Anti-Cancer Drugs, 4, pp. 231–240

The cytotoxicity of heterocyclic thiosemicarbazones and their metal complexes on human and murine tissue culture cells

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Heterocyclic thiosemicarbazones, thioureas and 2-substituted pyridine *N*-oxides as well as representative nickel, cobalt and copper complexes were shown to be potent antineoplastic/cytotoxic agents. The cytotoxicity was demonstrated against single cell leukemia as well as cell lines derived from solid tissue (colon adenocarcinoma, HeLa, KB, skin, bronchogenic lung, bone osteosarcoma and glioma). In L1210 cells, DNA synthesis and subsequently RNA synthesis were particularly inhibited by the agents. IMP dehydrogenase activity and thus purine *de novo* synthesis was reduced significantly by the agents. Dihydrofolate reductase, ribonucleoside reductase, nucleoside kinase and DNA polymerase α activities were inhibited by the agents. d(NTP) pool levels were reduced by most of the agents. DNA strand scission was present with all of the derivatives; however, there was no evidence of intercalation, cross linking or alkylation/binding to bases of DNA. This new group of compounds may offer novel exploratory derivatives for future investigations in the treatment of cancer.

Key words: Antineoplastic, antileukemic, Co, Cu, IMP dehydrogenase, metal complexes, Ni, purine inhibitors, thiosemicarbazones.

Introduction

Thiosemicarbazones have previously been tested for antitumor,[1] anti-viral,[2] anti-bacterial,[3] anti-malarial[4] and antileprotic activities.[5] Many of these biological screens have been limited to unsubstituted thiosemicarbazones (Figure 1). Consequently, the metal complexes were also not screened. Recently the inhibition of 2-substituted pyridine and pyrazine heterocylic ^{4}N-substituted (^{4}N-aklyl-, ^{4}N-aryl-, ^{4}N-dialkyl- and 3-azacyclo-) thiosemicarbazones against *Aspergillus niger* and *Paecilomyes variotii* growth was reported.[6–12] Only the thiosemicarbazone derivatives of 2-acetylpyridine with 3-azacyclo or ^{4}*N*-dialkyl substitutions were active against *A. niger* growth. 2-Acetylpyridine ^{4}*N*-alkyl- and ^{4}*N*-arythiosemicarbazones showed very little activity. Thiosemicarbazones derived from 2-formylpyridine and acetylpyrazine with bulky ^{4}*N*-substitutions, e.g. dialkyl or azacyclo, have good inhibitory activity; consequently, thiosemicarbazone-selected metal complexes of stoichiometry $[M(HL)X_2]$,[7,11] $[M(L)X]$[6,8–12] and $[M(L2)]$[8,12] were tested. The nickel(II) complexes demonstrated similar activity as their precursor thiosemicarbazone,[8] but the copper(II) complexes possessed more inhibition against the growth of *A. niger*.[6,11] Many thiosemicarbazones as well as their nickel(II) and copper(II) complexes were active against the growth of *P. variotii*.

Thiosemicarbazones have affected ribonucleotide reductase activity and thus DNA synthesis.[13] The metal complexes of thiosemicarbazones disrupted DNA. [Cu(4DP)Cl] and [Cu(Pzhexim)Cl] were shown to inhibit growth on non-small cell lung cancer HOP 62, colon cancer (COLO205) and leukemia (K562) at 10^{-6} M.[14] Due to these observed inhibitions in growth, we initiated studies to determine if any of these agents blocked growth of murine or human tumor cells.

Materials and methods

Source of compounds

The compounds selected for this study were previously reported; their structures may be found in Figure 1. 2-Acetylpyridine-^{4}N-cyclohexylthiosemicarbazone **1** (HL4CH),[15] 2-acetylpyridine-3-hexamethyliminylthiosemicarbazone **2**

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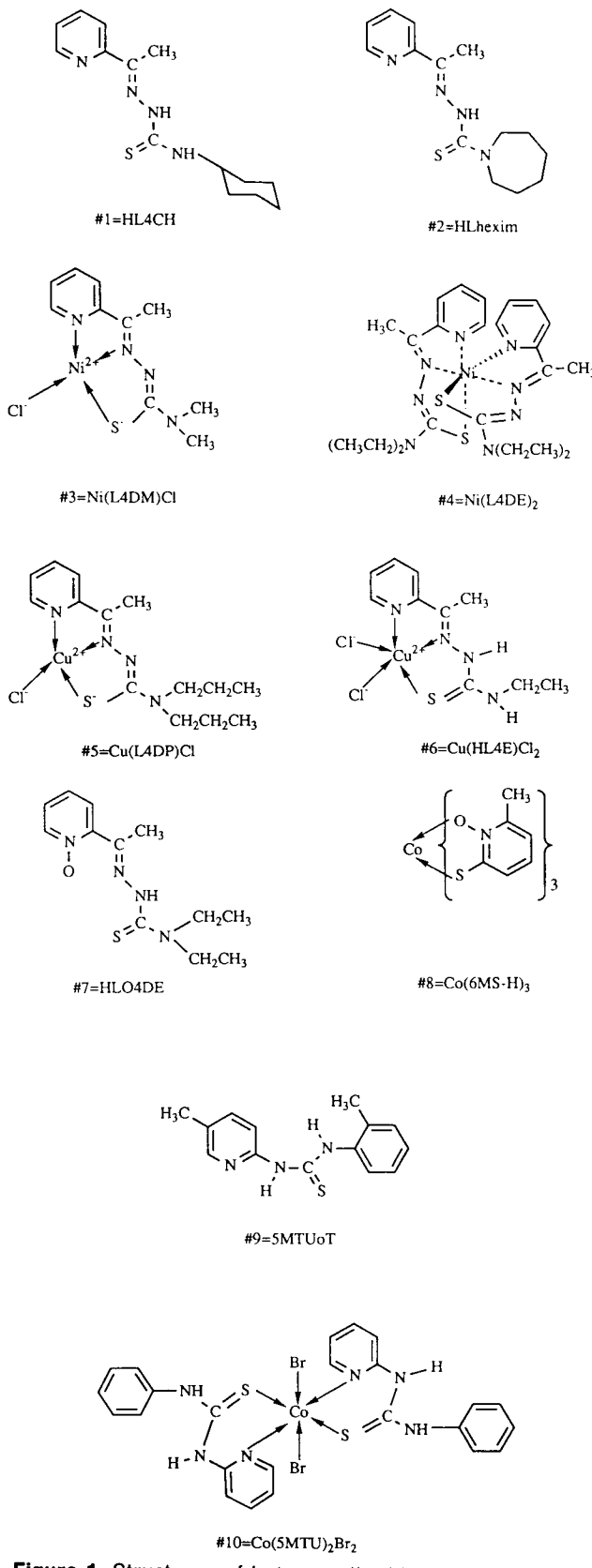

