EFFECT OF CHELATING AGENTS UPON THE SYNTHESIS OF NUCLEIC ACIDS AND PROTEIN: INHIBITION OF DNA SYNTHESIS BY 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE

Alan C. Sartorelli
Department of Pharmacology
Yale University School of Medicine
New Haven, Connecticut 06510

Received February 23, 1967

A series of heterocyclic aldehyde thiosemicarbazones that form coordination compounds with divalent iron, cobalt, nickel, copper and zinc have been prepared and tested for antineoplastic potency; several of these agents exhibit pronounced carcinostatic properties against a wide spectrum of transplanted neoplasms (Brockman et al., 1956; French and Blanz, 1965, 1966, 1966a). One of the most potent members of this new class of growth-inhibitory compounds is 1-formylisoquinoline thiosemicarbazone (IQ-1). Since the molecular basis for the activity of these agents is unknown, the present study was designed to elucidate some of the metabolic effects induced by IQ-1, a representative member of this series. The results showed that in sarcoma 180 ascites cells IQ-1 caused marked inhibition of the synthesis of DNA, while the formation of RNA and protein was considerably less sensitive.

MATERIALS AND METHODS

CD-1 mice bearing 6-day growths of sarcoma 180 ascites cells were given a single intraperitoneal injection of a suspension of IQ-1 (suspended by homogenization in absolute ethanol and 2-3 drops of 20% Tween 80; the final solution was 5% with respect to ethanol) at selected times before each mouse received either 200 μg of thymidine-^{3}H (6.7×10^{3} counts/min/μg), 200 μg of uridine-^{3}H (7.1×10^{3} counts/min/μg), 125 μg of DL-leucine-1-^{14}C (1.7×10^{4}

counts/min/μg), 90 μg of sodium formate-^{14}C (1.2×10^5 counts/min/μg), 50 μg of adenine-8-^{14}C (2.6×10^4 counts/min/μg) or 660 μg of deoxycytidine-^{3}H (1.1×10^3 counts/min/μg); 1 h was allowed for the metabolic utilization of each isotopic compound.

In experiments that involved the use of formate-^{14}C, deoxycytidine-^{3}H or thymidine-^{3}H, sodium nucleates were isolated by the method of Tyner et al. (1953) and hydrolyzed for 1.5 h with 70% perchloric acid (Marshak and Vogel, 1951). The desired purine and pyrimidine bases were then purified and analyzed as described by Danneberg et al. (1958). Thymine nucleotides in the cold perchloric acid-soluble extracts were separated by column chromatography on ECTEOLA-cellulose and analyzed for radioactivity (Sartorelli and Booth, 1965). In the case of adenine-8-^{14}C, the dried sodium nucleates were subjected to alkaline hydrolysis to separate deoxyribonucleotides from ribonucleotides and the adenine and guanine of both fractions were then purified and analyzed (Bieber and Sartorelli, 1964). When uridine-^{3}H was employed, the cold acid-insoluble fraction was extracted with 4% perchloric acid at 90° for 15 min, and aliquots were analyzed both for radioactivity and for ribose using adenosine as the standard (Schneider, 1955). After exposure of cells to leucine-1-^{14}C, residual protein was isolated and analyzed as previously described (Booth and Sartorelli, 1961). Radioactivity in residual protein was measured with a Nuclear-Chicago gas-flow counter equipped with a "Micromil" window, and in all other instances radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The influence of IQ-1 on the incorporation of thymidine-^{3}H, uridine-^{3}H and leucine-1-^{14}C into DNA, RNA and protein, respectively, is shown in Table I. IQ-1 in concentrations of 6.25 mg/kg of body weight caused greater than 90% inhibition of the formation of DNA, as measured by the incorporation of thymidine-^{3}H into the thymine of DNA; considerably less inhibition of the formation of RNA and protein was induced by IQ-1 at levels up to 25 mg/kg, a

