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Cytotoxicity of Cobalt Complexes of Furan Oximes in Murine and Human Tissue-cultured Cell Lines

Iris H. Hall,[1]* Ken F. Bastow,[1] Amy E. Warren,[1] Cheryl R. Barnes[1] and Gilles M. Bouet[2]

[1]Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27559-7360, USA

[2]Laboratoire de Chimie de Coordination, Faculté de Pharmacie, 16 Boulevard Daviers, F-49100, Angers, France

The copper complexes of 2-furaldehyde and furan oximes have previously demonstrated potent cytotoxicity, L1210 DNA synthesis inhibition, DNA topoisomerase II inhibition and DNA fragmentation. Currently a series of cobalt metal complexes of 2-furaldehyde oximes were compared with copper complexes of furan oximes to determine whether the type of metal is important to the cytotoxicity and mode of action of the complexes. The cobalt complexes of furan oximes, like the copper complexes, were shown to be cytotoxic to suspended tumor cell lines, e.g. leukemias, lymphomas, acute monocytic leukemia and HeLa-S^3 uterine carcinoma. The cobalt complexes did not demonstrate dramatic cytotoxicity against the growth of tumors derived from solid human tumor lines. The cobalt complexes preferentially inhibited L1210 DNA synthesis, followed by inhibition of RNA and protein synthesis from 25 to 100 μM over 60 min. These agents, like the copper complexes of 2-furaldehyde and furan oximes, were inhibitors of DNA polymerase α activity and *de novo* purine synthesis with marginal inhibition of ribonucleoside reductase and dihydrofolate reductase activities with DNA fragmentation. Unlike the copper complexes, the cobalt complexes did not inhibit L1210 DNA topoisomerase II activity but did reduce thymidylate synthetase activity. Thus, varying the type of metal within the complexes of 2-furaldehyde and furan oximes produces differences in both cytotoxicity and mode of action.

Keywords: cobalt; cytotoxicity; complexes; 2-furaldehyde; furan oximes; DNA inhibition; fragmentation

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INTRODUCTION

Based on the cytotoxicity of other copper, cobalt and iron metal complexes of trimethylamine carboxyboranes[1,2] and copper and nickel complexes of heterocyclic thiosemicarbazones,[3,4] we undertook a study of the metal complexes of 2-furaldehyde and furan oximes to determine their ability to retard tumor cell growth in tissue-culture cells. The copper complexes of 2-furaldehyde and furan oxime derivatives were found to be potent cytotoxic agents in both murine and human suspended tissue-cultured cell lines as well as those derived from solid tumors.[5,6] Mode-of-action studies in murine L1210 lymphoid leukemia cells showed that the compounds suppressed DNA, RNA and protein syntheses after 60 min at 100 μM in a concentration-dependent manner.

Inhibition of purine and pyrimidine *de novo* syntheses, as well as inhibition of ribonucleoside reductase and nucleoside kinase activities with DNA strand scission, occurred with the agents.[5,6] All of these effects of the drug are probably additive in their ability to cause cell death. Most important was the inhibition of DNA topoisomerase II activity, which causes apoptosis.[5,6] IC_{50} values of these agents were 1.75–24.9 μM, lower than those

* Correspondence to: Iris H. Hall, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27559-7360, USA.

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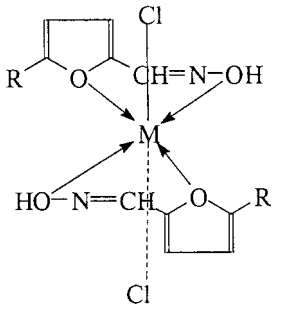
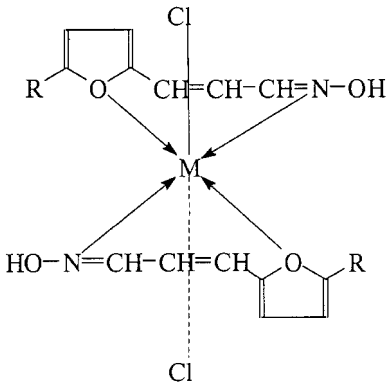


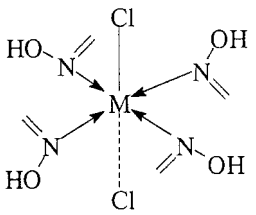
ML_2Cl_2 complexes (L = M5FDH or E5FDH)

1: M = Co , L = M5FDH



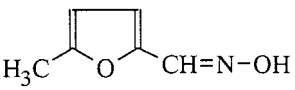
ML_2Cl_2 complexes (L = FAOH or M5FAOH)

4: M = Co , L = FAOH
6: M = Co , L = M5FAOH

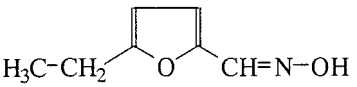


ML_4Cl_2 complexes (all ligands)

