solution of the test compound or standard in 0.2 mL of sterile water was injected subcutaneously in the interscapular region of the mice at 0.5 and 4 h past challenge, using four different dose levels, with ten mice per dose level. Oral doses, prepared as described above were administered by gavage. Otherwise the procedures were the same as for the subcutaneous doses. The nonmedicated control animals and the test animals were observed twice daily, and deaths were recorded per observation period up to 72 h past challenge. PD_{50} for 72 h survival was calculated using probit analysis.

Acknowledgment. We are indebted to Ms. S. Hitt, Dr. A. Braemer, and Dr. T. Matthews for the antibacterial assays, to Dr. J. Muchowski for supplying the 1,2,3-thiadiazole starting materials, to Dr. Muchowski and Dr. J. Edwards for helpful discussions, and to Mrs. J. Nelson and Dr. M. Maddox for NMR measurements.

Note Added in Proof. Subsequent in vivo testing has shown that the bacterial challenge used to obtain the oral ED_{50} data in Table II was approximately 40 times the LD_{50} for untreated mice. When ca. 1600 LD_{50} doses were administered, and the initial dose of drug was given for 1 h, rather than 0.5 h, past challenge, ED_{50} values increased ca. tenfold.

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Comparative Cytotoxic and Biochemical Effects of Ligands and Metal Complexes of α-*N*-Heterocyclic Carboxaldehyde Thiosemicarbazones

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Several α-*N*-heterocyclic carboxaldehyde thiosemicarbazones and their iron and copper complexes have been tested for their cytotoxicity and inhibiting activity against DNA synthesis under controlled metal conditions. No ligands show cytotoxicity against Ehrlich cells at the concentrations tested, while some iron and copper complexes are active. In contrast, the ligands inhibit DNA synthesis at much lower concentrations than used above. Similarly, the metal complexes are effective inhibitors at concentrations much below those necessary to demonstrate cytotoxicity. In addition, the iron complexes of 1-formylisoquinoline thiosemicarbazone, 2-formylpyridine thiosemicarbazone, and 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone were shown to be three- to sixfold more active than their free ligands as inhibitors of partially purified ribonucleotide reductase to which no iron has been added. The copper complex of 2-formylpyridine thiosemicarbazone was slightly more active than the free ligand against the reductase.

Bis(thiosemicarbazones) and α-*N*-heterocyclic carboxaldehyde thiosemicarbazones comprise two interesting classes of experimental cancer chemotherapeutic agents in that both are strong metal chelating agents.[1-4] The thiosemicarbazones are potent inhibitors of ribonucleotide reductase in vitro.[5] This enzyme catalyzes a critical and possibly rate-limiting step in DNA synthesis and cell division. There is substantial evidence in the case of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) and related compounds that their copper complexes are the active in vivo forms of these agents.[6-8] Although studies have demonstrated in vivo and in vitro cytotoxic activity for iron and copper complexes of 1-formylisoquinoline thiosemicarbazone and certain 5-substituted 2-formylpyridine thiosemicarbazones, as well as in vivo activity for a number of the free ligands, there has been no concerted attempt to sort out the relationship of ligands and metal complexes which underlies these observations.[9,10] Thus, the present investigation compares the cytotoxic properties of a number of thiosemicarbazone ligands and their copper and iron complexes in a metal-free assay system involving Ehrlich ascites tumor cells. The comparison is extended to examine the inhibition of DNA synthesis and, more specifically, of ribonucleotide reductase, thought to be the

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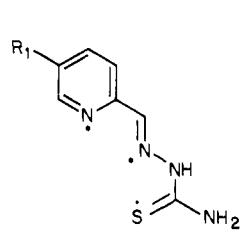
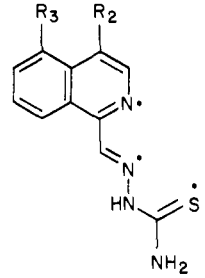
Table I. Structures of Compounds[a]



5-substituted 2-formylpyridine thiosemicarbazone



4,5-substituted 1-formylisoquinoline thiosemicarbazones

substituent			compound numbers		
R_1	R_2	R_3	ligand	iron complex	copper complex
H			1	6	11
CH_3			2	7	12
OH			3	8	13
	H	H	4	9	14
	CH_3	NH_2	5	10	15

[a] Locations of atoms involved in metal binding are indicated by dots. All copper complexes have 1:1 metal–ligand stoichiometry. All iron complexes have 1:2 metal–ligand stoichiometry.

site of cellular toxicity, and the properties of uptake of these compounds into Ehrlich cells.