Figure 1. Structures of heterocyclic thiosemicarbazones and their metal complexes.

(HLhexim),[16] a planar diamagnetic nickel(II) complex **3** (Ni[L4DM]Cl),[7] and octahedral nickel(II) complex **4** (Ni[L4DE]$_2$),[8] a planar copper(II) complex **5** (Cu[L4DP]Cl),[6] a 5-coordinate copper-(II) complex **6** (Cu[HL4E]Cl$_2$),[7] 2-acetylpyridine *N* - oxide ^4N - diethylthiosemicarbazone **7** (HLO4DE),[17] an octahedral cobalt(III) complex, tris(2-thiolato-6-picoline *N*-oxide)cobalt(III) **8** (Co[6MS-H]$_3$),[18] *N*-2-(5-picolyl)-*N'*-*o*-tolythiourea **9** (5-MTUoT)[19] and an octahedral picolyl thiourea cobalt(II) complex **10** (Co[5MTU]$_2$Br$_2$).[19]

All radioisotopes were purchased from New England Nuclear (Boston, MA) unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with correction for quenching. Substrates and cofactors for enzyme assays were obtained from Sigma (St Louis, MO).

Pharmacological methods

In vivo *antineoplastic studies.* Compounds **1–10** were tested for *in vivo* antineoplastic activity in the Ehrlich ascites carcinoma screen in CF$_1$ male mice (~28 g) at 8 mg/kg/day, i.p. 6-Mercaptopurine was used as a standard.[20]

Cytotoxic studies. Compounds **1–10** (Table 1) were tested for cytotoxic activity by preparing a 1 mM solution of the drugs in 0.05% Tween 80/H_2O by homogenization. The drug solutions were sterilized by passing them through an Acrodisc (45 μm). The following cell lines were maintained by literature techniques: murine L1210 lymphoid leukemia,[21] P388 lymphocytic leukemia,[21] rat UMR bone osteosarcoma, human Tmolt$_3$ acute lymphoblastic T cell leukemia, colorectal adenocarcinoma SW480,

Table 1. *In vivo* antineoplastic activity at 8 mg/kg/day (i.p.) in CF$_1$ male mice

Compounds ($N = 6$)		Ehrlich ascites carcinoma inhibition of growth (%)	LD_{50} CF$_1$ mice (mg/kg, i.p.)
1	HL4CH	33.3	330
2	HLhexim	88.6	280
3	Ni(L4DM)Cl	67.8	142
4	Ni(L4DE)$_2$	66.9	>500
5	Cu(L4DP)Cl	99.9	21
6	Cu(HL4E)Cl$_2$	81.8	<10
7	HLO4DE	93.6	280
8	Co(6MS-H)$_3$	81.5	>500
9	5MTUoT	87.3	>500
10	Co(5MTU)$_2$Br$_2$	64.7	>500

lung bronchogenic MB-9812, osteosarcoma TE418, KB epidermoid nasopharynx, A431 skin epidermoid carcinoma, HeLa-S^3 suspended and solid cervical carcinoma, and glioma EH 118 MG. The protocol used to assess cytotoxicity was that of Geran *et al.*[21] Standards were determined in each cell line. ED_{50} values for the drug's cytotoxicity were determined by the Trypan blue exclusion technique. The ED_{50} is the drug concentration (μg/ml) which inhibits 50% of the cell growth; a value less than 4 μg/ml was required for significant activity of growth inhibition. Solid tumor cytotoxicity was determined by staining cells using 0.2% crystal violet in methanol.[22] Measurements were made using a Molecular Devices 96 Well Plate Reader at 580 nm.