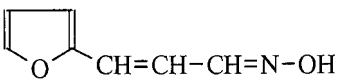
2: M = Co , L = M5FDH
3: M = Co , L = E5FDH
5: M = Co , L = FAOH



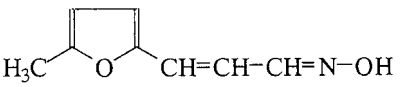
5-methyl-2-furaldehyde oxime (M5FDH)



5-ethyl-2-furaldehyde (E5FDH)



3-(2-furyl)prop-2-ene aldoxime (FAOH)



3-(5-methyl-2-furyl)prop-2-ene aldoxime (M5FAOH)

Figure 1 Structures of the complexes and ligands.

afforded by the standard VP-16, which has an IC_{50} value of 25 μM. Inhibition of DNA topoisomerase II activity in human cancer cells by antineoplastic agents has led to agents with higher specificity for cancer cells, e.g. etoposide, VP-16, and have a higher log(kill) of the cancer cells.[5,6] We have noted different effects on nucleic acid metabolism and DNA topoisomerase II inhibition, depending on which metal is within the complexes, i.e. copper thiosemicarbazones are potent DNA topoisomerase inhibitors, whereas nickel thiosemicarbazones are not inhibitors. Sodium, calcium and iron(II) complexes of amine carboxyboranes are not DNA topoisomerase II inhibitors, whereas copper, cobalt,

iron(III) and chromium(III) complexes of amine carboxyboranes are inhibitors. Since we had available cobalt complexes of 2-furaldehyde and furan oximes, we investigated their cytotoxicity, effects on nucleic acid metabolism and DNA topoisomerase II activity to compare these with the previously reported copper 2-furaldehyde and furan oximes.[5,6]

MATERIALS AND METHODS

All six compounds had been synthesized previously and the chemical and physical characteristics reported[7,8] (Fig. 1). All radioisotopes were purchased from New England Nuclear (Boston, MA, USA) unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with correction for quenching. Substrates and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cytotoxicity

Compounds **1–6** were tested for cytotoxic activity by homogenizing drugs as a 1 mg ml^{-1} solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passing them through an Acrodisc (45 μm) and tested serially from 0.2 to 15 $\mu g\,ml^{-1}$ against each cell line. The following cell lines were maintained by literature techniques[9] and the growth media and growth conditions were according to American Type Culture Collection protocols: murine L_{1210} lymphoid leukemia and P388 lymphocytic leukemia, rat UMR-106 osteosarcoma, human $Tmolt_3$ and $Tmolt_4$ acute lymphoblastic T cell leukemia, Hl-60 leukemia, HuT-78 lymphoma, THP-1 acute monocytic leukemia, HeLa-S^3 suspended cervical carcinoma, HeLa solid cervical carcinoma, KB epidermoid nasopharynx, Sk-Mel-2 malignant melanoma, colorectal adenocarcinoma SW480, HCT-8 ileocecal adenocarcinoma, lung bronchogenic MB-9812, A549 lung carcinoma and glioma HS683. Geran *et al.*'s protocol[9] was used to assess the suspended cell cytotoxicity of the compounds and standards in each cell line. Cell numbers were determined by the Trypan Blue exclusion technique after three days' incubation. Solid tumor cytotoxicity was determined by Leibovitz *et al.*'s method[7] utilizing Crystal Violet/methanol and read at 562 nm (Molecular Devices)[10] after 4–5 days' incubation when the controls had converged. Values for cytotoxicity were expressed as ED_{50} ($\mu g\,ml^{-1}$), i.e. the concentration of the compound inhibiting 50% of cell growth. A value of less than 4 $\mu g\,ml^{-1}$ was required for significant activity of growth inhibition.[9]

Incorporation studies

The effects of drugs on the incorporation of radiolabeled precursors into [^{3}H]DNA, [^{3}H]RNA or [^{3}H]protein for 10^6 L1210 cells at 25, 50 and 100 μM for inhibition of DNA, RNA and protein synthesis was determined for 60-min incubations.[11] The acid-insoluble labeled DNA, RNA or protein was collected on disks which were counted in a Packard β-counter. The incorporation of [^{14}C]glycine (53.0 mCi $mmol^{-1}$) into purines was obtained by the method of Cadman *et al.*[12] Incorporation of [^{14}C]formate (53.0 mCi $mmol^{-1}$) into pyrimidines was determined by the method of Christopherson *et al.*[13] The final purines or pyrimidines were separated by thin-layer-chromatography (TLC) from starting components using the appropriate standard nucleoside bases, and counted.