Results and Discussion

Cytotoxicity. The compounds studied are listed in Table I. The choice of ligands was based upon the past and present interest in 5-hydroxy-2-formylpyridine thiosemicarbazone (**3**), 1-formylisoquinoline thiosemicarbazone (**4**), and 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (**5**) and the previous demonstration of in vitro cytotoxicity by iron and copper complexes of 2-formylpyridine thiosemicarbazone (**6** and **11**) and 5-methyl-2-formylpyridine thiosemicarbazone (**7** and **12**).[5,11-14] The initial in vitro incubation of cells and drugs provided a common set of conditions for all of the studies reported here.

With the concentrations of drugs used for this work, there is no significant activity of any of the ligands in prolonging the life of the host mice (Table II). In contrast, some of the iron and copper complexes have a marked inhibitory effect upon the capacity of Ehrlich cells to grow in these animals. As seen earlier, the copper complexes are effective at lower concentrations than their iron counterparts, either in terms of the total drug used or that which is taken up by the cells during a 1-h incubation.[10] A surprising finding was that none of the compounds, **5**, **10**, or **15**, showed activity in this assay system at the concentrations used, despite the excellent antitumor effects seen with the free ligand in vivo against the Sarcoma 180 ascites tumor.[14]

Table II provides uptake data for a number of these drugs which can be used to refine the analysis of the results of the cytotoxicity test. In most cases, concentrations of drug were used which approximated those used in the cytotoxicity tests. In a few instances, lower concentrations were used or experiments were not carried out because of the insolubility of the compounds.

None of the ligands is well absorbed by Ehrlich cells. This was an unexpected finding, for compound **1** itself has a 1-octanol/H_2O partition coefficient of about 5. In contrast, all of the copper complexes, which carry a 1+ charge, and two of the iron complexes are readily taken up. Thus, it might be argued that the insensitivity of Ehrlich cells to these ligands results from their inability to reach critical sites within the cells in adequate concentrations.

Table II

	cytotoxicity		uptake		inhibn of DNA synth	
no.[f]	concn, nmol/mg[a]	T/C[b]	concn, nmol/mg[c]	% in cells	50%, nmol/mg	90% nmol/mg
1	1800 (>13)[d]	1.1	6	10	1.4	8
			10	14		
			20	10		
			100	13		
2	200[d,e]	1.1				
3	200	1.1				
4	500[d,e]	1.1			0.35	7.5
5	500 (125)	1.1	465	25	0.4	2.5
			930	28		
6	530 (55)	1.5	93	10	0.05	0.50
			480	12		
			960	14		
7	215 (>9)[d]	1.2	90	10	1.85	18.5
	360 (37)	2.2	176	9		
			330	11		
8	540[d,e]	0.9				
9	360 (>83)[d]	1.6	10	69	0.06	1.0
			50	55		
			100	70		
			130	64		
10	360 (>59)[d]	1.1	4	64	0.45	4.5
			16	71		
			38	81		
			68	87		
11	50 (>12)	1.6	13	87	0.70	2.8
12	10 (7)	1.85	10	72	5.0	12.0
			23	82		
			48	84		
			85	42		
13	50[d,e]	1.03	0.7	40		
	138[d,e]	2.8	1.2	33		
14	11 (8)	1.4	8	72	>0.60	
	48 (38)	2.8	19	83		
			36	82		
			94	53		
15	6 (4)	1.0	6	72	>16	
	25 (>10)[d]	1.0	12	66		

[a] In parentheses is the minimum amount of soluble compound in solution according to uptake studies. [b] T/C = average survival of animals with treated cells/average survival of controls; C = 18 days. Experiments terminated after 50 days. T/C maximum = 2.8. [c] All compounds were soluble at the levels used in incubation. [e] Highly insoluble compound. [f] Structures of drugs are given in Table I.

