Europ. J. Cancer Vol. 16, pp. 87–92
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0014-2964/80/0101-0087 $02.00/0

Mechanism of the Antimetastatic Action of *N*-Diazoacetylglycinamide in Mice Bearing Lewis Lung Carcinoma*

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Abstract—N-*diazoacetylglycinamide* (*DGA*) *sharply reduced the formation of spontaneous pulmonary metastases in mice bearing subcutaneous Lewis lung carcinoma. At the same time, it caused no significant and parallel inhibition of primary tumor growth. The inhibition of metastasis formation, thus, appears to be due to selective antimetastatic effects, different from cytotoxicity for tumor cells. This is supported by the low effectiveness of the late treatment of mice having spontaneous metastases, and also by the absence of significant effects on the formation of artificial metastases obtained by i.v. injection of tumor cells. The lack of any cytotoxic effect for tumor cells localized in the lungs and subcutaneously was also shown by examining the effects of the treatment with DGA on the fractional incorporation of* ^{3}H-*TdR in tumor cells. A proposed mechanism for the antimetastatic effects of DGA is the inhibition of tumor cell detachment from the primary implant and their access to the blood stream. This mechanism is also consistent with the fact that DGA caused the greatest reduction of spontaneous metastasis formation when administered at the same time as the peak of dissemination of tumor cells in the blood stream.*

INTRODUCTION

N-DIAZOACETYLGLYCINE derivatives have been shown to reduce the growth of some rodent transplantable tumors. Different degrees of activity were observed, depending on the structure of the compounds examined and the experimental system used [1]. Recently, *N*-diazoacetylglycinamide (DGA) has been found capable of markedly inhibiting the formation of lung metastases in mice bearing Lewis lung carcinoma. Two analog compounds, *N*-diazoacetylglycine hydrazide and the ethyl ester, were shown to be practically inactive against metastases [2, 3]. The depression of pulmonary metastasis formation caused by DGA was attributed to selective antimetastatic effects [2, 3], since all of the three substances caused no significant inhibition of subcutaneous tumor growth: similar to a selective antimetastatic agent, ICRF 159 [4], and unlike a purely cytotoxic agent, cyclophosphamide [5].

The purpose of this investigation was to examine the mechanism of the antimetastatic effects of DGA in the same experimental system. This was performed by examining the treatment schedule dependency of the effects on subcutaneous tumor growth and lung colony formation. Furthermore, the possible occurrence of cytotoxic effects on small lung tumors, and also on the subcutaneous tumor, has been examined by determining the effects of DGA on the fractional incorporation of ^{3}H-TdR in tumor cells in both locations. Additionally, the effects of DGA on artificial metastases, obtained by injecting i.v. a tumor cell suspension, were examined. The results obtained are hereafter reported.

Accepted 28 May 1979.

*Supported by Consiglio Nazionale delle Ricerche Grants CT 77.01110.03, CT 77.01370.04 and by a Grant from the University of Trieste.

Abbreviations:
DGA = *N*-diazoacetylglycinamide;
ICRF 159 = (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane (Razoxane);
^{3}H-TdR = thymidine-6-H^3.

MATERIALS AND METHODS

Synthesis

The synthesis of DGA was performed following previously reported procedures [6].

Animal treatment

The compound was administered i.p. as freshly prepared aqueous solution in 0.1 N $NaHCO_3$ in volumes of 0.1 ml/10 g of body weight. Control animals only received the solvent.

Tumor transplantation and evaluation

For studying the effects of treatment on the formation of spontaneous metastases, Lewis lung carcinoma was transplanted in C57BL C.R. mice by aseptically implanting s.c. tumor fragments in the axillary region [7]. Primary tumor weight was determined by caliper measurements, assuming tumor density to be equal to 1, as the volume of the rotation ellipsoid having the long and short axes equal to a and b respectively:

$$(1) \qquad \text{tumor weight} = \pi/6 \times a^2 \times b$$

The number of lung metastases was determined at sacrifice using a dissection magnifying lens. The mass of metastases was estimated as the sum of their individual weights, determined using equation (1). Artificial metastases were obtained by injecting i.v. a single cell suspension (2.5×10^5 viable cells per mouse) prepared enzymatically as described by Hill and Stanley [8].

Measurement of fractional incorporation

C57BL C.R. mice received a s.c. and i.v. implant as described above. Thirteen days later, the fractional incorporation of ^{3}H-TdR into DNA of pulmonary and subcutaneous tumors (^{3}H in DNA at 1 hr/total ^{3}H in the tissue) was estimated by the methods described by Houghton and Taylor [9].