Enzyme assays

The effects of the cobalt complexes on nucleic acid metabolism were determined at 25, 50 and 100 μM of compounds **1–4** after 60-min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler *et al.*'s method.[14–15] The DNA polymerase α assay was described by Sawada *et al.*[16] with ^{3}H 2-deoxyribothymidine-5′-triphosphate (TTP). Messenger-, ribosomal- and transfer-RNA polymerase nuclei enzymes were isolated with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using ^{3}H-uridine-5′-triphosphate (UTP).[17,18] The following enzyme activities were determined using L1210 homogenates. Ribonucleoside reductase activity was measured using [^{14}C]-cytidine-5′-diphosphate (CDP) with dithioerythritol.[19] [^{14}C] 2′-Deoxyribocytidine- 5′-diphosphate was separated from the ^{14}C-CDP by TLC on polyethyleneimine cellulose (PEI) plates. Thymidine, thymidine-5′-monophosphate (TMP) and thymidine-5′-diphosphate (TDP) kinase activities were determined using [^{3}H]thymidine (58.3 mCi $mmol^{-1}$) in the medium of Maley and Ochoa[20] and separated by TLC. Carbamyl phosphate synthetase activity was determined by the method of Kalman *et al.*[21] and the product citrulline was determined colorimetri-

cally. Aspartate transcarbamylase activity was measured using the incubation medium of Kalman *et al.*;[21] the product carbamyl aspartate was determined colorimetrically by the method of Koritz and Gohen[23] Thymidylate synthetase activity was analyzed by Kampf *et al.*'s method.[24] The ^3H_2O separated by charcoal was proportional to the amount of TMP formed from [^{3}H]-2′-deoxyribouridine-5′-monophosphate [dUMP]. Dihydrofolate reductase activity was determined by the NADH-disappearance spectrophotometric method of Ho *et al.*[25] at 340 nm. Phosphoribosyl-pyrophosphate (PRPP)-amidotransferase activity was determined by Spassova *et al.*'s method as the generation of NADH;[26] inosine-5′-monophosphate (IMP) dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi mmol^{-1}) (Amersham, Arlington Heights, IL, USA), after separating ^{14}C-xanthosine-5′-monophosphate (XMP) on PEI plates (Fisher Scientific) by thin-layer chromatography [TLC][27] which was then counted. Protein content was determined for the enzymic assays by the Lowry technique.[28]

DNA studies

After deoxyribonucleoside triphosphates (d[NTP]) had been extracted,[29] their levels were determined by the method of Hunting and Henderson[30] with calf thymus DNA, *E. coli* DNA polymerase I (non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed) and either 0.4 mCi of [^{3}H-*methyl*]dTTP or [5-^{3}H]dCTP. Thus, 2′-deoxyriboadenosine-5′-triphosphate (dATP), 2′-deoxyriboguanosine-5′-triphosphate (dGTP), 2′-deoxyribocytidine-5′-triphosphate (dCTP) and thymidine-5′-triphosphate (dTTP) levels were determined after incubation with the drugs for 60 min at 100 μM.

The effects of compounds **1–4** on DNA strand scission were determined by the methods of Suzuki *et al.*,[31] Pera *et al.*[32] and Woynarowski *et al.*[33] L1210 lymphoid leukemia cells were incubated with 10 μCi [H^3-*methyl*]thymidine (84.0 Ci mmol^{-1}) for 24 h at 37 °C. L1210 cells (10^7) were harvested and then centrifuged at 600 g × 10 min in phosphate-buffered saline (PBS). They were later 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 M KCl and 0.01 M EDTA); this was followed by 0.2 ml of the cell preparation. After the gradient had been incubated for 2.5 h at room temperature, it was centrifuged at 12000 g × 17 h at 8 °C. Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3 M HCl, and measured for radioactivity.

Thermal calf thymus DNA denaturation studies, changes in DNA UV absorption at 220–340 nm and DNA viscosity studies were conducted after incubation of compounds **1–4** at 100 μM at 37 °C for 24 h.[34]

Human DNA topoisomerase inhibition

Sample drugs were prepared in dimethyl sulfoxide (DMSO) so that the stock final concentration was 5 mM (w/v). The enzyme assay consisted of test drugs at 50 μg ml^{-1}, 0.5 unit of human topoisomerase II (p170 isoform) (TopoGen, Columbus, OH, USA), *ca* 0.1 mg of supercoiled PBR322 DNA in 50 mM Tris buffer, pH 7.5, 15 mM β-mercaptoethanol, 30 mg ml^{-1} bovine serum albumin, 1 mM ATP, 10 mM $MgCl_2$ and 150 mM KCl. After 30 min incubation at 37 °C the reaction was terminated with 0.75% (v/v) SDS and 0.5 mg ml^{-1} proteinase K. After an additional hour of incubation, aliquots were applied to a 0.8% (v/v) agarose TBE gel containing 0.5 μg ml^{-1} ethidium bromide and 1% (w/v) sodium dodecyl sulfate (SDS). Following overnight electrophoresis at 30 V (constant), the gel was destained and photographed using a UV-transilluminator and Polaroid film.