Table II also compares the short-term inhibition of DNA synthesis by these substances. Surprisingly, almost all of the compounds are highly effective inhibitors, including the metal-free ligands. In three series, based on the ligands **1**, **2**, and **4**, the iron complex is a substantially better inhibitor than either the copper complex or ligand, based on the total nanomoles of drug in the cell suspension. It is clear that the ligands are active on the biochemical level but they do not sustain a long-term cytotoxic effect. Extending the comparison, the effects of ligands and their metal complexes on ribonucleotide reduction have been studied, using partially purified ribonucleotide reductase to which no external iron has been added. Table III summarizes the results of inhibitor studies on ribonucleotide reductase. Iron complexes are three to six times as potent as free ligands in the suppression of ribonucleotide reduction. These results agree with a report of Agrawal et al. that the iron complex **9** is five times as

Table III. Effect of Ligands and Metal Complexes of α-N-Heterocyclic Carboxaldehyde Thiosemicarbazones on Ribonucleotide Reductase

drug	ID_{50}, μM[a]
1	0.90
4	0.40
5	0.60
6	0.20
9	0.15
10	0.10
11	0.75

[a] ID_{50} = micromolar concentration required to inhibit the enzyme by 50%.

effective an inhibitor as the free ligand when an external iron source is left out of the assay mixture.[9] In the presence of iron, ligands are equally as good as the complexes. According to a previous study, either the ligand 1 or its iron complex exists as the iron complex in this assay medium and is unlikely to be dissociated by interaction with the enzyme.[2] Therefore, it may be that the intact complex reacts with and inhibits the enzyme. It is known from previous work that, in the assay for ribonucleotide reductase, complex 11 is reduced by dithiothreitol and dissociated to yield the free ligand.[2] Hence, the similar response of the catalytic system to thiosemicarbazone 1 and its copper complex is due to the presence of the ligand in each case. Whether the activity of compound 1 is due to the free ligand or to the formation of the iron complex from the iron which contaminates the preparation is not known. However, the presence of freely chelatable iron within Ehrlich cells is not expected. Therefore, the ligands may be inhibiting DNA synthesis by a mechanism different from iron complex, such as by binding to the presumed iron site in ribonucleotide reductase. Similarly, it is known that copper complex 11 is not dissociated in Ehrlich cells to yield the ligand, as occurs in the above assay system.[15,16] Thus, the interaction of the copper chelate with the pathways leading to DNA synthesis is expected to differ from that of the metal-free ligand.

The combination of Tables II and III together with the preceding discussion bring into question the nature of the cytotoxic species in vivo. There seem to be two views which can be taken. First, the ligands alone may simply not have cytotoxic properties despite their inhibition of DNA synthesis. Complexation to yield iron or copper complexes must occur to form the active entity. Alternatively, the ligands and the metal complexes each may comprise a distinct class of cytotoxic agents. That is, the cytotoxicity assay used here involves a 1-h exposure of cells to drug. Active compounds must either be nonspecific with respect to the cell cycle or must persist for a long period in the cells and gradually attack them as they enter a critical phase of the cycle. Some of the iron and copper complexes fit into this category. Perhaps the inactivity of the ligands at the cellular level, despite their strong effects biochemically, is due to a cell-cycle specificity to their activity and to a reversible nature to their interactions with cells. If this is so, then a continual exposure of cells to ligand would need to be done over the course of the cell cycle. In either hypothesis, metal complexes must be viewed at least as distinct, pharmacologically active compounds and perhaps as the only active forms of the thiosemicarbazones.