RESULTS

Effects of DGA on spontaneous metastasis formation

The effects of the treatment with DGA for 8 consecutive days, starting on day 1 after tumor transplantation, are reported in Table 1. The highest dose used was the maximum tolerated dose for this treatment schedule. The total number and the number of small nodules was significantly reduced at the two highest dosages employed. At the same dosages, no large metastases were found in any of the treated animals, and the number of large metastases was also significantly reduced at the next lowest dosages. The estimated weight of metastases was markedly and significantly reduced at these three highest dosages. The treatment with DGA also caused the absence of macroscopically detectable lung tumors in 3 and 2 of the 8 animals at each of the highest two doses employed.

The effects observed using different treatment schedules are reported in Table 2. With any of the schedules employed, the formation of lung metastases was markedly and significantly reduced. The greatest depression was observed for the treatment on days 8–14. Using these schedules no large metastases were found in any of the treated animals and the mass of lung tumors was dramatically reduced; also 2 and 4 of the 8 treated animals were found free of macroscopically detectable lung colonies.

Effects of DGA on artificial metastases

The results obtained testing DGA on the formation of lung tumors, produced by i.v. injection of tumor cells, are reported in Table 3. No statistically significant reduction either of the number or weight of metastases was

Table 1. Dose dependency of the effects of DGA on pulmonary metastasis formation

Dose (mg/kg/day)	Average No. of metastases per mouse ±S.E.			Average weight ±S.E. (mg)	Animals free of metastases
	Small*	Large	Total No.		
Controls	33.1±2.5	5.7 ±0.9	38.1±3.3	77.5±17.1	0/8
150	24.5±2.85	5.76±1.28	28.2±3.28	73.6±16.3	0/8
212	25.5±2.65	3.14±0.79	28.2±2.97	50.4±10.7	0/8
300	30.1±4.30	2.51±0.67†	32.0±4.84	45.7±10.7†	0/8
424	12.9±3.87†	0 0	11.8±3.46†	2.33±1.55†	3/8
600	12.6±4.41†	0 0	11.0±3.87†	6.98±2.71†	2/8

*Diameter smaller than 2 mm.

Groups of 8 mice were treated daily i.p. with DGA at the doses indicated, on days 1–8 after tumor transplantation. Sacrifice and lung examination for metastases were performed on day 21. Statistical analysis performed is the Student–Neumann–Keul test [22]; † means significantly different from the controls ($P < 0.05$).

Table 2. Effects of treatment with DGA using different schedules on pulmonary metastasis formation

Inclusive days of treatment	Average No. of metastases per mouse ±S.E.			Average weight ±S.E. (mg)	Animals free of metastases
	Small*	Large	Total No.		
Controls	17.5±4.0	7.2 ±2.4	23.5±6.0	35.4±14.2	0/8
1–7	7.18±4.53†	0 0	8.70±5.41†	1.26±0.86†	2/8
8–14	2.45±0.93†	0 0	3.06±1.13†	0.20±0.08†	4/8
15–21	7.53±2.28†	2.38±0.00†	9.40±2.80†	7.07±3.25†	0/8

*Diameter smaller than 2 mm.
Groups of 8 mice were treated daily i.p. with DGA (500 mg/kg) as indicated. Sacrifice and lung examination for metastases were performed on day 22. Statistical analysis performed is the Student–Neumann–Keul test [22]; † means significantly different from controls ($P<0.05$).

Table 3. Effects of DGA on artificial metastases

Treatment	Average No. of metastases per mouse ±S.E.			Average weight ±S.E.	Animals free of large metastases
	Small*	Large	Total No.		
–	8.4 ±2.2	3.7 ±1.0	11.4±2.9	41.0±15.5	2/10
+	7.30±1.10	2.50±0.60	8.80±1.50	16.6±5.00	4/10

*Diameter smaller than 2 mm.
Groups of 10 animals implanted i.v. with 2.5×10^5 single viable tumor cells, were treated daily for 8 days with 500 mg/kg DGA, starting 24 hr after tumor cell injection. Sacrifice and lung examination for metastases were performed on day 15. None of the values is significantly different from controls (one-way analysis of variance [23], $P<0.05$).

Table 4. Effects of DGA on primary tumor growth

Daily dose (mg/kg)	Treatment schedule	Average tumor weight ±S.E. (mg)	
		Controls	Treated
150	1–8	593±41	492±90
212	1–8		385±60
300	1–8		397±86
424	1–8		397±71
600	1–8		267±50*
500	1–7	654±171	423±95
500	8–14	1157±197	972±255
500	15–21	1783±352	1364±330

The primary tumor weight of the same animals used for the experiments reported in Tables 1 and 2, was determined 24 hr after the last drug administration. The statistical analysis performed is a one-way analysis of variance [23]: * means significantly different from the controls, $P<0.05$.

observed: the number of animals free of large metastases was increased from 2 to 4 of the 10 mice used in each group.

