product was collected by filtration, washed (H_2O), and dried in vacuo to give 3.8 g (92% yield) of **7b** as red crystals: mp 144–147°. An analytical sample was obtained by recrystallization from $CHCl_3$–CCl_4 as fine red needles: mp 158–160°. Anal. ($C_{12}H_{10}O_6$) C, H.

2,3-Bis[[(aminocarbonyl)oxy]methyl]naphthazarin (7c). To a gently stirred solution of 7 g of **7b** in 2000 ml of CH_2Cl_2 was added 9.1 g of NaOCN followed by 20 ml of CF_3CO_2H. Stirring was continued for 23 hr at room temperature followed by addition of 50 ml of H_2O. The mixture was stirred for 15 min and the resulting solid collected by filtration. It was washed with CH_2Cl_2 and air-dried to give 3.1 g (33% yield) of **7c** as a red powder. An analytical sample was prepared by reprecipitation from Me_2SO–$CHCl_3$ as a red powder which did not melt up to 300°. Anal. ($C_{14}H_{12}N_2O_8$) C, H, N.

2,3-Bis[[(methylsulfonyl)oxy]methyl]naphthazarin (7d). A mixture of 5.9 g of **6b** and 12.8 g of silver mesylate in 80 ml of CH_3CN was stirred at room temperature for 24 hr. The CH_3CN was removed in vacuo at 40°; the residue was triturated with 100 ml of CH_2Cl_2, filtered, and washed with 2 × 50 ml of CH_2Cl_2. The combined CH_2Cl_2 solution was concentrated to a small volume (25 ml) in vacuo, filtered from some insoluble material, and diluted with 100 ml of C_6H_6 to give 4.9 g (76% yield) of **7d** as a red powder. Depending on the rate of heating, a decomposition point of the **7d** was observed at ca. 150°. An analytical sample was prepared as a red powder by two recrystallizations from C_6H_6–CH_2Cl_2: mp 158–160°. Anal. ($C_{14}H_{14}O_{10}S_2$) C, H.

Naphthazarin Diacetate (8b). To a warm (30–40°) solution of 2.4 g of naphthazarin (**8a**) in 30 ml of Ac_2O was added six drops of concentrated H_2SO_4. The mixture was stirred overnight at room temperature and poured into 300 ml of ice water. The resulting yellow solid was collected by filtration, washed with H_2O (2 × 10 ml), and dried to give 2.4 g (70% yield) of **8b**: mp 193–195°. An analytical sample was recrystallized from H_2O–MeOH: mp 193.5–195° (lit.[17] mp 195°).

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Inhibition of Tumor Cell Transplantability by Iron and Copper Complexes of 5-Substituted 2-Formylpyridine Thiosemicarbazones

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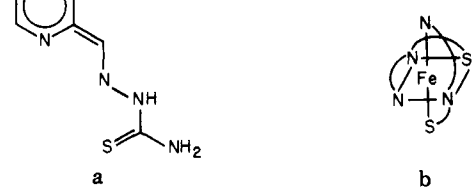
The cytotoxicity of copper and iron complexes of 5-substituted 2-formylpyridine thiosemicarbazones against Ehrlich ascites tumor cells has been measured. Brief in vitro incubation of cells and drugs is followed by implantation into host mice. Subsequent degree of tumor development is a measure of cytotoxicity. A spectrum of activities for the iron complexes is observed, starting with the least active as designated by its 5-substitution: OH < $OCOCH_3$ ~ $N(CH_3)_2$ < H < CH_3 ~ Cl ~ CF_3. The last three complexes can prevent completely tumor growth in the new host. Copper complexes of 5-H and 5-CH_3 also prevent successful tumor cell transplantation.

The possibility that metal complexes of α-*N*-formyl heterocyclic thiosemicarbazones may be cytotoxic to tumor cells has been raised recently in preliminary communications.[1,2] Experimental findings together with relevant background information are reported here in support of this proposal.

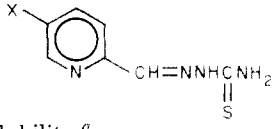
Thiosemicarbazones of 1-formylisoquinoline and 2-formylpyridine and a multitude of their derivatives have been examined for activity against a variety of transplanted animal tumors.[3,4] A number of studies have indicated that these compounds (L) can act at a molecular level by inhibiting ribonucleoside diphosphate reductase, an obligatory enzyme in the pathway of synthesis of precursors of DNA.[5-7] Furthermore, it has been hypothesized that the basis of the inhibition is the binding of iron at the active site of the enzyme (E) in an E·Fe·L complex which prevents catalysis.[8,9] In fact, Ablov and Belichuk have

Chart I. (a) 2-Formylpyridine Thiosemicarbazone and (b) Octahedral Binding of Iron by Ligand[10]



a b

Table I. Cytotoxicity of Metal Complexes of 5-Substituted 2-Formylpyridine Thiosemicarbazone