Table I

INCORPORATION OF THYMIDINE-^{3}H, URIDINE-^{3}H AND LEUCINE-1-^{14}C INTO DNA, RNA AND PROTEIN, RESPECTIVELY, OF 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE-TREATED SARCOMA 180 ASCITES CELLS

Conc. of IQ-1 (mg/kg)	counts/min/μmole x 10^{-2} DNA	RNA	counts/min/mg Residual protein
0	107.7 $\pm$ 6.1	11.4 $\pm$ 1.4	4600 $\pm$ 580
6.25	3.3 $\pm$ 0.6	9.0 $\pm$ 0.8	2760 $\pm$ 570
12.5	2.1 $\pm$ 0.9	10.0 $\pm$ 1.9	3600 $\pm$ 270
25	0.9 $\pm$ 0.2	6.4 $\pm$ 0.8	3160 $\pm$ 340

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of IQ-1. Twelve h later, either thymidine-^{3}H (6.7 x 10^{3} counts/min/μg) at a level of 200 μg per animal, uridine-5,6-^{3}H (7.1 x 10^{3} counts/min/μg) at a level of 200 μg per animal or DL-leucine-1-^{14}C (1.7 x 10^{4} counts/min/μg) at a level of 125 μg per animal was administered and 1 h was allowed for metabolic utilization. Each value represents the mean ($\pm$ the standard error) of results obtained with 3-16 mice.

concentration of IQ-1 that produced essentially complete blockade of the utilization of thymidine-^{3}H.

The duration of blockade of the formation of DNA, RNA and protein caused by 25 mg of IQ-1/kg is shown in Fig. 1. Essentially complete inhibition of the incorporation of thymidine-^{3}H into DNA occurred when the isotope was administered 15 min after the IQ-1. This degree of blockade persisted for up to 12 h after IQ-1; however, by 24 h after the chelating agent, inhibition of this pathway was relieved completely. In contrast, the onset of inhibition of the formation of RNA and protein occurred later; maximum inhibition of synthesis (RNA about 50% and protein about 30%) was attained 3 h after exposure of the cells to the drug.

To determine whether the inhibition by IQ-1 of thymidine incorporation into DNA actually was attributable to a decrease in the rate of formation of

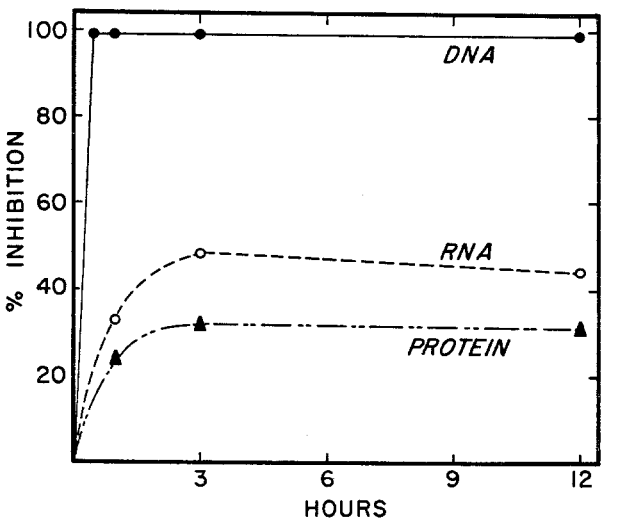

Fig. 1. Duration of inhibition of the synthesis of DNA, RNA and protein by IQ-1. Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 25 mg of IQ-1 per kg. At selected time intervals thereafter, either thymidine-^{3}H (6.7×10^{3} counts/min/μg) at a level of 200 μg per animal, uridine-5,6-^{3}H (7.1×10^{3} counts/min/μg) at a level of 200 μg per animal, or DL-leucine-1-^{14}C (1.7×10^{4} counts/min/μg) at a level of 125 μg per animal was administered and 1 h was allowed for metabolic utilization. Each value represents the mean of results obtained with 3-16 mice.

