Biochemical Pharmacology, Vol. 21, pp. 3035–3039. Pergamon Press, 1972. Printed in Great Britain.

EFFECTS OF DIAZOACETYL-GLYCINE AMIDE ON PURINE NUCLEOTIDE AND DEOXYRIBONUCLEIC ACID SYNTHESIS IN EHRLICH ASCITES TUMOR CELLS

T. GIRALDI, G. STEPPANI and L. BALDINI

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

(*Received* 1 *February* 1972; *accepted* 23 *June* 1972)

Abstract—Ascites tumour cells derived from diazoacetyl-glycine amide-treated animals show a reduced incorporation of labelled thymidine, whereas the incorporation of labelled uridine and leucine is unchanged with respect to control cells. The *in vivo* incorporation of both thymidine-H^3 and adenine 8-C^{14} into DNA of tumour cells, is also inhibited by a previous injection of diazoacetyl-glycine amide into the animals. The utilization of adenine 8-C^{14} for RNA synthesis is not affected under these conditions, whereas the amount of formate-C^{14} recovered in both DNA and RNA fractions is significantly reduced. These results suggest that the antitumor drug diazoacetyl-glycine amide is capable of inhibiting the purine nucleotide and DNA biosynthesis.

THE AMIDE of diazoacetyl-glycine, $N_2{=}CH{-}CO{-}NH{-}CH_2{-}CO{-}NH_2$, has been recently shown to interfere with a number of biological processes, such as immune response[1] and tumour growth.[2] In particular, it has been shown to exert a marked antitumour activity on the ascitic forms of Sarcoma 180 and Ehrlich carcinoma and on the Galliera rat sarcoma, and a less pronounced but still evident inhibiting activity on the solid forms of Sarcoma 180 and Ehrlich carcinoma, and on the Harding–Passey melanoma.[2]

In order to gain some information on the possible mechanisms governing the antitumour activity of DGA, we have investigated its effects on DNA, RNA and protein synthesis in Ehrlich ascites carcinoma cells. The results so far obtained suggest that DGA has an inhibitory activity on the biochemical processes leading to DNA formation.

Incorporation of thymidine methyl-H^3, uridine-H^3 and DL-*leucine-H^3 into Ehrlich ascites carcinoma cells* in vitro

Male Swiss albino mice weighing 20–25 g were inoculated i.p. with 5×10^6 cells of EAC. From the fifth day on, the animals were injected i.p. with 0·1 ml of DGA in 0·1 N $NaHCO_3$ (pH 8·4) each day for 4 days (400 mg/kg/day). The control animals received only the $NaHCO_3$ solution. Twenty-four hr after the last administration, the animals were killed by cervical fracture, and the ascitic fluid was collected and centrifuged at 500 *g* for 5 min. The supernatant was discarded, and the packed cells were suspended in BSS to the original volume. After counting in a hemocytometer, the cells were diluted with BSS to a concentration of 1×10^6 cells/ml. One μCi of the appropriately labelled precursor (0·1 ml aqueous solution of thymidine methyl-H^3,

Abbreviations used: DGA, diazoacetyl-glycine amide; TCA, trichloroacetic acid; EAC, Ehrlich ascites carcinoma; PCA, perchloric acid; BSS, Hanks balanced salt solution; i.p.: intraperitoneally; PPO, 2,5-diphenyloxazole; dimethyl POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

5 Ci/mM, or of uridine-H^3, 5·4 Ci/mM, or of D,L-leucine-H^3, 19·4 Ci/mM: purchased from the Radiochemical Centre, Amersham, England), was added to 2 ml of the cell suspensions, which were then incubated in a water-bath at 37° with stirring for 30 min. The incubation was stopped by freezing at −20°.

The technique employed for the measurement of the incorporation of radioactivity was a modification of the paper disc method.[3] The frozen cell suspensions were thawed by immersion in a water bath at 37° for 5 min. Aliquots of 0·1 ml were pipetted onto discs of Whatman 3 MM chromatographic paper (20 mm dia.), which were allowed to dry for 10 min at room temperature. The discs were then 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 allowed to dry. They were finally put in scintillation vials containing 5 ml of a scintillator fluid made of 0·4% PPO and 0·01% dimethyl POPOP in toluene, and counted in a Packard Tri-Carb Spectrometer.

MATERIALS AND METHODS

Incorporation of thymidine methyl-H^3, adenine-8-C^{14} and formate-C^{14} into nucleic acids of Ehrlich ascites carcinoma cells in vivo

Male Swiss albino mice weighing 20–25 g were inoculated i.p. 5 days before each experiment with 5×10^6 cells of EAC and on the day of the experiment they were treated i.p. with DGA (1·5 g/kg). The single dose of 1·5 g of DGA/kg, used in these experiments, is approximately equitoxic to a dose of 400 mg/kg/day for 4 days, administered to tumour bearing mice employed for the *in vitro* experiments. After 30 min–2 hr, they were injected i.p. with 10 μCi of the labelled precursor dissolved in 0·1 ml of isotonic NaCl solution (thymidine methyl-H^3, 5 Ci/mM, or adenine-8-C^{14}, 60 Ci/M or sodium formate, 56 Ci/M: purchased from the Radiochemical Centre, Amersham, England). Two or six hr later, the animals were killed by cervical fracture, and the ascitic fluid collected through an incision of the abdominal wall.