Statistical analysis

Data are displayed in tables and figures as the means ± standard deviations of the mean expressed as percentage of control. *N* is the number of samples per group. Student's '*t*'-test was used to determine the probable level of significance (*P*) between test samples and control samples.

RESULTS

The cobalt complexes of 2-furaldehyde and furan oximes demonstrated potent cytotoxicity against the growth of suspended tumor cells, with ED_{50} <4 μg/ml^{-1} (Table 1). Compounds **1–6** were significantly active against the growth of murine L1210 lymphocytic leukemia, human $Tmolt_4$ leukemia, HL-60 leukemia, THP-1 monocytic leukemia and HuT-78 lymphoma. In the Hela-S^3

Table 1 Cytotoxicity of cobalt complexes of furaldehyde and furan oximes, ED_{50} ($\mu g\ mg^{-1}$)

(a) *Suspended tumors*

	Murine		Human					
Compound[a]	P388	L1210	$Tmolt_3$	$Tmolt_4$	HuT-8	THP-1	Hl-60	HeLa uterine S^3
1	3.87	1.10	1.58	2.14	2.06	0.81	0.68	2.43
2	4.70	1.89	2.90	1.03	1.68	0.94	1.69	2.94
3	4.01	2.04	1.98	0.95	1.68	0.81	1.24	2.81
4	5.24	2.07	4.09	1.19	1.42	0.62	1.58	4.86
5	3.69	2.49	2.64	2.14	1.81	0.81	1.35	3.78
6	4.28	3.91	2.13	0.87	1.68	0.80	1.80	1.54
Standard agents for comparison								
6-MP	2.43	9.13	1.62	2.67	1.63	3.03	6.36	2.12
Ara-C	2.43	0.86	2.67	2.36	2.50	2.54	3.90	2.13
HU	2.67	2.87	4.47	6.68	3.87		5.22	1.96
5-FU	1.41	3.52	2.14	2.75	1.50	0.49	5.28	2.47
VP-16	1.83	3.57	—	1.92	1.13	3.27	3.49	7.87

(b) *Tumors derived from solid tumors*

	Human								Rat
Compound	Melanoma SK-Mel2	Solid HeLa	KB naso-pharynx	SW480 colon	HCT-8 ileum	Lung MB9812	Lung A549	Glioma HS683	UMR-106 osteosarcoma
1	6.65	8.21	4.86	13.80	5.75	4.09	3.24	4.43	4.67
2	7.36	7.14	4.40	4.90	7.28	4.00	3.29	4.43	0.20
3	8.02	8.59	3.97	7.40	6.29	4.27	3.95	4.61	8.00
4	6.71	7.89	4.13	6.00	9.52	4.63	3.92	4.22	6.81
5	6.82	7.04	7.72	5.80	5.21	5.00	3.55	4.31	6.57
6	7.45	5.97	7.79	9.20	8.45	4.99	3.59	4.25	2.65
Standard agents for comparison									
6-MP	6.86	5.61	11.04	3.61	1.15	4.29	4.71	4.46	3.42
Ara-C	10.53	4.74	2.84	3.42	2.54	6.16	6.28	1.88	0.92
HU		8.12	5.27	7.33	1.77	7.18	8.89	2.27	3.21
5-FU	5.93	4.11	1.25	3.09	1.12	5.64	3.58	1.28	0.61
VP-16	3.53	3.05	3.32	3.34	3.78	3.50	4.74	2.44	0.71

[a] Compounds; **1**. $[CoCl_2(M_5FDH_2)]$, mol. wt 384; **2**. $[CoCl_2(M_5FDH_4)]$, mol. wt 630; **3**. $[CoCl_2(E5FDH)_4]$, mol. wt 710; **4**. $[CoCl_2(FAOH)_2]$, mol. wt 404; **5**. $[CoCl_2(FAOH)_4]$, mol. wt 678; **6**. $[CoCl_2(M_5FAOH)_2]$, mol. wt 432. $N = 4$.

suspended uterine carcinoma and $Tmolt_3$ leukemia screens, compounds **1–3, 5** and **6** were significantly active. All of the compounds were moderately active against lung A549 growth, with ED_{50} values between 3 and 4 $\mu g/ml^{-1}$. Compound **3** was marginally active against human KB nasopharynx growth, with an ED_{50} value of 3.97 $\mu g/ml^{-1}$, and compounds **2** and **6** were active in the rat UMR-106 osteosarcoma screen, with ED_{50} values of 0.20 and 2.65 $\mu g/ml^{-1}$, respectively. None of the compounds were active against the growth of human melanoma, glioma HS683, lung MB-9812, adenocarcinoma of the colon SW480 and of the ileum HCT-8 or HeLa solid uterine carcinoma. All of the remaining ED_{50} values were between 4 and 13.80 $\mu g/ml^{-1}$.