Incorporation Studies. Incorporation of labeled precursors into [^{3}H]DNA, [^{3}H]RNA and [^{3}H]protein for 10^6 L1210 cells was determined by the method of Liao *et al.*[23] A concentration (10, 25, 50 and 100 μM) response for inhibition of DNA, RNA and protein synthesis was determined with compounds **1**, **3**, **4**, **9** and **10** at 60 min. [^{14}C]Glycine (53.0 mCi/mmol) incorporation into purines was determined by the method of Cadman *et al.*[24] [^{14}C]Formate (53.0 mCi/mmol) incorporation into pyrimidines was determined by the method of Christopherson *et al.*[25]

Enzyme assay studies. Inhibition of various enzyme activities was carried out by first preparing the appropriate L1210 cell homogenate or subcellular fraction, then adding the drug to be tested during the enzyme assay. For the concentration response studies, the inhibition of enzyme activity was determined at 10, 25, 50 and 100 μM of compounds **1**, **3**, **4**, **9** and **10** for 60 min incubations. DNA polymerase α activity was determined in a cytoplasmic extract isolated by the method of Eichler *et al.*[26] Nuclear DNA polymerase β was determined by isolating nuclei.[27] The polymerase assay for both α and β was that of Sawada *et al.*[28] with [^{3}H]TTP. Messenger-, ribosomal- and transfer-RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate and the individual RNA polymerase activities were determined using [^{3}H]UTP.[29,30] Ribonucleoside reductase activity was measured with [^{14}C]CDP with and without dithioerythritol.[31] The deoxyribonucleotides [^{14}C]dCDP were separated from the ribonucleotides by thin layer chromatography (TLC) on PEI plates. Thymidine, TMP and TDP kinase activities were determined using [^{3}H]thymidine (58.3 mCi/mmol) and the medium of Maley and Ochoa.[32] Carbamyl phosphate synthetase activity was determined by the method of Kalman *et al.*[33] and citrulline was determined colorimetrically.[34] Asparate transcarbamylase activity was determined by the method of Kalman *et al.*[33] and carbamyl aspartate was determined colorimetrically.[35] OMP decarboxylase activity was determined using orotidine-5-monophosphate [carboxyl-^{14}C] (34.9 μCi/mmol) by the method of Appel.[36] Thymidylate synthetase activity was analyzed by the method of Kampf *et al.*[37] The ^3H_2O measured was proportional to the amount of TMP formed from [^{3}H]dUMP. Dihydrofolate reductase activity was determined by the spectrophotometric method of Ho *et al.*[38] PRPP amidotransferase activity was determined by the method of Spassova *et al.*[39] and IMP dehydrogenase activity was determined with 8-[^{14}C]IMP (54 mCi/ mmol) (Amersham, Arlington Heights, IL) where XMP was separated on PEI plates (Fisher Scientific) by TLC.[40] Protein was determined for all of the enzymatic assays by the Lowry *et al.* technique.[41]

Deoxyribonucleoside triphosphates were extracted by the method of Bagnara and Finch.[42] Deoxyribonucleoside triphosphates were determined by the method of Hunting and Henderson[43] with calf thymus DNA, *Escherichia coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 μCi of [^{3}H-methyl]dTTP or [5-^{3}H]dCTP.

Effects of the compounds on DNA strand scission were determined by the methods of Suzuki *et al.*,[44] Pera *et al.*[45] and Woynarowski *et al.*[46] L1210 lymphoid leukemia cells were incubated with 10 μCi [methyl-^{3}H]thymidine, 84.0 Ci/mmol for 24 h at 37°C. After the L1210 cells (10^7) were harvested, they were centrifuged at 600 *g* for 10 min in phosphate buffered saline (PBS), washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml: 0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100 and 2.5% sucrose) was layered onto a 5-20% alkaline-sucrose gradient (5 ml: 0.3 M NaOH, 0.7 KCl and 0.01 M EDTA) followed by 0.2 ml cell preparation. After incubation for 2.5 h at room temperature, the gradient was centrifuged at 17 000 r.p.m. at 20°C for 17 h (Beckman rotor SW60). Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3N HCl and measured for radioactivity. Thermal calf thymus DNA denaturation studies and DNA viscosity studies were conducted after incubation of

Table 2. Cytotoxicity of compounds in rodent and human tissue culture lines (ED_{50} = μg/ml)

Compound	Rodent			Human								
	murine lymphoid L1210	murine T leukemia $Tmolt_3$	rat UMR-106	colon adenocarcinoma	HCT-8 ileum mucosa	uterine HeLa-S^3	HeLa solid	KB nasopharynx	skin A431	bronchogenic lung	bone osteosarcoma	glioma brain
1	2.97	4.27	2.31	0.89	3.29	3.06	1.72	1.04	2.60	1.78	1.36	1.20
2	2.42	2.63	2.54	0.40	0.30	2.04	2.09	0.81	1.61	1.98	1.35	0.77
3	2.59	2.27	0.14	0.06	0.28	2.28	0.14	0.24	0.26	1.96	1.64	0.30
4	2.59	3.66	2.32	1.05	0.30	2.74	2.02	1.36	1.56	1.92	0.64	0.68
5	1.76	2.74	0.29	0.41	0.30	2.58	0.05	0.25	0.01	1.47	0.72	0.81
6	2.44	2.61	0.43	0.25	0.38	2.59	0.06	0.06	0.01	0.64	0.82	1.58
7	1.30	1.83	2.61	1.15	0.38	2.67	1.80	1.01	1.92	0.81	0.53	1.03
8	3.08	2.35	2.41	0.75	0.47	2.04	2.69	1.34	6.23	1.28	1.29	2.10
9	2.45	2.22	3.89	2.19	3.12	2.67	3.33	0.59	0.01	1.44	3.39	2.06
10	2.30	3.05	2.05	4.18	6.44	2.43	7.73	4.82	0.97	7.62	5.00	6.56
5-FU	1.41	2.14	—	3.09	—	2.47	—	1.25	—	5.69	—	1.28
ARA C	2.76	2.67	—	3.42	—	2.13	—	2.84	—	4.60	—	1.88
Hydroxyurea	2.67	3.18	—	4.74	—	1.96	—	5.29	—	7.37	7.57	2.27

compounds **1**, **3**, **4**, **9** and **10** at 100 μM at 37°C for 24 h.[46]

Statistics

The mean and standard deviation are designated by '$\bar{X} \pm SD$'; the number of animals per group by 'N'. The probable differences between test and control samples were determined by the Student's *t*-test with raw data.

