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SHORT COMMUNICATIONS

Strand-scission of Sarcoma 180 tumor cell DNA induced by 1-formylisoquinoline thiosemicarbazone

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The α-(*N*)-heterocylic carboxaldehyde thiosemicarbazones have demonstrated potential as both antineoplastic and antiviral agents (see, for appropriate references [1, 2]). The primary biochemical lesion induced by these agents is on the synthesis of DNA, with the enzyme ribonucleoside diphosphate reductase being the major site of blockade in the biosynthetic pathways leading to these macromolecules [3–8]. However, although studies of structure–activity relationships have indicated the requirement for inhibition of ribonucleoside diphosphate reductase activity for antineoplastic potency [2], several investigations have suggested that the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones cause a second metabolic alteration on the DNA biosynthetic pathways distinct from reductase blockage [9, 10]. The purpose of this communication is to provide evidence for a direct interaction between one of the most potent members of this series, 1-formylisoquinoline thiosemicarbazone (IQ-1), and DNA and the apparent induced scission of DNA strands of Sarcoma 180 ascites cells after exposure to this agent.

Thymidine-methyl-^{3}H (27·8 mCi/m-mole) and Aquasol were purchased from New England Nuclear, Boston, Mass. Sephadex G-25 was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J. Millipore nitrocellulose filters (pore size, 0·45 μm) were obtained from Millipore Corp., Bedford, Mass. Calf-thymus DNA was purchased from Sigma Chemical Co., St. Louis, Mo. DNA-^{3}H was isolated and purified from Sarcoma 180 cells after exposure to thymidine-methyl-^{3}H according to the procedure of Marmur [11]. 3′-^{14}C-1-formylisoquinoline thiosemicarbazone (IQ-1-3′-^{14}C) was synthesized in this laboratory as previously described [12].

CD-1 female mice (Charles River Breeding Laboratories, North Wilmington, Mass.) were used for all experiments. The animals were inoculated i.p. with 5×10^6 Sarcoma 180 ascites cells; 5 days after implantation, the DNA of the tumor cells was labeled by injecting i.p. 70 μCi thymidine methyl-^{3}H (27·8 mCi/m-mole) two times at 12-hr intervals. Twelve hr after the final injection of thymidine methyl-^{3}H, IQ-1, as an aqueous suspension prepared by homogenization in 5% ethanol containing two drops of 20% Tween 80, was injected i.p. either once or three times, at doses each of 30 mg/kg of body weight every 4 hr. Four hr after the last IQ-1 injection, cells were collected into small beakers containing phosphate-buffered saline (8·0 g NaCl, 0·2 g KCl, 2·16 g $Na_2HPO_4.7H_2O$ and 0·2 g KH_2PO_4 in 1 liter of H_2O, pH 7·4). Erythrocytes were removed by repeated washing of cells with phosphate-buffered saline. The number of neoplastic cells was determined with a model B Coulter Counter and the suspension was adjusted to contain 1×10^6 cells/ml.

Centrifugation of cells through alkaline sucrose was carried out essentially according to the method of Horwitz and Horwitz [13] as modified by Abelson and Penman [14]. Differences included the replacement of CsCl with 40% neutral sucrose as a cushion, and the composition of the alkaline sucrose gradient employed was 5–20% instead of 15–30%. The alkaline sucrose gradient was prepared by placing 1·0 ml of 40% neutral sucrose in the bottom of a centrifuge tube and overlaying this with 1·0 ml of 25% alkaline sucrose followed by an alkaline sucrose gradient of from 5 to 20% sucrose containing 1·0 M NaCl, 0·01 M EDTA and 0·2 M NaOH. A lysing solution (0·5 ml) of 1% sarcosyl in 0·2 M NaOH and 5% sucrose was carefully layered on top of the gradient. Sarcoma 180 ascites cells (0·5 ml of 1×10^6 cells/ml) were layered over the lysing solution in a medium containing 0·02 M EDTA, 0·075 M NaCl, and 0·05 M Tris-HCl, pH 7·5. A small quantity (0·1 ml) of the lysing medium containing 0·25% sarcosyl was added on top of the layer containing the cells to ensure complete lysis. Centrifugation was carried out at 160,000 *g* for 3·5 hr at 5° using a Beckman SW 40 Ti rotor in a Spinco model L2-65B ultra-centrifuge.