Mode-of-action studies for L1210 lymphoid leukemia cells showed that DNA synthesis was preferentially inhibited after 60 min in a concentration-dependent manner with greater than 65% inhibition at 100 μM (Tables 2–5). This was followed by significant inhibition of both RNA and protein syntheses under the same incubation conditions. In order to establish whether the cobalt complexes interfered with DNA template activity for DNA or RNA synthesis, the individual polymerases were examined with excess d[NTP]s. DNA polymerase α activity was suppressed by 47% to

Table 2 Effects of compound **1** on L1210 lymphoid leukemia metabolism

	Percentage of control			
Assay ($N = 4$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^{a}	79 ± 5	63 ± 4*	28 ± 2*
RNA synthesis	100 ± 6^{b}	95 ± 4	89 ± 4	65 ± 3
Protein synthesis	100 ± 5^{c}	38 ± 3*	30 ± 2*	28 ± 3*
DNA polymerase α	100 ± 6^{d}	69 ± 4*	55 ± 5*	42 ± 3*
mRNA polymerase	100 ± 7^{e}	102 ± 6	127 ± 5*	226 ± 8*
rRNA polymerase	100 ± 4^{f}	133 ± 5*	88 ± 5	71 ± 4*
tRNA polymerase	100 ± 7^{g}	104 ± 6	95 ± 5	74 ± 5*
Ribonucleoside reductase	100 ± 5^{h}	79 ± 4*	72 ± 3*	66 ± 3*
Dihydrofolate reductase	100 ± 5^{i}	63 ± 4*	58 ± 5*	54 ± 4*
Purine *de novo* synthesis	100 ± 5^{j}	58 ± 5*	58 ± 4*	42 ± 3*
PRPP amidotransferase	100 ± 6^{k}	86 ± 5	69 ± 5*	66 ± 4*
IMP dehydrogenase	100 ± 5^{l}	86 ± 6	79 ± 4*	76 ± 3*
Pyrimidine *de novo* synthesis	100 ± 5^{m}	85 ± 5	77 ± 5*	77 ± 4*
Carbamyl phosphate synthetase	100 ± 8^{n}	115 ± 6	115 ± 7	108 ± 6
Aspartate transcarbamylase	100 ± 6^{o}	101 ± 5	104 ± 6	105 ± 5
Thymidylate synthetase	100 ± 5^{p}	97 ± 3	81 ± 3*	72 ± 4*
Thymidine kinase	100 ± 6^{q}	156 ± 6*	126 ± 5	73 ± 3*
Thymidine monophosphate kinase	100 ± 7^{r}	135 ± 5*	123 ± 5	103 ± 4
Thymidine diphosphate kinase	100 ± 6^{s}	80 ± 4*	77 ± 3*	71 ± 3*
d[ATP]	100 ± 5^{t}			60 ± 4*
d[GTP]	100 ± 6^{u}			98 ± 5
d[CTP]	100 ± 5^{v}			100 ± 5
d[TTP]	100 ± 4^{w}			95 ± 4

* $P < 0.001$; control values based on 10^6 L1210 cells.

[a] 25 153 dpm
[b] 4851 dpm
[c] 7461 dpm
[d] 47 804 dpm
[e] 1502 dpm
[f] 4239 dpm
[g] 6400 dpm
[h] 2744 dpm
[i] 0.868 OD units net
[j] 92551 dpm
[k] 0.121 OD units net
[l] 76058 dpm
[m] 19758 dpm
[n] 0.392 μmol citrulline
[o] 1.064 mol *N*-carbamyl aspartate
[p] 18463 dpm
[q] 1317 dpm
[r] 1179 dpm
[s] 1891 dpm
[t] 6.17 pmol
[u] 5.27 pmol
[v] 6.87 pmol
[w] 6.94 pmol

58% by the compounds at 100 μM. mRNA polymerase (II) activity was elevated by the agents by 31% to 134% at 100 μM after 60 min incubation, while rRNA polymerase (I) activity was reduced by 27% to 34% and t-RNA polymerase (III) activity was inhibited by 26% to 40% by compounds **1, 2** and **4** but compound **3** caused a 122% increase in activity.

Since template utilization by the polymerase was not inhibited by enough to account for the observed suppression of either DNA or RNA synthesis, a series of metabolic pathways and regulatory enzymes of those pathways were examined to determine whether the cobalt complexes were antimetabolites of nucleic acid metabolism. Ribonucleoside reductase activity was reduced by 21% to 34% and dihydrofolate reductase activity was suppressed by 48% to 60% at 100 mM after 60 min. *De novo* purine synthesis was reduced by 43% to 52% after 60 min but PRPP-amido transferase activity was only reduced by 15% to 43% and IMP dehydrogenase activity was inhibited by 11% to 35% after 60 min at 100 μM. *De novo* pyrimidine synthesis was reduced by 23% by compound **1** and by 37% by compound **4**. The early regulatory enzymes in this pathway were not affected by the compounds but thymidylate synthetase activity was suppressed by 27% to 54% after 60 min incubation at 100 μM. Thymidine kinase activity was reduced by 27% and 31% by compounds **1** and **3**, respectively; TMP kinase activity was reduced by 55% and 64% by compounds **3** and **4**, respectively and TDP kinase activity was suppressed by 29% to 64% after 60 min incubation at 100 μM. d[ATP] pool levels were lowered by 40% to 52% by compounds **1–3** but only by 18% by compound **4**. d[GTP] pool levels were reduced by 46% by compounds **2** and **4**. d[CTP] pool levels were reduced by 35% by compound **3**. d[TTP] pool levels were suppressed by 47% by compound **2**. To