Results

Antineoplastic *in vivo* activity

In vivo Ehrlich ascites carcinoma growth was inhibited significantly by HLhexim **2**, Cu(L4DP)Cl **5**, $Cu(HL4E)Cl_2$ **6**, HLO4DE **7**, $Co(6MS\text{-}H)_3$ **8** and 5MTUoT **9** with greater than 80% inhibition at 8 mg/kg/day, i.p. (Table 1). The LD_{50} values varied over a wide range, but the nickel and cobalt complexes did appear to be safe agents. The two copper complexes **5** and **6** possess relatively low LD_{50} values.

Cytotoxicity

All compounds **1–10** were active against the growth of murine L1210 lymphoid leukemic cells with very little difference in the ED_{50} values, i.e. 1.76–3.08 μg/ml (Table 2). Rat UMR-106 osteosarcoma growth was inhibited by all of the compounds. Compounds **3**, **5** and **6** afforded ED_{50} values of less than 1 μg/ml. In human tumor cell cultures, $Tmolt_3$ T cell leukemia growth was inhibited significantly by all of the compounds except **1**, HL4CH. HLO4DE **7** afforded the best activity yielding an ED_{50} value of 1.83 μg/ml. In the colon adenocarcinoma screen a number of the derivatives demonstrated potent activity, e.g. **1**, **2**, **3**, **5**, **6** and **8** all afforded ED_{50} values of less than 1 μg/ml. HCT-8 ileum mucosa growth was inhibited significantly by compounds **2–8** whereas compound **10** was inactive. HeLa-S^3 suspended uterine carcinoma growth was inhibited by all of the compounds significantly. HeLa solid uterine carcinoma growth was inhibited significantly by **3**, **5** and **6**, with ED_{50} values less than 1 μg ml. Compound **10** was inactive. KB nasopharyngeal carcinoma growth was significantly inhibited by all of the compounds except $Co(5MTU)_2Br_2$ **10**. $Cu(HL4E)Cl_2$ **6** was especially potent with an ED_{50} of 0.06 μg/ml. Epidermoid skin A431 growth was inhibited by all of the compounds except **8**. Compounds **3**, **5**, **6** and **9** demonstrated particularly good activity. Bronchogenic lung carcinoma was inhibited by all compounds except $Co(5MTU)_2Br_2$ **10**. $Cu(HL4E)Cl_2$ **6** and HLO4DE **7** were particularly active with ED_{50} values less than 1 μg/ml. Human bone osteosarcoma growth was significantly inhibited by **4–7**, with ED_{50} values less than 1 μg/ml. $Co(5MTU)_2Br_2$ **10** was not active in the bone osteosarcoma screen. Human brain glioma growth was significantly inhibited by HLhexim **2**, both nickel complexes **3** and **4**, and Cu(L4DP)Cl **5** with ED_{50} values less than 1 μg/ml. Compound **10** was inactive against glioma growth.

Mode of action

Selected compounds were tested for their mode of action in the L1210 lymphoid leukemia cell model (Tables 3–5). HL4CH **1**, Ni(L4DM)Cl **3**, and $Co(5MTU)_2Br_2$ **10** caused a concentration dependent reduction of DNA synthesis from 25 to 100 μM, with greater than 80% reduction at 100 μM. $Ni(L4DE)_2$ **4** caused only 20% reduction of DNA synthesis at 100 μM and 5MTUoT **9** only 58% at 100 μM. RNA synthesis in L1210 cells was inhibited to a lesser degree by all five compounds. HL4CH **1** and the nickel complexes **3** and **4** caused greater than 50% inhibition at 100 μM. 5MTUoT **9** and $Co(5MTu)_2Br_2$ **10** were potent L1210 protein synthesis inhibitors with greater than 85% reduction at 100 μM. Whereas HL4CH **1** and HLO4DE **7** were not as potent in the inhibition of protein synthesis, $Ni(L4DE)_2$ **4** actually stimulated protein synthesis 3-fold at 100 μM.

DNA polymerase α activity was inhibited 36–41% at 100 μM by all the compounds except 5MTUoT **9** which stimulated DNA polymerase α activity by 78%. mRNA polymerase activity was inhibited 16–42% by the compounds at 100 μM with HL4CH **1** and Ni(L4DM)Cl **3** being most active and 5MTUoT **9** and $Co(5MTU)_2Br_2$ **10** being the least active. rRNA polymerase activity was not significantly affected by the compounds, but tRNA polymerase activity was stimulated by the nickel (**3** and **4**) and cobalt complexes (**9** and **10**). Ribonucleoside reductase activity was inhibited by 50–89% at 100 μM with $Co(5MTu)_2Br_2$ **10** affording the greatest inhibition.