Effects of DGA on primary tumor growth

Data reported in Table 4 indicate that DGA caused a significant reduction of primary tumor weight at the end of treatment only when a dose of 600 mg/kg was given daily on days 1–8.

Effects of DGA on the fractional incorporation of ^{3}H-*TdR*

The effects of a single maximum tolerated dose of DGA on the fractional incorporation of ^{3}H-TdR in tumor cells are shown in Table 5. Twenty-four or 48 hr after treatment, no significant reduction of the fractional incorporation was observed in tumor cells either subcutaneously or in the lungs of treated animals, when compared with controls.

Table 5. Effects of DGA on the fractional incorporation of ^{3}H-TdR into subcutaneous and pulmonary Lewis lung tumors

Tumor location	Treatment	24 hr		48 hr	
		FI	T/C %	FI	T/C %
Subcutaneous	−	38±3.2		30±1.4	
	+	39±1.9	102±8.6	29±3.0	95±9.8
Pulmonary	−	56±2.0		47±2.2	
	+	59±0.6	106±1.1	49±1.6	105±3.4

Twenty-four or 48 hr after treatment with 1.3 g/kg DGA, groups of 8 animals received i.p. 10 μCi of ^{3}H-TdR. One hr later they were sacrificed and the tumor tissues were processed as indicated in the experimental section. T/C is the per cent ratio of the average (±S.E.) for the treated group to that of the controls. None of the values is significantly different from controls (one-way analysis of variance [23], $P<0.05$).

DISCUSSION

The results reported so far indicate that DGA markedly reduced the formation of spontaneous lung metastases in mice bearing Lewis lung carcinoma. The antimetastatic effects were strictly dose dependent, and their magnitudes rapidly decreased with lower dosages. Using an optimal dosage and different treatment schedules, the greatest antimetastatic effects were observed when the treatment was performed in the early phases of primary tumor growth. The hypothesis put forward in preliminary investigations that the antimetastatic effects of DGA are not due to any cytotoxic effect of this substance for tumor cells localized in the lungs [2, 3] is supported by the following findings. The late treatment of animals having spontaneous metastases has very little effect. As previously reported [2, 3], DGA markedly inhibited the formation of lung colonies when administered at dosages and using treatment schedules which caused no significant reduction of primary tumor growth. Furthermore, an examination of any possible cytotoxic effects in tumor cells performed by examining the fractional incorporation of ^{3}H-TdR into DNA of tumor cells has been made. Using this technique for Lewis lung carcinoma, a strict correlation has already been found between inhibition of subcutaneous and pulmonary tumor growth caused by cyclophosphamide or ^{60}Co radiation, and fractional incorporation [9]. In the present set of experiments no such inhibition was observed. Finally, DGA did not significantly reduce the formation of lung colonies after i.v. injection of tumor cells.

The above reported data indicate that DGA at the highest dosage caused significantly reduced primary tumor growth: it also caused a tendency towards a reduction, though statistically insignificant, on the formation of artificial metastases. On the other hand, the effects of DGA on the formation of spontaneous pulmonary metastases were much more pronounced and were evident also in conditions where primary tumor growth was unaffected. Furthermore, no inhibition of the fractional incorporation of ^{3}H-TdR in tumor cells localized in the lungs or subcutaneously was observed using a single maximum tolerated dose. It thus appears that at least the greatest part of the inhibitory effects caused by DGA on the formation of spontaneous metastases are due to selective antimetastatic effects of this substance. The suggested mechanism might consist in the inhibition of detachment of tumor cells from the primary tumor and their access to the blood stream. Indeed, the maximum concentration of tumor cells in the blood stream has already been shown to occur on days 10–12 after s.c. implantation of Lewis lung carcinoma [10]. This is consistent with the fact that the greatest antimetastatic effects were observed when DGA was administered on days 8–14. Furthermore, the effects of DGA in animals having artificial metastases, where the process of tumor cell detachment and access to the blood stream is absent, were not significant. The mechanism of inhibition by DGA of tumor cell detachment and access to the blood stream might consist in the inhibition of proteolytic enzymes, which are considered responsible for this process [11–14]. In fact, DGA belongs to the class of α-diazocarbonyl derivatives of amino acids, which are irreversible inhibitors directed at the active site of

thiol [15, 16] and acid proteinases [17, 18], and DGA also inhibits neutral proteinases [19]. Furthermore, inhibitors of neutral proteinases have been found capable of inhibiting metastasis formation in mice bearing Lewis lung carcinoma [20, 21]. An investigation into whether DGA actually inhibits proteinases in the primary tumor, as well as an examination of the therapeutic effectiveness of DGA as an adjuvant to surgical treatment of solid animal tumors in terms of survival time is in progress.

