopped after 30 min. The above values represents the mean ±S.E. of cold TCA insoluble radioactivity (values in parentheses are per cent of controls): two groups of five mice were employed.

by DGA. Thus, these facts and the other data reported here would suggest that DGA causes an inhibition of DNA synthesis by acting on the final steps of that process. Its activity, however, does not seem to be very selective as far as the biochemical target is concerned. In fact, we have previously shown[4] that this drug is also able to inhibit the incorporation of labelled formate into DNA and RNA. Specific pathways leading to the synthesis of nucleic acid precursors are, therefore also affected by DGA. The precise nature and localization of the step at which the inhibition is excerted, are at present under investigation.

Acknowledgements—The authors would like to thank Prof. D. Romeo for valuable discussion during the preparation of the manuscript, also G. Fonzari for excellent technical assistance.

REFERENCES

1. G. Brambilla, S. Parodi, M. Cavanna and L. Baldini, *Transplantation* **10,** 100 (1970).
2. L. Baldini, G. Brambilla, M. Cavanna, C. E. Carraceni and S. Parodi, *Transplantation* **13,** 224 (1972).
3. L. Baldini and G. Brambilla, *Cancer Res.* **26,** 1754 (1966).
4. T. Giraldi, G. Steppani and L. Baldini, *Biochem. Pharmac.* **21,** 3035 (1972).
5. G. Schmidt and S. J. Tannhauser, *J. biol. Chem.* **161,** 83 (1945).
6. J. H. Peters and P. Hausen, *Eur. J. Biochem.* **19,** 502 (1971).
7. G. A. Bray, *Analyt. Biochem.* **1,** 279 (1970).
8. H. J. Grav and R. M. S. Smellie, *Biochem. J.* **94,** 518 (1965).
9. H. J. Grav and R. M. S. Smellie, *Biochem. J.* **89,** 486 (1963).
10. R. Roychoudhury and D. P. Bloch, *J. biol. Chem.* **244,** 3359 (1969).
11. J. B. Shepherd and H. M. Keir, *Biochem. J.* **99,** 443 (1966).
12. A. G. Cornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177,** 751 (1949).
13. R. W. Ruddon and J. M. Johnson, *Molec. Pharmac.* **4,** 258 (1968).
14. C. C. Price, G. M. Gaucher, P. Koneru, R. Shibakawa, J. R. Sowa and M. Yamaguchi, *Ann. N.Y. Acad. Sci.* **163,** 593 (1969).
15. H. Busch, *An Introduction to the Biochemistry of the Cancer Cell,* p. 321. Academic Press, New York (1911).
16. J. A. Stock, in *Experimental Chemotherapy—IV* (Eds R. J. Schnitzer and F. Hawking) p. 277. Academic Press, New York (1966).
17. A. C. Sartorelli and B. Booth, *Molec. Pharmac.* **3,** 71 (1967).
18. G. T. Cramer and A. C. Sartorelli, *Biochem. Pharmac.* **18,** 1355 (1969).
19. L. L. Bennett Jr., F. M. Schabel Jr. and H. E. Skipper, *Archs Biochem. Biophys.* **64,** 423 (1956).
20. R. Dulbecco and M. Vogt, *J. exp. Med.* **99,** 167 (1954).
21. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).