Purine biosynthesis as [^{14}C]glycine incorporation was reduced 55–77% with $Ni(L4DE)_2$ **4** having

Table 3. The effects of thiosemicarbazone metal complexes on L1210 cell metabolism after 60 min incubation

Assay ($N = 6$)	Percent of control ($\bar{X} \pm$ SD)						
		compound **1**			compound **3**		
	Control	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	14 ± 3*	12 ± 2*	12 ± 2*	14 ± 3	12 ± 2*	12 ± 2*
RNA synthesis	100 ± 6[b]	50 ± 3*	48 ± 4*	46 ± 5*	50 ± 5	48 ± 4*	46 ± 5*
Protein synthesis	100 ± 4[c]	78 ± 3*	67 ± 5*	63 ± 6*	78 ± 6*	67 ± 6*	63 ± 4*
DNA polymerase α	100 ± 5[d]	70 ± 3*	63 ± 4*	59 ± 5*	92 ± 7	74 ± 5*	64 ± 5*
mRNA polymerase	100 ± 6[e]	79 ± 4*	70 ± 3*	58 ± 4*	88 ± 6	74 ± 5*	68 ± 6*
rRNA polymerase	100 ± 5[f]	118 ± 7	105 ± 6	89 ± 5	117 ± 7	158 ± 9*	103 ± 6
tRNA polymerase	100 ± 8[g]	93 ± 7	103 ± 8	105 ± 6	113 ± 6	127 ± 8	142 ± 7*
Ribonucleoside reductase	100 ± 6[h]	112 ± 5	76 ± 6*	71 ± 7*	79 ± 5*	55 ± 6*	50 ± 4*
Purine *de novo* synthesis	100 ± 7[i]	58 ± 6*	53 ± 5*	45 ± 6*	60 ± 5*	46 ± 7*	45 ± 4*
PRPP amido transferase	100 ± 8[j]	72 ± 7*	130 ± 6	152 ± 7*	86 ± 7	90 ± 6	102 ± 8
IMP dehydrogenase	100 ± 5[k]	64 ± 5*	50 ± 4*	46 ± 4*	75 ± 5*	66 ± 6*	42 ± 4*
Pyrimidines *de novo* synthesis	100 ± 7[l]	138 ± 8	155 ± 7	139 ± 9	137 ± 6	143 ± 5*	105 ± 6
Carbamyl phosphate synthetase	100 ± 7[m]	144 ± 6*	101 ± 8	89 ± 7	93 ± 6	89 ± 7	79 ± 5*
Aspartate transcarboxylase	100 ± 6[n]	113 ± 7	102 ± 9	70 ± 5*	109 ± 7	110 ± 8	72 ± 6*
OMP decarboxylase	100 ± 7[o]	108 ± 9	106 ± 5	108 ± 7	93 ± 8	130 ± 7*	173 ± 6*
Thymidine kinase	100 ± 6[p]	50 ± 5*	40 ± 5*	37 ± 3*	63 ± 6*	55 ± 6*	36 ± 5*
TMP kinase	100 ± 5[q]	77 ± 5*	65 ± 6*	63 ± 4*	18 ± 3*	15 ± 2*	11 ± 3*
TDP kinase	100 ± 5[r]	91 ± 6	87 ± 7	86 ± 6	103 ± 6	98 ± 7	45 ± 4*
Thymidylate synthetase	100 ± 6[s]	79 ± 5*	77 ± 6	59 ± 7*	98 ± 6	90 ± 7	85 ± 6
Dihydrofolate reductase	100 ± 4[t]	105 ± 6	85 ± 6	73 ± 4	84 ± 8	69 ± 7*	37 ± 5*
d(ATP)	100 ± 6[u]			52 ± 5*			91 ± 9
d(GTP)	100 ± 6[v]			24 ± 3*			141 ± 6*
d(CTP)	100 ± 7[w]			74 ± 6*			142 ± 8*
d(TTP)	100 ± 5[x]			79 ± 6*			75 ± 6*

Control values for 10^6 cells/h: [a]26152 d.p.m., [b]4851 d.p.m., [c]7164 d.p.m., [d]47804 d.p.m., [e]1502 d.p.m., [f]4239 d.p.m., [g]6400 d.p.m., [h]2744 d.p.m., [i]92551 d.p.m., [j]0.121 ΔOD 340/h/mg protein, [k]76058 d.p.m., [l]13680 d.p.m., [m]0.392 mol citrulline, [n]1.064 mol, [o]44743 d.p.m., [p]0.867 ΔOD 340/h/mg protein, [q]0.625 ΔOD 340/h/mg protein, [r]0.121 ΔOD 340/h/mg protein, [s]18463 d.p.m., [t]0.868 ΔOD units/h/mg protein, [u]6.17 pmol, [v]5.27 pmol, [w]6.87 pmol, [x]6.94 pmol.

the most inhibition at 100 μM. PRPP amidotransferase activity was not inhibited by HL4CH **1** and Ni(L4DM)Cl **3** but Ni(L4DE)$_2$ **4**, 5MTUoT **9** and Co(5MTU)$_2$Br$_2$ **10** were very potent. IMP dehydrogenase activity was reduced 42–62% by the compounds in a concentration dependent manner. Pyrimidine synthesis as [^{14}C]formate incorporation was stimulated with the compounds except Co(5MTU)$_2$Br$_2$ **10** which caused a 45% reduction at 100 μM. Carbamyl phosphate synthetase activity was only affected marginally (0–21%) by the compounds. Aspartate transcarbamylase activity was not affected significantly by HL4CH **1**, Ni(L4DM)Cl **3** and Ni(L4DE)$_2$ **4**. 5MTUoT **9** and Co(5MTU)$_2$Br$_2$ **10** caused greater than 50% reduction of the transcarbamylase activity at 100 μM. OMP decarboxylase activity was suppressed only by [Co(5MTU)$_2$Br$_2$] **10**. Thymidine kinase activity was inhibited by HL4CH **1** and Ni(L4DM)Cl **3** at 100 μM. TMP kinase activity was inhibited significantly at 37–83% by the compounds. TDP kinase activity was inhibited greater than 50% at 100 μM by Ni(L4DM)Cl **3**, 5MTUoT **9** and Co(5MTU)$_2$Br$_2$ **10**. Thymidylate synthetase activity was inhibited by HL4CH **1** by 45%. The remaining compounds were not as active against the synthetase. Dihydrofolate reductase activity was reduced 37–63% by the compounds.