Table II

INCORPORATION OF FORMATE-^{14}C AND DEOXYCYTIDINE-^{3}H INTO THE NUCLEIC ACIDS OF 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE-TREATED SARCOMA 180 ASCITES CELLS

		counts/min/μmole x 10^{-2}			
Isotopic substrate	IQ-1	DNA thymine	NA* cytosine	NA adenine	NA guanine
Formate-^{14}C	-	69.9 $\pm$ 4.2		78.0 $\pm$ 7.2	117.0 $\pm$ 18.9
	+	2.5 $\pm$ 0.6		36.8 $\pm$ 7.8	51.9 $\pm$ 9.7
Deoxycytidine-^{3}H	-	12.8 $\pm$ 1.1	4.9 $\pm$ 0.2		
	+	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1		

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 25 mg of IQ-1 per kg body weight. Twelve h later, either formate-^{14}C (1.2×10^{5} counts/min/μg) at a level of 90 μg per animal or deoxycytidine-^{3}H (1.1×10^{3} counts/min/μg) at a level of 660 μg per animal was administered and 1 h was allowed for metabolic utilization. Each value represents the mean ($\pm$ the standard error) of results obtained with 3-8 mice.

*Nucleic acid (mixed DNA and RNA).

DNA or was unique to this isotopic tracer, other labeled precursors of DNA were employed. The data obtained with formate-^{14}C and deoxycytidine-^{3}H (Table II) and adenine-8-^{14}C (Table III) as metabolic tracers indicate that this heterocyclic aldehyde thiosemicarbazone was capable of retarding the incorporation of each of these isotopic substrates into DNA. In agreement with the data obtained using uridine-^{3}H as a measure of the rate of formation of RNA, the utilization of adenine-^{14}C for the synthesis of these molecules was considerably less sensitive to the chelating agent (Table III).

Table III

INCORPORATION OF ADENINE-8-^{14}C INTO THE DNA AND RNA PURINES OF 1-FORMYL-ISOQUINOLINE THIOSEMICARBAZONE-TREATED SARCOMA 180 ASCITES CELLS

	counts/min/μmole x 10^{-2}			
Treatment	DNA adenine	DNA guanine	RNA adenine	RNA guanine
None	35.7 ± 2.8	3.2 ± 1.1	88.0 ± 8.8	13.2 ± 2.3
IQ-1	12.7 ± 1.4	0.7 ± 0.1	67.2 ± 8.8	12.0 ± 2.1

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 25 mg of IQ-1 per kg body weight. Twelve h later, adenine-8-^{14}C (2.6×10^{4} counts/min/μg) at a level of 50 μg per animal was administered and 1 h was allowed for metabolic utilization. Each value represents the mean (± the standard error) of results obtained with 7-8 mice.

To determine whether the inhibition of the synthesis of DNA induced by IQ-1 was related to a decrease in the activities of either thymidine kinase, thymidine monophosphate kinase, or DNA nucleotidyltransferase, the cellular levels of which relate to the rate of growth, extracts were prepared from cells 12 h after exposure to 25 mg of IQ-1/kg and were assayed for enzymatic activities. No decrease in the cellular activities of these enzymes was caused by the pre-treatment with IQ-1, indicating that the inability of the cells to fabricate DNA was not the result of either loss of or decrease in the catalytic activity of one of these enzymes. Because of the extreme insolubility of IQ-1, the direct inhibitory effects of this compound on these enzymes could not be tested;

therefore, the conversion of thymidine-^{3}H to its nucleotide forms in intact cells exposed to IQ-1 was measured. The results indicated that IQ-1-treated cells incapable of incorporating thymidine into DNA were able to fabricate thymidine triphosphate.