The separation of RNA and DNA was carried out with minor modifications of the method of Schmidt and Thannhauser.[4–5] About 10^8 cells were transferred into a centrifuge tube and centrifuged at 500 *g* for 5 min. The supernatant fluid was discarded and the cells were resuspended in 3 ml of isotonic NaCl solution. This suspension was centrifuged again at 500 *g* for 5 min and the supernatant discarded. Three ml of 0·5 N PCA were then added to the packed cells, and the precipitate was washed with 3 ml of water, followed by absolute ethanol, ethanol–ether 3 : 1 (v/v) and ether. All these operations were carried out at 4°. After desiccation, the dry precipitate was suspended in 0·5 N KOH, and incubated at 37° for 18 hr. The samples were then acidified with 6 N PCA (final pH of 3) at 4° and centrifuge after 30 min. The supernatant contained hydrolysed RNA. The DNA present in the precipitate was hydrolysed in 0·5 N PCA at 70° for 20 min. The specific radioactivity of RNA and DNA hydrolysates was assayed in the following manner. The nucleotide concentration was determined spectrophotometrically, comparing the extinction of the hydrolysates at 260 and 270 nm, respectively, with that of standards of yeast RNA and salmon sperm DNA (Sigma Chemical Co.) hydrolysed under the same experimental conditions. After neutralization of the samples with NaOH, their radioactivity was measured in a Bray's scintillation fluid,[6] with a Packard Tri-Carb scintillation spectrometer.

The cross-contamination between RNA, DNA and protein in the isolated fractions was checked by administering 10 μCi of thymidine methyl-H^3 or uridine-H^3 to 2 lots

of tumour bearing mice. The specific radioactivity of the RNA fraction derived from EAC cells of thymidine-H^3 treated mice, was 0·85 per cent of that measured in the corresponding fraction derived from uridine-H^3 treated animals. The specific radioactivity of the DNA fraction isolated from uridine-H^3 treated mice was 11·7 per cent of that extracted from thymidine-H^3 treated animals. This apparent higher contamination might be ascribed to a metabolic conversion of UMP to CMP, as reported for HeLa cells.[7] The radioactivity of the remaining protein fraction was 0·3 per cent of that resulting from the sum of the total radioactivities of the DNA and RNA fractions.

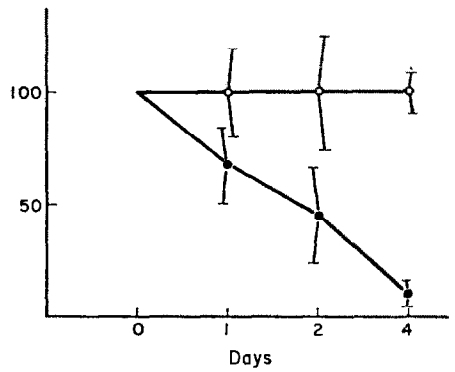

FIG. (a).

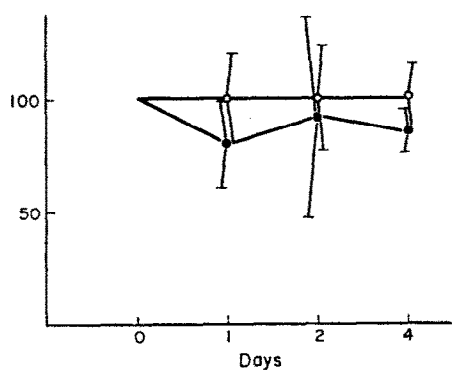

FIG. (b).

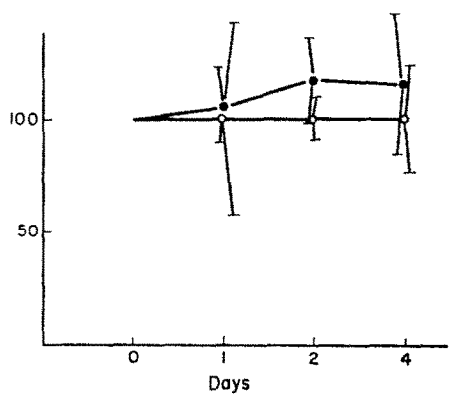

FIG. (c).