L1210 d(NTP) pools were affected by the compounds. dTTP pools were reduced significantly 21–42% except by Ni(L4DE)$_2$ **4**, which was reduced only 10%. dATP and dGTP pool levels were reduced by HL4CH **1**. dGTP pool levels were elevated by **3** but reduced by the other compounds; dCTP pools were elevated by **3**, **4** and **10**, and reduced by **1** and **9**.

When the compounds were reacted with cDNA there were no observable changes in the UV absorption at 260 nm, and cDNA's melting point (T_m value) was not altered by the compounds. The viscosity of cDNA was decreased by all of the compounds, e.g. Control: 271 s, **1**: 233 s, **3**: 240 s,

Table 4. The effects of thiosemicarbazone metal complexes on L1210 cell metabolism after 60 min incubation

Assay ($N = 6$)	Percent of control ($\bar{X} \pm$ SD)						
		compound **4**			compound **9**		
	Control	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	150 ± 6*	112 ± 7*	80 ± 5*	144 ± 7*	104 ± 6	42 ± 4*
RNA synthesis	100 ± 6[b]	94 ± 7*	82 ± 5*	75 ± 5*	79 ± 6*	68 ± 6*	57 ± 5*
Protein synthesis	100 ± 4[c]	125 ± 8	216 ± 4*	394 ± 7*	15 ± 3	12 ± 3	11 ± 2*
DNA polymerase α	100 ± 5[d]	112 ± 7	67 ± 6*	60 ± 5*	138 ± 6	134 ± 7	178 ± 7
mRNA polymerase	100 ± 6[e]	140 ± 8	87 ± 7	70 ± 6*	126 ± 7	113 ± 7	82 ± 6
rRNA polymerase	100 ± 5[f]	119 ± 7	97 ± 5	88 ± 8	98 ± 5	96 ± 6	95 ± 6
tRNA polymerase	100 ± 8[g]	123 ± 5	161 ± 6*	163 ± 7*	155 ± 8	134 ± 8	79 ± 5*
Ribonucleoside reductase	100 ± 6[h]	105 ± 6	93 ± 5	80 ± 6	80 ± 7	58 ± 5*	54 ± 6*
Purine *de novo* synthesis	100 ± 7[i]	74 ± 6*	44 ± 5*	23 ± 4*	55 ± 5*	44 ± 5*	30 ± 3*
PRPP amido transferase	100 ± 8[j]	66 ± 5*	56 ± 7*	32 ± 4*	58 ± 6*	32 ± 4*	17 ± 2*
IMP dehydrogenase	100 ± 5[k]	66 ± 8*	59 ± 3	58 ± 5*	56 ± 4*	56 ± 4*	38 ± 2*
Pyrimidines *de novo* synthesis	100 ± 7[l]	118 ± 8	96 ± 9	103 ± 4	164 ± 7*	137 ± 6*	117 ± 5
Carbamyl phosphate synthetase	100 ± 7[m]	86 ± 5	85 ± 6	84 ± 7	85 ± 6	82 ± 7	80 ± 8
Aspartate transcarboxylase	100 ± 6[n]	98 ± 8	93 ± 7	91 ± 5	72 ± 8*	72 ± 6*	48 ± 5*
OMP decarboxylase	100 ± 7[o]	120 ± 9	179 ± 8*	122 ± 7*	115 ± 6	119 ± 7	134 ± 8*
Thymidine kinase	100 ± 6[p]	112 ± 6	103 ± 5	84 ± 6	137 ± 7	104 ± 5	83 ± 6
TMP kinase	100 ± 5[q]	54 ± 4*	25 ± 3*	17 ± 8*	66 ± 5*	64 ± 6*	51 ± 5*
TDP kinase	100 ± 5[r]	75 ± 6*	69 ± 5*	64 ± 7*	59 ± 7*	56 ± 7*	48 ± 5*
Thymidylate synthetase	100 ± 6[s]	126 ± 8	104 ± 7	90 ± 8	132 ± 6	118 ± 8	101 ± 7
Dihydrofolate reductase	100 ± 4[t]	85 ± 6	75 ± 5*	59 ± 6*	67 ± 5*	50 ± 5*	45 ± 6*
d(ATP)	100 ± 6[u]			87 ± 7			127 ± 8
d(GTP)	100 ± 6[v]			67 ± 6*			30 ± 4*
d(CTP)	100 ± 7[w]			105 ± 8			49 ± 5*
d(TTP)	100 ± 5[x]			90 ± 7			63 ± 5*

Control values for 10^6 cells/h: [a]26152 d.p.m., [b]4851 d.p.m., [c]7164 d.p.m., [d]47804 d.p.m., [e]1502 d.p.m., [f]4239 d.p.m., [g]6400 d.p.m., [h]2744 d.p.m., [i]92551 d.p.m., [j]0.121 OD 340/h/mg protein, [k]76058 d.p.m., [l]13680 d.p.m., [m]0.392 mol citrulline, [n]1.064 mol, [o]44743 d.p.m., [p]0.867 OD 340/h/mg protein, [q]0.625 OD 340/h/mg protein, [r]0.121 OD 340/h/mg protein, [s]18463 d.p.m., [t]0.868 OD units/h/mg protein, [u]6.17 pmol, [v]5.27 pmol, [w]6.87 pmol, [x]6.94 pmol.

4: 230 s, **9**: 231 s, **10**: 229 s. When DNA strand scission of L1210 was examined 24 h after incubation at 100 μM all of the compounds except $Co(5MTU)_2Br_2$ **10** caused massive DNA fragmentation with smaller weight DNA appearing lower in the gradient (Figures 2–6).