Acknowledgement—The skillful technical assistance of Mr. G. Fonzari is gratefully appreciated.

REFERENCES

1. L. Baldini and G. Brambilla, Antineoplastic activity of diazoacetyl-glycine derivatives. *Cancer Res.* **26,** 1754 (1966).
2. T. Giraldi, C. Nisi and G. Sava, Antimetastatic effects of *N*-diazoacetyl-glycine derivatives in C57BL mice. *J. nat. Cancer Inst.* **58,** 1129 (1977).
3. T. Giraldi, A. M. Guarino, C. Nisi and L. Baldini, Selective antimetastatic effects of *N*-diazoacetyl-glycine derivatives in mice. *Europ. J. Cancer* **15,** 603 (1979).
4. K. Hellmann and K. Burrage, Control of malignant metastases by ICRF 159. *Nature* (*Lond.*) **224,** 273 (1969).
5. F. Spreafico and S. Garattini, Selective antimetastatic treatment—current status and future prospects. *Cancer Treat. Rev.* **1,** 239 (1974).
6. T. Curtius and J. Thompson, Einwirkung von salpetriger saure auf polyglycinester. III. Abhandlung. Einwirkung von ammoniak auf diazoacetyl-glycinester und diazoacetyl-glycyl-glycinester. *Chem. Ber.* **39,** 1383 (1906).
7. R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher and B. J. Abbott, Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemother. Rep.* **3** part 3, 13 (1972).
8. R. P. Hill and J. A. Stanley, The lung-colony assay: extension to the Lewis lung tumor and the B16 melanoma—radiosensitivity of B16 melanoma cells. *Int. J. Radiat. Biol.* **27,** 377 (1975).
9. P. J. Houghton and D. M. Taylor, Fractional incorporation of [^{3}H] thymidine and DNA specific activity as assays of inhibition of tumor growth. *Brit. J. Cancer* **35,** 68 (1977).
10. A. J. Salsbury, K. Burrage and K. Hellmann, Histological analysis of the antimetastatic effect of (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane. *Cancer Res.* **34,** 843 (1974).
11. G. C. Easty, Invasion by cancer cells. In *The Biology of Cancer.* (Edited by E. J. Ambrose and F. J. C. Roe) p. 58. Ellis Horwood, London (1975).
12. A. R. Poole, Tumor lysosomal enzymes and invasive growth. In *Lysosomes in Biology and Pathology.* (Edited by J. T. Dingle) Vol. 3, p. 303. North Holland Publishing Company, Amsterdam (1973).
13. A. J. Salsbury, The significance of circulating cancer cell. *Cancer Treat. Rev.* **2,** 55 (1975).
14. B. Sylvén, Biochemical and enzymatic factors involved in cellular detachment. In *Chemotherapy of Cancer Dissemination and Metastasis.* (Edited by S. Garattini and G. Franchi) p. 129. Raven Press, New York (1973).
15. R. Leary, D. Larsen, H. Watanabe and E. Shaw, Diazomethyl ketone substrate derivatives as active-site-directed inhibitors of thiol proteases. Papain. *Biochemistry* **16,** 5857 (1977).
16. R. Leary and E. Shaw, Inactivation of Cathepsin B1 by diazomethyl ketones. *Biochem. biophys. Res. Commun.* **79,** 926 (1977).
17. G. R. Delpierre and J. S. Fruton, Specific inactivation of pepsin by diazo ketone. *Proc. nat. Acad. Sci.* (*Wash.*) **56,** 1817 (1966).
18. O. K. Kim, J. Spona and G. A. Hamilton, A reactive aspartyl residue of pepsin. *Biochem. biophys. Res. Commun.* **30,** 489 (1968).
19. M. Kopitar, J. Babnik, I. Kregar and A. Suhar, Neutral proteinases and inhibitors of leucocyte cells. In *Movement, Metabolism and Bactericidal Mechanism of Phagocytes.* (Edited by F. Rossi, P. Patriarca and D. Romeo) p. 117. Piccin, Padova (1977).

20. T. Giraldi, M. Kopitar and G. Sava, Antimetastatic effects of a leucocyte intracellular inhibitor of neutral proteases. *Cancer Res.* **37,** 3834 (1977).
21. T. Giraldi, C. Nisi and G. Sava, Lysosomal enzyme inhibitors and antimetastatic effects in the mouse. *Europ. J. Cancer* **13,** 1321 (1977).
22. G. W. Snedecor and W. G. Cochran, *Statistical Methods*. Sixth Edition. Chapter 10, p. 258. Iowa State University Press, Ames, Iowa (1967).
23. R. R. Sokal and F. J. Rohlf, Single classification analysis of variance. In *Biometry*. p. 204. Freeman, San Francisco (1969).