Compound	5-X	Dose, mg/ml	Solubility,[a] mg/ml	T/C[b]	Tumor incidence[c]	Day[d]	Long-term survivors,[e] days
	-H	1.0[f]		0.74	3/5	20	
		0.5		1.37	7/7	14	
FeL_2^+							
	-H	1.1	4.5	0.55	2/3 (2, 18)	21	
		1.05		0.10	0/5	23	3/5 (45)
		1.05		0.53	5/5	19	
		0.105		0.89	4/4 (1, 3)	17	
	-OH	1.00	0.5	1.16	6/6	14	
		1.00		0.75	4/4 (1, 19)	20	
	$-OCOCH_3$	1.00	0.9	0.60	3/3 (2, 22)	22	
	$-N(CH_3)_2$	1.00	2.5	0.50	3/4 (1, 14)	22	
	-Cl	1.05	1.5	0.15	0/5	23	5/5 (45)
	$-CF_3$	1.10	1.3	0.17	0/5	23	5/5 (75)
	$-CH_3$	1.10	1.9	0.42	1/5	21	2/5 (75)
		1.00		0.12	0/5	23	5/5 (45)
		0.100		0.89	4/5	20	
CuL^+							
	-H	0.75	0.1	0.29	0/5	21	5/5 (75)
		0.80		0.22	0/5	23	5/5 (75)
		0.050		0.13	1/5	22	
	$-CH_3$	0.75	0.1	0.24	0/5	20	
		0.050		0.20	0/4 (1, 19)	20	

[a] Solubility in pH 7.5 Tris with an estimated uncertainty of ±20%. [b] (Δ wt/wk treated)/(Δ wt/wk control). [c] Determined by analysis of weight changes with groups on day of final observation. Deaths of tumor-bearing animals occurred as shown in parentheses (number, day). [d] Day of final observation. [e] Animal groups with tumors were sacrificed at conclusion of period of observation. Others were maintained and analyzed as in footnote *b* for tumor development. [f] Drug delivered to 30-g mouse; 13 mg/kg.

prepared the iron complex of 2-formylpyridine thiosemicarbazone as well as its cobalt and nickel complexes (Chart I).[10] Other workers have reported the x-ray structure of 1-formylisoquinoline thiosemicarbazonatonickel(II).[11] In each of these cases a 1:2 metal to ligand stoichiometry has been found.

Although there have been no detailed studies heretofore to examine the validity of the mechanism of cellular inhibition described above including ligand specificity for iron, there is ample physiological evidence that compounds with the constellation of metal binding groups shown in Chart I (a) markedly disrupt iron metabolism in animals and man, with the probable complexation of significant quantities of iron in vivo.[12-14]

It may be argued, therefore, that it is the iron complex, itself, which is cytotoxic to tumor cells in vivo. This is the case with 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone), which is activated in vivo by Cu^{2+}.[15] However, as will be described elsewhere, the ligand, 2-formylpyridine thiosemicarbazone, also has a large formation constant with Cu^{2+}.[1,16] Hence the possibility is not excluded that this chelating agent may be activated by copper.

Preliminary communications have outlined chemical and biochemical evidence to support the plausibility of formation and stability of iron and copper complexes of 2-formylpyridine thiosemicarbazone in vivo, the centrality of the iron(II) complex in the inhibition of ribonucleoside diphosphate reductase, as well as the antitumor activity of iron, copper, and zinc complexes of 1-formylisoquinoline thiosemicarbazone.[1,2] In the present work the effects of iron and copper complexes of selected 5-substituted 2-formylpyridine thiosemicarbazones upon Ehrlich ascites tumor cell transplantability are examined.

Results and Discussion

As an initial screen for compounds which are toxic to tumor cells after short exposures, a combination of in vitro incubation of test material and tumor cells followed by implantation of cells into the host organism has been used. This procedure provides information on drug-induced tumor cell toxicity which is relatively uncomplicated by attendant host responses to the compounds.

In this study a number of iron and copper complexes of 5-substituted 2-formylpyridine thiosemicarbazones were examined. Table I summarizes the results of several experiments. Average change of weight of treated vs. controls served as a quantitative basis of comparison of cytotoxic effects upon tumor and animal host. Experiments were repeated using many of the compounds with qualitatively similar results. Only in the case of the iron complex of 5-H was there a marked difference of results among the runs.

An ordering of the relative effectiveness of the iron complexes from the least effective at 1 mg/ml in vitro incubation is $OH < N(CH_3)_2 \sim OCOCH_3 < H < Me \sim Cl \sim CF_3$. At this concentration of drug, about 13 mg/kg is delivered to an average 30-g mouse. Neither 5-H nor 5-Me, examined at 0.1 mg/ml, had activity. Strikingly, the 5-OH complex was least active, in contrast to its outstanding in vivo activity against a variety of transplanted tumors including sarcoma-180 ascites tumors.[3,4] In part this may be due to its lower solubility in the aqueous solution used for incubation. However, the correlation fails with other compounds which are considerably more soluble (Table I). The ordering of complexes on this basis from least soluble is $OH < OCOCH_3 < CF_3 \sim Cl < CH_3 < N(CH_3)_2 < H$.