Table 3 Effects of compound **2** on L1210 lymphoid leukemia metabolism

	Percentage of Control			
Assay ($N = 4$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	72 ± 5*	44 ± 3*	32 ± 4*
RNA synthesis	100 ± 6	67 ± 5*	52 ± 4*	32 ± 3*
Protein synthesis	100 ± 5	83 ± 5	57 ± 5*	48 ± 4*
DNA polymerase α	100 ± 6	67 ± 4*	50 ± 4*	43 ± 4*
mRNA polymerase	100 ± 7	107 ± 6	196 ± 7*	234 ± 8*
rRNA polymerase	100 ± 4	106 ± 5	77 ± 5*	73 ± 4*
tRNA polymerase	100 ± 7	128 ± 5*	125 ± 5*	67 ± 4*
Ribonucleoside reductase	100 ± 5	106 ± 6	87 ± 5	70 ± 4*
Dihydrofolate reductase	100 ± 5	57 ± 5*	50 ± 4*	40 ± 3*
Purine *de novo* synthesis	100 ± 5	61 ± 5*	57 ± 4*	51 ± 3*
PRPP amidotransferase	100 ± 6	98 ± 5	60 ± 4*	59 ± 4*
IMP dehydrogenase	100 ± 5	93 ± 6	79 ± 4*	65 ± 4*
Pyrimidine *de novo* synthesis	100 ± 5	101 ± 6	92 ± 5	92 ± 5
Carbamyl phosphate synthetase	100 ± 8	111 ± 7	103 ± 5	102 ± 6
Aspartate transcarbamylase	100 ± 6	105 ± 6	105 ± 5	106 ± 7
Thymidylate synthetase	100 ± 5	83 ± 6	79 ± 4*	73 ± 4*
Thymidine kinase	100 ± 6	237 ± 8*	121 ± 6	117 ± 5
Thymidine monophosphate kinase	100 ± 7	142 ± 6*	126 ± 7*	89 ± 4*
Thymidine diphosphate kinase	100 ± 6	87 ± 5	69 ± 4*	59 ± 3*
d[ATP]	100 ± 5			51 ± 3*
d[GTP]	100 ± 6			54 ± 4*
d[CTP]	100 ± 5			85 ± 5
d[TTP]	100 ± 4			53 ± 4*

* $P \leq 0.001$, control values based on 10^6 L1210 cells.

determine whether the cobalt complexes targeted the DNA molecule, a number of studies were performed. *In vitro* ct-DNA studies demonstrated that all four compounds caused an UV hyperchromic shift to a higher wavelength, suggesting interaction of the agents with the individual nucleoside bases of DNA. Thermal denaturation studies showed that the T_m values for the control and compound **3** were 93.5 °C, for compounds **1** and **2** 87.5 °C, and for compound **4** 83.5 °C, which do not strongly support the concept that the cobalt complexes caused intercalation between the base pairs of DNA. ct-DNA viscosity was 456 s for the control, 445 s for compound **1**, 442 s for compound **2** 473 s for compound **3** and 470 s for compound **4**, which suggest that the cobalt complexes did not directly cause DNA fragmentation using isolated ct-DNA. The L1210 DNA strand scission study after a 24 h incubation at 100 μM indicated that the compounds caused DNA fragmentation, i.e. higher radioactivity present in fractions 11–18 (Fig 2). Compound **2** appears to have caused cross-linking of the DNA strands, i.e. higher radioactivity in fraction 1, with some DNA fragmentation (Fig. 2).

The agents at 50 μg ml^{-1} did not afford significant effects on DNA topoisomerase, i.e. inhibition of strand-passing or DNA topoisomerase II activity nor did DNA double-or single-stranded protein linked breaks occur when the human enzyme system was used (data not shown). Thus, the cobalt complexes had no inhibitor effects on DNA topoisomerase II activity.