After centrifugation, gradients were fractionated by infusion of 60% neutral sucrose into the bottom of the tube; 20-drop fractions were collected from the top of the gradient. Each fraction was precipitated with 4·0 ml of cold 5% trichloroacetic acid (TCA). The precipitate was collected onto millipore nitrocellulose filters (pore size, 0·45 μm) and washed four times with 5 ml each of cold 5% TCA. The filters were then placed in vials, dried, and after addition of 10 ml Aquasol, radioactivity was determined with a Packard Tri-carb liquid scintillation spectrometer.

Interaction of IQ-1 with DNA *in vitro* was measured by gel filtration on columns (50 × 1·0 mm) of Sephadex G-25 equilibrated with 0·01 M Tris-HCl, pH 7·0. ^{3}H-labeled purified Sarcoma 180 DNA ($3·5 \times 10^5$ cpm) plus 350–550 μg calf-thymus DNA to serve as a carrier were mixed with a 5% dimethyl sulfoxide solution of 4×10^{-5} M IQ-1-3′-^{14}C ($3·8 \times 10^5$ cpm) at room temperature for 10 min in a final volume of 1·0 ml. In some experiments, 1×10^{-5} M $FeCl_3$ was also added. After incubation, the entire mixture was added to a column of Sephadex G-25 and eluted with 0·01 M Tris-HCl, pH 7·0. Fractions of 3·0 ml were collected and absorbance at 260 nm and radioactivity were determined for each fraction.

Karon and Benedict [15] have shown that 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP) caused chromatid breaks, principally during the S phase, in hamster fibroblasts. In an effort (a) to determine whether other members of this class created lesions at the level of DNA, and (b) to translate effects at the chromatid level into more molecular terms, the effects of IQ-1 on the DNA of a susceptible tumor, Sarcoma 180, were determined by sedimentation of DNA prelabeled with thymidine methyl-^{3}H in alkaline sucrose gradients. The results shown in Fig. 1 demonstrate that the DNA of untreated Sarcoma 180 cells migrated primarily to the bottom of the tube. A single exposure of Sarcoma 180 cells to IQ-1 resulted in a slight increase in lower molecular weight fragments, whereas the DNA of cells treated with three doses of 30 mg/kg each of IQ-1 showed the presence of extensive lower molecular weight single-strand fragments.

That the exposure of Sarcoma 180 cells to three doses of IQ-1 did not result in a significant decrease in cellular viability is suggested by the finding, shown in Table 1,

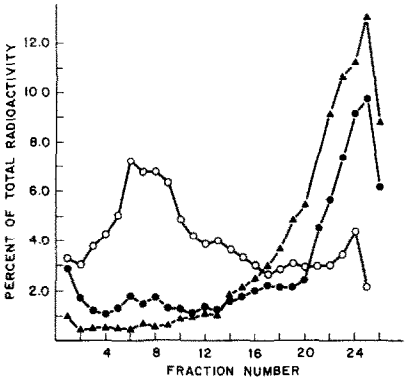

Fig. 1. Radioactivity profiles of thymidine methyl-^{3}H labeled Sarcoma 180 DNA in alkaline sucrose gradients after treatment with IQ-1. Mice bearing 5-day implants of Sarcoma 180 ascites cells were injected i.p. twice at 12-hr intervals with 70 μCi thymidine methyl-^{3}H (27·8 mCi/mmole). Twelve hr after the final injection of thymidine-^{3}H, either one or three doses of IQ-1, 30 mg/kg each, were injected i.p. at intervals of 4 hr. ^{3}H-labeled Sarcoma 180 cells from untreated and treated mice were layered onto alkaline sucrose gradients and centrifuged immediately at 160,000 g for 3·5 hr. Fractions were collected, and the radioactivity present in each fraction was measured. Per cent of total radioactivity refers to total counts present on the gradient. Fraction number zero is indicative of the top of the gradient. Key: ▲——▲, untreated Sarcoma 180 cells; ●——●, IQ-1-treated Sarcoma 180 cells (1 ×); and O——O, IQ-1-treated Sarcoma 180 cells (3 ×).