FIG. 1. *In vivo* treatment of EAC cells with DGA. Figure 1 represents cold TCA insoluble radioactivity from cells obtained from animals untreated (○——○) and treated (●——●) with DGA (400 mg/Kg/day injected i.p. for the indicated number of days). The cells were incubated *in vitro* for 30 min with 1 μCi of H^3-labelled thymidine (Fig. 1a), uridine (Fig. 1b) or L-leucine (Fig. 1c), respectively. Each point represents the mean derived from five animals (with confidence limits at $P = 0{\cdot}05$), expressed as percentage of incorporation in cells obtained from untreated animals (100 per cent).

RESULTS AND DISCUSSION

Figure 1 shows the *in vitro* incorporation of thymidine-H^3, uridine-H^3 and leucine-H^3 into DNA, RNA and protein of EAC cells derived from mice treated with DGA, in comparison with that of cells isolated from control animals. Parallel to the increase of the length of the treatment with DGA, there is a remarkable fall in the utilization of

thymidine for DNA synthesis. On the contrary, the incorporation of uridine and leucine into RNA and protein respectively, is not affected by the drug.

Further evidence suggesting a possible inhibitory activity of DGA on DNA biosynthesis is provided by the results of experiments in which the synthesis of DNA and RNA of EAC cells was followed at short times after the injection of the drug into animals (Table 1). After a 2 hr contact between DGA and cells the incorporation of thymidine-H^3 into EAC cell DNA is reduced to 34·2 per cent with respect to control cells. Also, the incorporation of adenine 8-C^{14} into DNA is significantly reduced after 6 hr, whereas the incorporation of this labelled precursor into RNA of EAC cells is virtually unaffected by the treatment of animals with DGA.

TABLE 1. SPECIFIC RADIOACTIVITY OF NUCLEIC ACIDS EXTRACTED FROM EAC CELLS DERIVED FROM ANIMALS TREATED WITH DGA AND LABELLED NUCLEIC ACID PRECURSORS

DGA	Labelled precursor	DNA specific radioactivity (dis./min/100 μg)	(%)	RNA specific radioactivity (dis./min/100 μg)	(%)
–	Thymidine methyl-H^3	10,360 (13,420–7300)	100		
+		3540 (5600–1480)	34*		
–	Adenine-8-C^{14}	14,264 (18,814–9714)	100	21,417 (29,337–13,457)	100
+		6801 (9100–4501)	48*	19,600 (27,246–11,954)	91†
–	Sodium formate-C^{14}	5288 (6658–3918)	100	11,167 (14,606–7728)	100
+		1730 (2176–1284)	33*	4982 (6008–3956)	45*

Each value is the mean obtained from eight animals (confidence limits at $P = 0{\cdot}05$ in parentheses). Animals were treated i.p. with 1·5 g/kg of DGA; 30 min later, they were injected i.p. with 10 μCi of labelled adenine or sodium formate, and after 6 hr killed by cervical fracture. Thymidine treated animals, received thymidine 2 hr after DGA, and were killed 30 min later.

* Mean significantly different (Student's *t*-test, $P < 0{\cdot}005$).

† Mean not significantly different (Student's *t*-test, $P < 0{\cdot}05$).

Data reported in Table 1 also show that DGA causes an inhibition of purine nucleotide metabolism, as indicated by the reduced amount of formate C^{14} recovered in both DNA and RNA of EAC cells derived from drug treated animals.

The experiments reported in Table 1 show that DGA does not exert its effect on DNA synthesis by merely arresting the progression of cells from the G_1 to the S phase. In fact the ascites cells employed in our experiments were in the logarithmic phase of growth up to the sixth day, with a doubling time of about 10–12 hr. Therefore, in 2 hr, only 20–25 per cent of cells enter the S phase. At this time, however, there is already a 66 per cent inhibition of thymidine-H^3 incorporation into DNA (Table 1).

In conclusion, by studying the *in vivo* effects of DGA on nucleic acids and proteins, we have observed that this anti-tumour drug is able to interfere with both the biosynthesis of purine nucleotides and the utilization of thymidine and adenine for DNA biosynthesis.

REFERENCES

1. G. BRAMBILLA, S. PARODI, M. CAVANNA and L. BALDINI, *Transplantation*, **10**, 100 (1970).
2. L. BALDINI and G. BRAMBILLA, *Cancer Res.* **26**, 1754 (1966).
3. R. J. MANS, G. D. NOVELLI, *Arch. Biochem. Biophys.* **94**, 48 (1961).

4. G. SCHMIDT and S. J. THANNHAUSER, *J. biol. Chem.* **161,** 83 (1945).
5. W. C. HUTCHINSON and H. N. MUNRO, *Analyst.* **86,** 768 (1961).
6. G. A. BRAY, *Analyt. Biochem.* **1,** 279 (1960).
7. K. HABEL and N. P. SALZMAN, *Fundamental Techniques in Virology.* Academic Press, New York (1969).