DISCUSSION

The cobalt complexes of the 2-furaldehyde and furan oxime derivatives demonstrated similar cytotoxicity to their comparable copper complexes,[5,6] in that they were very active against the growth of suspended murine and human leukemias and lymphomas as well as HeLa-S^3 uterine carcinoma. The cobalt derivatives demonstrated activity against the growth of human HuT-78 lymphoma, Tmolt$_4$ leukemia and THP-1 acute monocytic leukemia. In these tumor screens the copper complexes were not tested. The cobalt

Table 4 Effects of compound **3** on L1210 Lymphoid Leukemia Metabolism

	Percentage of Control			
Assay ($N = 4$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	64 ± 4*	31 ± 3*	30 ± 2*
RNA synthesis	100 ± 6	62 ± 3*	61 ± 2*	43 ± 3*
Protein synthesis	100 ± 5	52 ± 6*	41 ± 3*	38 ± 3*
DNA polymerase α	100 ± 6	63 ± 4*	62 ± 4*	53 ± 3*
mRNA polymerase	100 ± 7	106 ± 5	140 ± 5*	150 ± 7*
rRNA polymerase	100 ± 4	92 ± 6	75 ± 4*	64 ± 45*
tRNA polymerase	100 ± 7	121 ± 6	179 ± 7*	222 ± 7*
Ribonucleoside reductase	100 ± 5	103 ± 5	97 ± 4	79 ± 4*
Dihydrofolate reductase	100 ± 5	65 ± 5*	48 ± 4*	40 ± 5*
Purine *de novo* synthesis	100 ± 5	77 ± 4*	58 ± 4*	48 ± 4*
PRPP amidotransferase	100 ± 6	113 ± 6	91 ± 5	85 ± 6
IMP dehydrogenase	100 ± 5	82 ± 5	78 ± 4*	76 ± 3*
Pyrimidine *de novo* synthesis	100 ± 5	92 ± 6	90 ± 5	88 ± 4
Carbamyl phosphate synthetase	100 ± 8	103 ± 7	111 ± 6	135 ± 5*
Aspartate transcarbamylase	100 ± 6	106 ± 6	106 ± 5	107 ± 4
Thymidylate synthetase	100 ± 5	103 ± 5	77 ± 3*	69 ± 3*
Thymidine kinase	100 ± 6	161 ± 6*	151 ± 5*	61 ± 4*
Thymidine monophosphate kinase	100 ± 7	98 ± 5	84 ± 5	45 ± 4*
Thymidine diphosphate kinase	100 ± 6	73 ± 4*	72 ± 4*	71 ± 3*
d[ATP]	100 ± 5			48 ± 3*
d[GTP]	100 ± 6			88 ± 4
d[CTP]	100 ± 5			65 ± 3*
d[TTP]	100 ± 4			98 ± 4

* $P \leq 0.001$; control values based on 10^6 L1210 cells.

complexes were active against the growth of lung A549, as were the copper complexes.[5,6] Neither cobalt nor copper complexes of 2-furaldehyde or furan oximes were active against the growth of glioma and lung MB-9812, but selected copper complexes did demonstrate activity in the human solid HeLa uterine carcinoma, skin epidermoid A431, ileum and colon adenocarcinomas and rat UMR-106 osteosarcoma screens. Thus, the type of metal within the complexes does have an impact on the cytotoxicity against tumors grown from human solid tumors but the cobalt and copper complexes demonstrated little difference in activity against growth of suspended tumors.

In the mode-of-action studies both the cobalt and copper complexes[5,6] preferentially inhibited the production of DNA, and to a lesser extent RNA, but both types of 2-furaldehye complexes were less effective in blocking protein synthesis after 60 min. The copper complexes of furan oximes were the least effective in the inhibition of protein synthesis.[6] However, the DNA template and its utilization by the polymerases for nucleic acid synthesis did not appear to be the main target of the cobalt complexes, which was similar to the effects of the copper complexes.[5,6] Nevertheless, DNA polymerase α was suppressed by the cobalt complexes by *ca* 50%, compared with *ca* 10–25% by the copper complexes.[5,6] d[NTP] pools would naturally become elevated if the DNA polymerase α activity was markedly suppressed by these compounds. After 60 min incubation the d[NTP] pool levels were not elevated but rather in general reduced, suggesting that the cobalt complexes cause a metabolic blockage or that they function as antimetabolites to suppress cancer growth.

Thus, a series of individual regulatory enzymes involved in nucleic acid synthesis were examined after first examining *de novo* purine and pyrimidine syntheses. Both types of complexes inhibited *de novo* purine synthesis with marginal inhibition of the two regulatory enzymes of the pathway, i.e. PRPP-amidotransferase and IMP dehydrogenase activities. The copper complexes were more effective in causing a reduction of IMP dehydrogenase activity. Another enzyme that plays a role in one-carbon transfer in purine synthesis is dihydrofolate reductase. The activity of this enzyme was