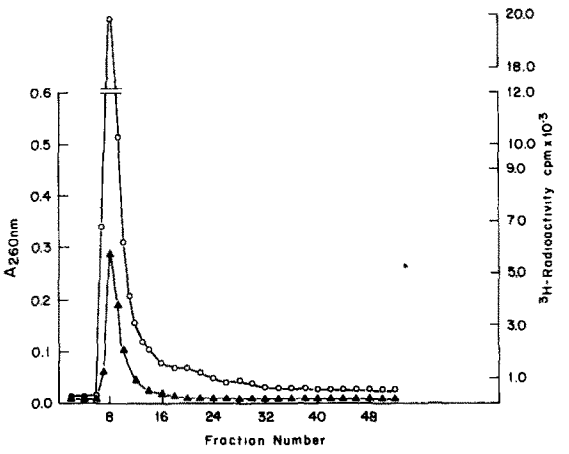

Fig. 2. Gel filtration of purified Sarcoma 180 DNA-^{3}H. A mixture containing Sarcoma 180 DNA-^{3}H ($2·5 \times 10^5$ cpm) diluted with 350 μg calf-thymus DNA as carrier, and 1×10^{-5} M $FeCl_3$ was added to a column of Sephadex G-25 equilibrated with 0·01 M Tris-HCl, pH 7·0. Elution was carried out with the same buffer. Radioactivity and absorbance at 260 nm were determined in 3·0-ml fractions which were collected. Key: O——O, ^{3}H-radioactivity from Sarcoma 180 DNA-^{3}H; and ▲——▲, A_{260} nm calf-thymus DNA.

that approximately 94 per cent of cells found in the peritoneal cavities of mice excluded trypan blue, regardless of the extent of drug treatment.

To provide evidence for direct interaction between Sarcoma 180 DNA and IQ-1, a series of experiments were conducted in which DNA-^{3}H (purified from Sarcoma 180 cells) was mixed with IQ-1 containing a small quantity of high specific activity IQ-1-3′-^{14}C in the presence or absence of Fe^{3+} ions. The mixture was placed onto a column of Sephadex G-25 and eluted with Tris-HCl. Figure 2 shows that Sarcoma 180 DNA was not readily retained by the column; furthermore, the major portion of the radioactive material migrated in one peak which contained the carrier calf-thymus DNA. However, as shown in Fig. 3, IQ-1 modified the pattern of chromatography of Sarcoma 180 DNA. Thus, ^{3}H-radioactivity from DNA exposed to IQ-1 occurred in three distinct bands. ^{14}C-radioactivity from IQ-1 was associated with each of these fractions suggesting interaction. A large portion of the ^{14}C-label appeared with the second DNA peak, which presumably represented fragments of DNA, and the remainder of the ^{14}C-label was retained by the column. The retention of ^{14}C on the column corresponds to the position of IQ-1 added alone on a similar column of Sephadex G-25. The presence or absence of Fe^{3+} ions did not alter the results significantly. Thus, the capacity of IQ-1 to form an iron chelate [16] did not appear to influence its ability to interact with DNA.

The results indicate that IQ-1 reacts with Sarcoma 180 DNA and induces single-strand breaks *in vivo*. Modifica-

Table 1. Per cent of trypan blue excluding Sarcoma 180 ascites cells treated with various doses of IQ-1 *in vivo**

IQ-1 doses	% Cells excluding trypan blue	
	Untreated	Treated
1	94·2	94·1
2		96·2
3	94·8	93·8

* Thirty mg/kg of IQ-1 was injected i.p. into mice bearing Sarcoma 180 ascites cells at 4-hr intervals and cells were isolated as described in the legend to Fig. 1. Aliquots of 0·5 ml of the cell suspension (2–3 × 10^6 cells/ml) were mixed with an equal volume of 0·4% trypan blue in a 0·9% NaCl solution and incubated for 5 min at 37° with shaking. The percentage of stained cells was determined with a hemocytometer.