To ascertain the stability of DNA in IQ-1-treated cells, the DNA was prelabeled with thymidine-^{3}H; 2 h later, one-half of the tumor-bearing mice were treated with 25 mg of IQ-1/kg, and the retention of label in DNA was compared with that of the DNA in the cells from untreated mice after 3, 24, 48 and 72 h. No detectable preferential loss of radioactivity from DNA occurred in IQ-1-treated cells, indicating that no extensive breakdown of these molecules occurred.

The mechanism by which this chelating agent inhibits the synthesis of DNA is unknown; three possibilities are presently under consideration as potential sites of action of this agent: (a) interference with the primer function of DNA, for although no extensive degradation of DNA was produced in IQ-1-treated cells, small drug-induced changes in the structure of DNA, not detected by the methods employed, may occur which interfere with such function, (b) direct inhibition of DNA nucleotidyltransferase, and (c) inhibition of the enzymatic reduction of ribonucleotides to deoxyribonucleotides. The latter potential site of action is made attractive by the finding of Moore and Reichard (1964) that ferric ions stimulated the activity of cytidine diphosphate reductase isolated from the Novikoff hepatoma. In this system the chelating agent EDTA inhibited enzymic activity; this inhibition was reversed by the addition of $FeCl_3$. That iron is involved in the mechanism of inhibition of the synthesis of DNA by IQ-1 was shown by the administration of $FeSO_4$ to sarcoma 180 tumor-bearing mice subsequent to IQ-1; such treatment resulted in partial reversal of the IQ-1-induced inhibition of the incorporation of thymidine-^{3}H into DNA, whereas $CuCl_2$ and $FeCl_3$ were inactive in this regard. Although the essential function of iron in the cytidine diphosphate reductase system has not been established, the involvement of a ferrous-ferric oxidation-reduction process in the mechanism

of action of the enzyme can be envisioned. Further evidence for a relationship between iron and DNA was shown by the finding that iron deficiency reduced the cellular content of DNA in the iron-requiring organism Mycobacterium smegmatis (Winder and O'Hara, 1962; Winder and Coughlan, 1966).

ACKNOWLEDGMENTS

The author is indebted to Dr. Frederick A. French, Mount Zion Hospital and Medical Center, for generous supplies of 1-formylisoquinoline thiosemicarbazone, and Miss Florence C. Dunmore, Miss Sheila J. Feld, Miss Andrea F. Gorske and Miss Lynn A. Bon Tempo for excellent assistance.

REFERENCES

Bieber, A. L. and Sartorelli, A. C., Cancer Res., 24, 1210 (1964).
Booth, B. A. and Sartorelli, A. C., J. Biol. Chem., 236, 203 (1961).
Brockman, R. W., Thomson, J. R., Bell, M. J. and Skipper, H. E., Cancer Res., 16, 167 (1956).
Danneberg, P. B., Montag, B. J. and Heidelberger, C., Cancer Res., 18, 329 (1958).
French, F. A. and Blanz, E. J., Jr., Cancer Res., 25, 1454 (1965).
French, F. A. and Blanz, E. J., Jr., J. Med. Chem., 9, 585 (1966).
French, F. A. and Blanz, E. J., Jr., Cancer Res., 26, 1638 (1966a).
Marshak, A. and Vogel, J., J. Biol. Chem., 189, 597 (1951).
Moore, E. C. and Reichard, P., J. Biol. Chem., 239, 3453 (1964).
Sartorelli, A. C. and Booth, B. A., Cancer Res., 25, 1393 (1965).
Schneider, W. C., in: S.P. Colowick and N.D. Kaplan (eds.), Methods in Enzymology, Vol. III, Academic Press, Inc., New York, 1955, p. 680.
Tyner, E. P., Heidelberger, C. and LePage, G. A., Cancer Res., 13, 186 (1953).
Winder, F. G. and O'Hara, C., Biochem. J., 82, 98 (1962).
Winder, F. G. and Coughlan, M. P., Biochem. J., 100, 39P (1966).