Table 5 Effects of compound A sp **4** on L1210 Lymphoid Leukemia Metabolism

	Percentage of Control			
Assay ($N = 4$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	51 ± 4*	30 ± 2*	15 ± 2*
RNA synthesis	100 ± 6	77 ± 5*	73 ± 3*	70 ± 3*
Protein synthesis	100 ± 5	63 ± 4*	46 ± 4*	41 ± 3*
DNA polymerase α	100 ± 6	74 ± 5*	65 ± 4*	46 ± 3*
mRNA polymerase	100 ± 7	121 ± 6	121 ± 5	131 ± 6*
rRNA polymerase	100 ± 4	117 ± 5	94 ± 5	72 ± 3*
tRNA polymerase	100 ± 7	94 ± 6	79 ± 4*	60 ± 5*
Ribonucleoside reductase	100 ± 5	118 ± 6	111 ± 6	72 ± 3*
Dihydrofolate reductase	100 ± 5	133 ± 6*	130 ± 5*	41 ± 4*
Purine *de novo* synthesis	100 ± 5	126 ± 5	86 ± 4	57 ± 4*
PRPP amidotransferase	100 ± 6	104 ± 6	99 ± 4	94 ± 5
IMP dehydrogenase	100 ± 5	108 ± 6	96 ± 5	89 ± 4
Pyrimidine *de novo* synthesis	100 ± 5	81 ± 5	73 ± 3*	63 ± 5*
Carbamyl phosphate synthetase	100 ± 8	123 ± 6	123 ± 7	98 ± 6
Aspartate transcarboxylase	100 ± 6	100 ± 5	104 ± 6	105 ± 75
Thymidylate synthetase	100 ± 5	96 ± 68	63 ± 5*	46 ± 3*
Thymidine kinase	100 ± 6	161 ± 7*	109 ± 6	81 ± 4*
Thymidine monophosphate kinase	100 ± 7	95 ± 4	63 ± 5*	36 ± 3*
Thymidine diphosphate kinase	100 ± 6	47 ± 4*	39 ± 3*	36 ± 4*
d[ATP]	100 ± 5			82 ± 4
d[GTP]	100 ± 6			54 ± 4*
d[CTP]	100 ± 5			96 ± 4
d[TTP]	100 ± 4			99 ± 4

* $P \leq 0.001$; control values based on 10^6 L1210 all.

reduced less by the cobalt complexes than by the copper complexes.[5,6] Ribonucleoside reductase activities were reduced more markedly by the cobalt derivatives; this enzyme is responsible in human cancer cells for converting ribonucleotides to deoxyribonucleotides and affects DNA synthesis markedly when blocked by antimetabolites, e.g. hydroxyurea (HU). Any one of these metabolic targets would account for the inhibition of purine synthesis and suppression of tumor cell growth. Suppression of purine synthesis by an agent would be reflected in both RNA and DNA syntheses but since in mammalian cells the d[NTP] pools compare about 10% and the r[NTP] pools 90%, suppression of syntheses would be most likely to appear first with DNA rather than RNA. Consequently, the fact that the agents inhibit *de novo* purine synthesis explains the rapid reduction in deoxypurine triphosphate pools generated from this pathway. There was no significant evidence that the early step of the *de novo* synthesis of pyrimidines was affected by the cobalt derivatives, but the copper complexes were effective in blocking *de novo* synthesis of pyrimidines. Both the cobalt and copper complexes reduced the activities of nucleoside kinase. In addition, the cobalt derivatives inhibited thymidylate synthetase activity, whereas the copper complexes did not. The biochemical effects of the complexes would lead to reduced deoxypyridine triphosphate pools, which was indeed the case with the cobalt derivatives but not the copper complexes.[5,6] These studies strongly indicate that the cobalt complexes of 2-furaldehyde and furan oximes are potent antimetabolites of the

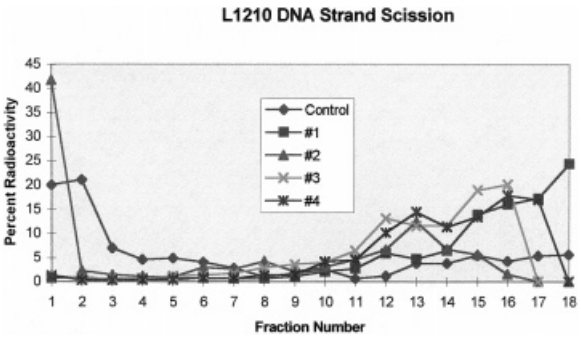


Figure 2 Effects of cobalt complexes of furan oximes on DNA fragmentation.

purine pathway and are not interacting with the DNA molecule itself via alkylation of nucleotide bases or intercalation between base pairs. The copper complexes were DNA topoiosmerase II inhibitors without inducing DNA-linked protein breaks but the cobalt complexes had no effects on either DNA topoisomerase II activity or protein-linked breaks although both complexes caused DNA fragmentation after a 24 h incubation.[5,6] A similar observation has been made with other metal complexes such as the aminecarboxyboranese/ copper, cobalt and iron complexes were DNA topoisomerase II inhibitors but chromium, sodium and calcium complexes were not DNA topoisomerase II inhibitors; however, all of these metal complexes caused DNA fragmentation by another mechanism.[1–4]

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