Discussion

These organic compounds and their nickel, copper and cobalt complexes demonstrated potent activity, particularly against solid tumors, e.g. lung bronchogenic, bone osteosarcoma, glioma and KB nasopharynx. This activity was also demonstrated *in vivo* in the Ehrlich ascites carcinoma screen. The L1210 lymphoid leukemia tissue culture model was selected because of its well-known growth properties. The major site of action of these derivatives was DNA synthesis. The purine pathway appeared to be the major metabolic pathway which was reduced by the agents. The regulatory site at IMP dehydrogenase appeared to be reduced by a magnitude which is consistent with the drug's ability to inhibit *de novo* synthesis of purines. The activities of nucleoside kinases were another area where the derivatives afforded significant inhibition. This inhibition of the kinase activity was of a magnitude to account for observed inhibition of DNA synthesis in L1210 cells. The inhibition of the thymidine kinase activities was also reflected in the observed lowered d(TTP) pools, necessary for incorporation into DNA. Dihydrofolate reductase and ribonucleoside reductase activities were moderately inhibited by the derivatives. The effects of inhibiting these two enzyme sites necessary for DNA synthesis would certainly be additive to the overall inhibition of DNA synthesis. The other d(NTP) pools were not lowered in a consistent manner and in fact they were elevated in certain cases. This may be due in part to the fact that DNA polymerase α activity was inhibited by the agents which would lead to the accumulation within the cells of d(NTP)s not incorporated into

Table 5. The effects of thiosemicarbazone metal complexes on L1210 cell metabolism after 60 min incubations

Assay ($N = 6$)	Control	Percent of control ($\bar{X} \pm$ SD)		
		compound **10**		
		25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	101 ± 6	26 ± 4*	16 ± 3*
RNA synthesis	100 ± 6[b]	101 ± 7	100 ± 7	79 ± 5*
Protein synthesis	100 ± 4[c]	22 ± 4*	13 ± 2*	9 ± 3*
DNA polymerase α	100 ± 5[d]	228 ± 8*	66 ± 7*	65 ± 4*
mRNA polymerase	100 ± 6[e]	93 ± 7	90 ± 6	84 ± 7
rRNA polymerase	100 ± 5[f]	118 ± 5	110 ± 6	107 ± 8
tRNA polymerase	100 ± 8[g]	84 ± 6	155 ± 7*	204 ± 8*
Ribonucleoside reductase	100 ± 6[h]	64 ± 3*	34 ± 4*	11 ± 4*
Purine *de novo* synthesis	100 ± 7[i]	81 ± 7*	78 ± 5*	38 ± 7*
PRPP amido transferase	100 ± 8[j]	45 ± 5*	31 ± 3*	15 ± 4*
IMP dehydrogenase	100 ± 5[k]	82 ± 6*	76 ± 4*	58 ± 5*
Pyrimidines *de novo* synthesis	100 ± 7[l]	134 ± 8	104 ± 7	55 ± 5*
Carbamyl phosphate synthetase	100 ± 7[m]	83 ± 7	79 ± 6*	79 ± 6*
Aspartate transcarboxylase	100 ± 6[n]	59 ± 6*	55 ± 5*	35 ± 3*
OMP decarboxylase	100 ± 7[o]	71 ± 5*	60 ± 4*	60 ± 3*
Thymidine kinase	100 ± 6[p]	110 ± 8	108 ± 7*	105 ± 7
TMP kinase	100 ± 5[q]	55 ± 4*	52 ± 5*	22 ± 5
TDP kinase	100 ± 5[r]	60 ± 5*	43 ± 6*	42 ± 4*
Thymidylate synthetase	100 ± 6[s]	101 ± 6	81 ± 7	80 ± 6*
Dihydrofolate reductase	100 ± 4[t]	50 ± 3*	46 ± 5*	37 ± 3*
d(ATP)	100 ± 6[u]			107 ± 6
d(GTP)	100 ± 6[v]			17 ± 4*
d(CTP)	100 ± 7[w]			120 ± 7
d(TTP)	100 ± 5[x]			58 ± 6*

Control values for 10^6 cells/h: [a]26152 d.p.m., [b]4851 d.p.m., [c]7164 d.p.m., [d]47804 d.p.m., [e]1502 d.p.m., [f]4239 d.p.m., [g]6400 d.p.m., [h]2744 d.p.m., [i]92551 d.p.m., [j]0.121 OD 340/h/mg protein, [k]76058 d.p.m., [l]13680 d.p.m., [m]0.392 mol citrulline, [n]1.064 mol, [o]44743 d.p.m., [p]0.867 OD 340/h/mg protein, [q]0.625 OD 340/h/mg protein, [r]0.121 OD 340/h/mg protein, [s]18463 d.p.m., [t]0.868 OD units/h/mg protein, [u]6.17 pmol, [v]5.27 pmol, [w]6.87 pmol, [x]6.94 pmol.

DNA. Inhibition of mRNA polymerase activity by the drugs would account in part for the observed reduction of RNA synthesis along with the inhibition by the agents of purine synthesis. There was no direct indication that the agents intercalated or specifically bound to the bases of cDNA. However, that cDNA viscosity was reduced after incubation with drug and that L1210 DNA strand scission occurred after drug inhibition suggested that the agents caused DNA fragmentation. This would explain the lower DNA synthesis and subsequent cytotoxicity of the agents.