This conclusion reinforces earlier results and discussion that in vivo antitumor studies need to be conducted using preformed iron and copper complexes.[2,10,17] In one brief experiment Agrawal et al. did report that complex 9 was as active against the Sarcoma 180 ascites tumor in mice as 40 times as much free ligand.[9] Similarly, their finding that this iron complex is more effective in the inhibition of partially purified ribonucleotide reductase than the free ligand when the assay is not supplemented with iron led them to suggest that the iron complex is the form of the ligand which reacts with the enzyme.[18] However, subsequent screening of second-generation thiosemicarbazones has not included an examination of iron or copper complexes for differences in activity and toxicity to the animals.[14,19]

The importance of such work resides in the possibility that the complexes may be more efficacious drugs against tumors. By using a bound ligand such as the iron complex, such effects, which may occur because of the substantial chelation of iron in vivo by the free ligand, might be avoided.[2,12,20,21] Finally, if free ligands must be activated by iron or copper chelation, then the nutritional iron and copper status of the host becomes a major factor in the success of chemotherapy with the ligands, as has been amply shown for bis(thiosemicarbazones).[6]

Experimental Section

Materials. All of the thiosemicarbazone ligands were gifts of Frederic A. French or the National Cancer Institute and have been previously characterized. Eagles minimal essential medium plus Earles salts (MEM) was obtained from Grand Island Biological Co. Tritiated precursors were purchased from Amersham/Searle Corp., and dimethyl sulfoxide was the Gold Label grade from Aldrich Chemical Co. The iron complexes were prepared according to published methods which the authors have previously used.[10] The copper complexes were also made according to an earlier synthesis.[22] In both cases, the complexes form quickly and completely under mild conditions. The ultraviolet–visible spectra of the complexes were checked to confirm complete chelation of the ligands.

Cytotoxicity. The assay for cytotoxicity combines an in vitro incubation of cells and drug with in vivo growth of these cells in mice to test for tumor viability.[10] The in vitro conditions minimize exposure of the drugs to extraneous metal ions. Thus, the form of a given drug, free ligand or metal complex, which is interacting with the tumor cells is unambiguous.

The Ehrlich ascites tumor was initially obtained from the Arthur D. Little Co. (Cambridge, Mass.) and is maintained in female HA/ICR mice (ARS Sprague–Dawley, Madison, Wis.) by weekly intraperitoneal transplantation of approximately 5×10^6 cells. For this assay, the cells in ascites fluid suspension were withdrawn under sterile conditions by syringe puncture and transferred to a sterile 15-mL capacity plastic tube containing one drop of heparin (1000 units/mL). The cell pellet was harvested by centrifugation at moderate speed in an International clinical centrifuge for 5 min. The cells were washed once with approximately 4 volumes of sterile MEM and resuspended at 5×10^7 cells/mL. The drugs were dissolved in Me_2SO, and 10–20 μL was added to 1 mL of cell suspension. Concentrations of drug per milligram of cell protein, as measured by the Biuret method, are given in Table II. In some experiments, the drugs were not completely soluble in the incubation medium. In these cases, particular care was used to continually resuspend the compound during the experiment. The drug–cell suspension was incubated at 37 °C for 1 h and centrifuged as before, and the cells were resuspended in the same volume of sterile MEM. After the addition of 50 μL of a sterile antibiotic solution (8 mg of penicillin and 5 mg of streptomycin in 1 mL of H_2O), 0.2 mL of the cell suspension containing 1×10^7 cells was injected in each of five animals per group. The cells of the control groups were only incubated with Me_2SO. Progress of tumor development was assessed by observing the increase in weight of tumor-bearing animals. The degree of inhibition of tumor growth by drugs is expressed as a ratio of average survival days of animal injected with drug-treated cells to average survival days of the control group. A ratio greater than 1.2 is indicative of significant prolongation of life.

DNA Synthesis and [^{3}H]Thymidine Uptake. These studies were carried out under the same conditions used for the cyto-

toxicity and uptake work. Short-term effects of various ligands and their iron and copper complexes on [^{3}H]thymidine utilization by Ehrlich ascites cells were measured by a modification of a procedure described previously.[23] Cells were suspended in MEM or Ringer's balanced salt solution at 37 °C for 10 to 15 min prior to the addition of various concentrations of drug in Me_2SO solution. Cells were exposed to drug for 30 min; then 0.5 μCi of [*methyl*-^{3}H]thymidine (1 mCi/mL; 2 Ci/mmol) per incubation was added, and aliquots were removed at 5, 10, and 20 min thereafter. Final thymidine concentration was approximately 42 μM. Controls containing up to 5% Me_2SO in place of drug showed little, if any, effect on labeled thymidine uptake into cells or incorporation into acid-precipitable matter under these conditions.