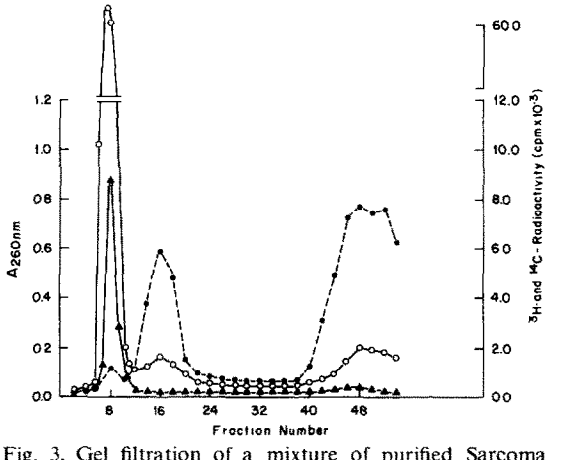

Fig. 3. Gel filtration of a mixture of purified Sarcoma 180 DNA-^{3}H and IQ-1-3′-^{14}C. A 1·0-ml mixture consisting of purified labeled Sarcoma 180 DNA-^{3}H ($3·5 \times 10^5$ cpm), 550 μg calf-thymus DNA, 4×10^{-5} M IQ-1-3′-^{14}C ($3·8 \times 10^5$ cpm) in an aqueous medium containing 5% dimethylsulfoxide, and 1×10^{-5} M $FeCl_3$ was added to a column of Sephadex G-25 equilibrated with 0·01 M Tris-HCl, pH 7·0, and processed as described in Fig. 2. Key: ●---●, ^{14}C-radioactivity from IQ-1-3′-^{14}C; O——O, ^{3}H-radioactivity from Sarcoma 180 DNA-^{3}H; and ▲——▲, A_{260} nm calf-thymus DNA.

tion of Sarcoma 180 DNA was also observed after mixing IQ-1 and purified DNA *in vitro*. The use of EDTA to minimize enzymatic degradation of DNA during the cell lysis on top of sucrose gradients, as well as in the alkaline sucrose environment employed for DNA denaturation, suggests that IQ-1 induces the fragmentation of Sarcoma 180 DNA *in vivo*, rather than on the sucrose gradient. The precise mechanism(s) responsible for fragmentation is unknown; however, it is conceivable that it is due to prolonged inhibition of the enzyme, ribonucleoside diphosphate reductase. No attempt was made to estimate the molecular weight of the IQ-1-induced DNA fragments for, although some reports have been published which indicate that a direct relationship exists between sedimentation distance and the molecular weight of single strands [17–19], the relationship appears to be more complicated [18, 20]. It has been demonstrated that the sedimentation velocity of DNA in an alkaline sucrose gradient is dependent upon the cell concentration [18, 21]. This relationship was also found to be of importance in this system, and the addition of $>1 \times 10^6$ Sarcoma 180 cells to the gradient led to aggregation of high molecular weight DNA.

The experiments employing gel filtration techniques demonstrate that binding between DNA and IQ-1 takes place, at least *in vitro*. The association of IQ-1 with a new fraction of apparently lower molecular weight DNA suggests that IQ-1 is primarily bound to modified DNA, possibly implying that the binding of IQ-1 to DNA increases its susceptibility to shearing as it passes through the Sephadex column. The IQ-1 retained by the Sephadex column is presumably either free or bound to DNA degradation products, such as nucleotides.

Regardless of the precise mechanism by which IQ-1 induces the breakage of Sarcoma 180 DNA, the finding of this second site of action appears to be of major significance to the cytotoxic mechanism of action of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones, since it creates a lesion in the genome which is reinforced by blockade of ribonucleoside diphosphate reductase.

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Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. 06510, *U.S.A.*

ASTERIOS S. TSIFTSOGLOU
KOU M. HWANG
KRISHNA C. AGRAWAL
ALAN C. SARTORELLI

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Effects of activators *in vitro* on rabbit lung and liver microsomal UDP-glucuronyltransferase activity

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UDP-glucuronate glucuronyltransferase (acceptor unspecific) (EC 2.4.1.17) activity toward several exogenous acceptor substrates has been reported in lung microsomes from various animal species [1–5]. Aitio [6] detected levels of microsomal UDP-glucuronyltransferase activity in rat and guinea pig lung equal to more than one-third the liver specific activity. Furthermore, the pulmonary enzyme exhibited a 2 to 3-fold activation by digitonin *in vitro*, suggesting a latent state. Activation of UDP-glucuronyltransferase *in vitro* by a number of materials has been previously described [7–14]. Recent work in our laboratory failed to demonstrate measurable "native" UDP-glucuronyltransferase activity in rabbit lung microsomes toward *p*-nitrophenol or phenolphthalein as acceptor substrates [15]. The present study was designed to determine if the enzyme was truly absent from rabbit lung or if it was present in