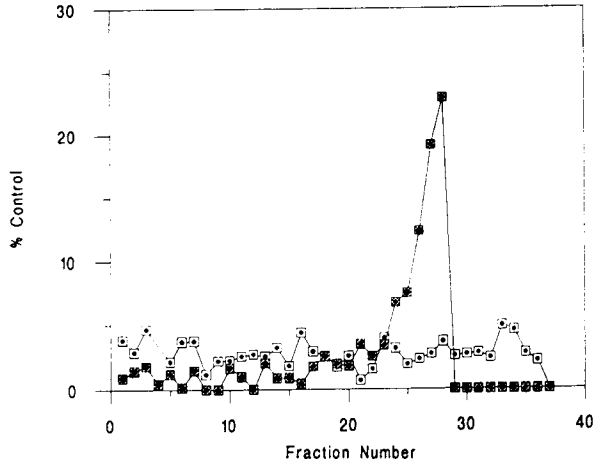

Figure 2. DNA strand scission: ◘, drug **1**; ▩, control.

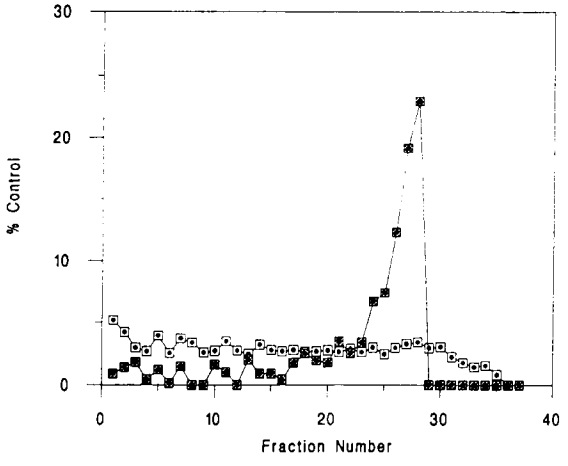

Figure 3. DNA strand scission: ◘, drug **3**; ▩, control.

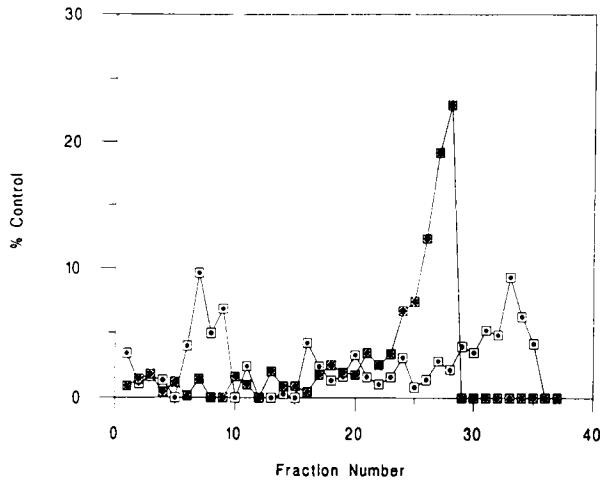

Figure 4. DNA strand scission: ▣, drug **4**; ▩, control.

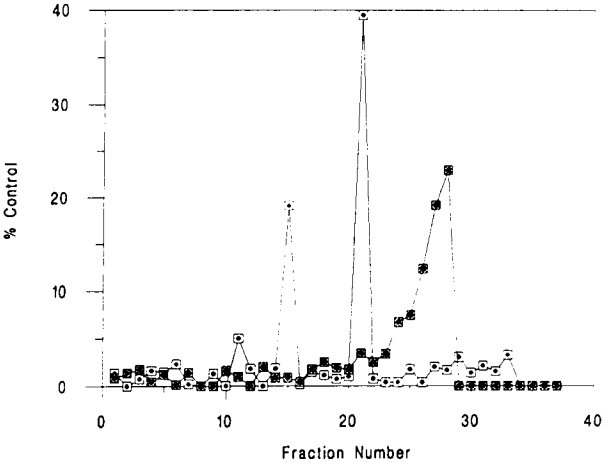

Figure 5. DNA strand scission: ▣, drug **9**; ▩, control.

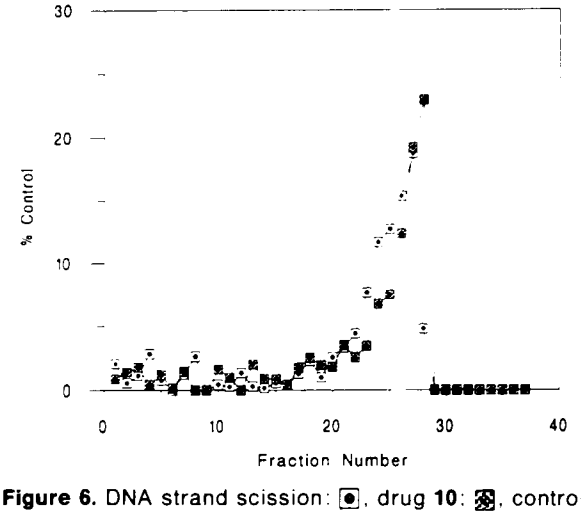

Figure 6. DNA strand scission: ▣, drug **10**; ▩, control.

There does not appear to be a single mode of action of these thiosemicarbazone derivatives, but the effects on cell metabolism appeared to be additive in bringing about cell death and thus antineoplastic activity. The nickel, copper and cobalt metal complexes did not appear to be a necessary requirement for cytotoxicity or metabolic inhibition. On the other hand, slightly different metals and/or chemical moieties would allow the molecules to bind differently to the catalytic portion of given enzymes and thus result in slightly different magnitudes of inhibition of enzyme activities.

Acknowledgment

The contributions of Mr John Klaric in manuscript preparation are much appreciated.

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(Received 4 January 1993; accepted 2 February 1993)