In general, the order of activity of these complexes differs from the patterns seen with their ligands with respect to several different tumor systems.[3,4] For instance, the qualitative order in L1210 leukemia is $H \sim Cl \sim CH_3 < CF_3 \sim OCOCH_3 < N(CH_3)_2 << OH$; in Sarcoma 180

ascites, H < Cl ~ CH_3 < CF_3 < $OCOCH_3$ < $N(CH_3)_2$ << OH; and in Lewis lung carcinoma, CH_3 < H << $N(CH_3)_2$ ~ OH < CF_3 ~ Cl ~ $OCOCH_3$. Since there appears to be no single pattern of reactivity for these different tumor systems, it is not possible at present to comment on the differences seen between ligands and complexes.[3] However, in the Ehrlich ascites system, the 5-OH ligand is very active.[3] Hence the lack of activity observed here for its iron complex suggests that the iron complexes may be distinguished from the parent ligands in their mode of action with this tumor.

The two copper complexes of 5-H and 5-Me are more active on a molar basis than their corresponding iron complexes. In fact, since the solubility of these copper complexes in Eagles medium is only about 0.1 mg/ml, it may be that their activity would extend to this lower total concentration of complex. The marked difference in activity between the 5-H ligand, its iron complex, and its copper complex further suggests that three different cytotoxic species are being observed here.

Of potential importance is the apparent rapidity with which these drugs inactivate tumor cells upon direct interaction. This would be a desirable property for agents used in conjunction with surgery to prevent metastasis from the site of tumor excision.

The 2-formylpyridine thiosemicarbazones and their 1-formylisoquinoline thiosemicarbazone relatives have been the subject of intense investigation as antitumor agents with the implication of their metal-binding capacity in proposed mechanisms of action. The present findings open up new possibilities for the design of active compounds which contain iron or copper.

Experimental Section

Materials. All substituted 2-formylpyridine thiosemicarbazone ligands were gifts of Frederic A. French or the National Cancer Institute and have been previously characterized.

Copper Complexes. As described elsewhere these ligands react rapidly and completely with copper ion in solution to form 1:1 complexes.[1,16] Solid materials are formed by the reaction of 121.6 mg of ligand dissolved in 15 ml of methanol and 189.0 mg of copper acetate monohydrate in 50 ml of methanol. The soluble reactants are mixed and stirred for 48 hr at room temperature. The product copper complex precipitates from solution and is purified by repeated washing in cold methanol. The elemental analysis (Galbraith Laboratories) for 2-formylpyridine thiosemicarbazonatocopper(II) acetate is consistent for a 1:1 complex in the solid state. Anal. Cu, C, H, N, S, O.

Iron Complexes. The titration of ligands with Fe^{3+} or Fe^{2+} is detailed elsewhere and shows that the iron complexes are 1:2 metal to ligand chelates in solution.[1,16] Solids for these studies were prepared by a published method, in which ferrous salts and ligands are allowed to react in hot methanol and recrystallized from this solvent, with care to exclude the presence of water.[10] Using $FeCl_2$ and 2-formylpyridine thiosemicarbazone, the dihydrochloride salt of bis(2-formylpyridine thiosemicarbazonato)iron(II) is formed having the following elemental analysis. Anal. Calcd: Fe, 11.46; C, 34.50; H, 3.31; N, 23.00; Cl, 14.55. Found (Galbraith Laboratories): Fe, 11.01; C, 34.62; H, 3.45; N, 22.72; Cl, 13.11.

Tumor Cell Studies. For the animal experiments, $Fe(NH_4)_2(SO_4)_2$ was used as the ferrous salt to make the iron complexes. The ferrous complex and ferric complexes product have identical uv–visible spectra with those of their chloride counterparts. Solutions of the iron(II) chelates oxidize slowly to the corresponding iron(III) species. After this has occurred, the in vitro incubation of complexes with tumor cells is carried out.

Methods. Incubation of Tumor Cells with Complexes. Ascites fluid from one mouse, usually 8–10 ml, containing about 10^9 cells (total) was spun at low speed to separate cells from the plasma. The cells were the resuspended with a volume of Eagles MEM equal to the Ascites fluid removed.

A given amount of drug (indicated in Table I) was then suspended or dissolved in 2 ml of Eagles MEM and this was then mixed with 2 ml of the cell suspension. The total volume of 4 ml of drug and cell was then incubated at 37° for 1 hr, after which 0.4 ml of this drug–cell mixture was injected into each of five mice per group. The same procedure was followed for each drug and each of several concentrations of the same drug. Thus the 0.4 ml of inoculum contained about 10^7 cells and no less than 10^6 viable cells. In every experiment a control group was used in which the inoculum was 0.4 ml of suspension prepared as described above but where Eagles MEM replaced the drug suspension or solution during the incubation period.

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 weight gain per week of animals with treated cells to that of control animals with untreated cells. Other control animals not injected with cells grow very little over the course of the experiment. The period of observation was approximately 20 days. At the end of this time tumor incidence was noted on the basis of weight change and gross appearance of animals. In cases where no tumor development was evident, the animals were kept for extended periods to see if they were free of the tumor.

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