Radioactivity in the external medium, in the perchloric acid soluble fraction, and in the perchloric acid insoluble fraction were measured by liquid scintillation techniques using a Nuclear Chicago Unilux III instrument. Aqueous 50-μL aliquots of these fractions were first dispersed in a solubilizing mixture containing 2 parts scintillation grade toluene and 1 part Beckman Bio-Solv BBS-3, to which 10 mL of a toluene–ethanol (6:4) based scintillation cocktail was added. PPO (6 g/L, Beckman) and Me_2POPOP (0.1 g/L, Beckman) were the primary and secondary fluors, respectively. Tritium counting efficiency was approximately 35%; no differences in the degree of quenching was observed for different samples.

Drug concentrations required to inhibit DNA synthesis by 50 and 90% compared to Me_2SO-treated controls were obtained from graphs summarizing the effects of each compound. Unlike the results for bis(thiosemicarbazone) complexes,[23] uptake of radiolabeled nucleoside is virtually unaffected at concentrations considerably in excess of those required to cause 50 and 90% inhibition of DNA synthesis. No effect on thymidine uptake by cells is observed for compounds **1**, **6**, or **11** at concentrations of 15, 20, and 4 nm of drug/mg of cell protein, respectively.

Uptake of Cu^{2+} and Fe^{3+} Complexes. The accumulation of complexes in Ehrlich cells was examined under conditions and drug concentrations which were similar to those used in the cytotoxicity study: 1×10^7 cells/mL were incubated, with various concentrations of the drugs, for 1 h at 37 °C. At the end of the incubation, the suspensions were spun down, the supernatant was collected, and the cells were washed once with the medium (MEM) and digested in acid (10:4:1 HNO_3–$HClO_4$–H_2SO_4). Requisite dilutions of the supernatant and cell digests were made, and the metal content in both was detected by the atomic absorption spectrophotometer. Protein content of the cells was estimated by the Biuret method; hence, uptake of the drug, expressed as nanomoles per milligram of protein, could be calculated.

Uptake of the Ligands. The accumulation of ligands in Ehrlich cells was examined under conditions and drug concentrations which were similar to those used in the cytotoxicity study. Cells were incubated with the ligands in Ringer's medium. The supernatants were scanned for the amounts of ligand left over, either by looking for the ligand directly or by Cu^{2+} titrations. Consequently, the percent of ligand within the cells could be computed. The controls used in the reference cuvette were comprised of cell supernatants incubated without the ligands. Furthermore, Cu^{2+} titrations were performed in both cuvettes.

Preparation and Assay of Ribonucleotide Reductase. Ribonucleotide reductase was partially purified from Novikoff hepatoma by a procedure similar to that previously outlined.[25] The 0–43% ammonium sulfate fraction was used in these experiments. Activity of the enzyme was assayed by monitoring the reductive conversion of CDP to dCDP by the use of a slightly modified assay procedure originally developed by Reichard et al.[24,25] The assay mixture (0.34 mL) contained 3 μCi of [^{3}H]CDP (specific activity 14 or 19 Ci/mmol), 2.2 mM ATP, 5.0 mM $MgCl_2$, 8.8 mM Hepes buffer (pH 7.5), 15 mM dithiothreitol, and enzyme protein between 0.4 and 1.3 mg. Incubation was at 30 °C for 40 min.

The inhibitors were dissolved in water or a water–dimethyl sulfoxide mixture. The maximum concentration of dimethyl sulfoxide in the assay mixture was 2%. At this concentration, dimethyl sulfoxide was not inhibitory. Each inhibitor was tested at a minimum of four concentrations. The ID_{50} (μM) was estimated from graphs summarizing the effects of each compound. ID_{50} is the concentration of drug required to reduce by 50% the observed activity of the partially purified ribonucleotide reductase of Novikoff rat liver tumor.

Acknowledgment. Leon A. Saryan is the recipient of a National Cancer Institute Individual Postdoctoral Fellowship (CA05528), under which a portion of this work was carried out. This research was supported by Grants CA21305 and CA16156 from the National Cancer Institute. The generosity of Frederic French and the National Cancer Institute in supplying the thiosemicarbazones is deeply appreciated.

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