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Cytotoxicity of copper and cobalt complexes of furfural semicarbazone and thiosemicarbazone derivatives in murine and human tumor cell lines

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The 2-furfural semicarbazone and thiosemicarbazone copper and cobalt complexes demonstrated potent cytotoxicity against the growth of suspended leukemias and lymphomas as well as human lung MB9812, colon SW480, ovary 1-A9 and HeLa-S^3 uterine carcinoma. In L1210 lymphoid leukemia cell the complexes inhibited preferentially DNA synthesis over 60 min at 25 to 100 μM. The copper and cobalt complexes functioned by multiple mechanisms to suppress synthetic steps in nucleic acid metabolism to reduce deoxynucleotide pools for incorporation into DNA. At high concentrations the complexes suppressed human DNA topoisomerase II activity with DNA nicks and DNA fragmentation but they did not alkylate the bases of DNA, cause intercalation between base pairs or cause cross-linking of DNA strands.

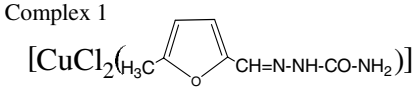
1. Introduction

The copper complexes of 2-furaldehyde and furan oximes previously demonstrated potent cytotoxicity, L1210 DNA synthesis inhibition, DNA topoisomerase II inhibition and DNA fragmentation [1–2]. A series of cobalt metal complexes of 2-furaldehyde oximes were compared to their copper complexes of furan oximes to determine if the type of metal is important to the complexes' cytotoxicity and mode of action [3]. The cobalt complexes of furan oximes like the copper complexes showed cytotoxicity 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 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 ribonucleotide 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 synthase activity. Varying the type of metal within the complexes of 2-furaldehyde and furan oximes produces differences in both cytotoxicity and mode of action. In order to examine this hypothesis, additional cobalt and copper complexes of 2-furfuraldehyde were examined for their activities. Furthermore since a number of thiosemicarbazones have demonstrated excellent cytotoxic action, a series of 2-furfural semicarbazones and thiosemicarbazones were also examined.

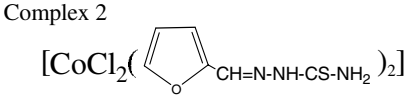
2. Investigations and results

All six of the 2-furfural metal complexes suppressed the growth of murine L1210 lymphoid and P388 lymphocytic leukemias, significantly, i.e. the ED_{50} values were < 4 μg/ml (Table 1). Significant ED_{50} values were also obtained in the human $Tmolt_3$ T cell and HL-60 leukemia, and THP-1 acute monocytic leukemia screens for the complexes. But in the $Tmolt_4$ screen only compounds **4**–**6** were active. Compounds **2**, **3** and **5** retarded lymphoma

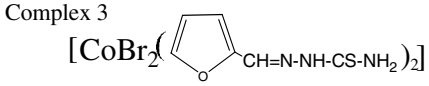
Complex 1



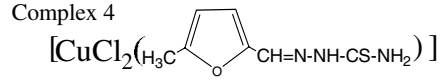
Complex 2



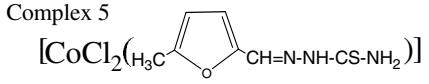
Complex 3



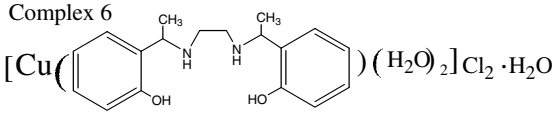
Complex 4



Complex 5



Complex 6



HuT-78 growth**.** HeLa-S^3 suspended uterine carcinoma growth was inhibited by all of the compounds. In the tumors derived from human solid tumors the complexes demonstrated more selectivity in their inhibition (Table 2). Compounds **1**–**4** and **6** demonstrated activity in the lung MB-9812 screen. Compound **4** only reduced Saos-2 osteo-

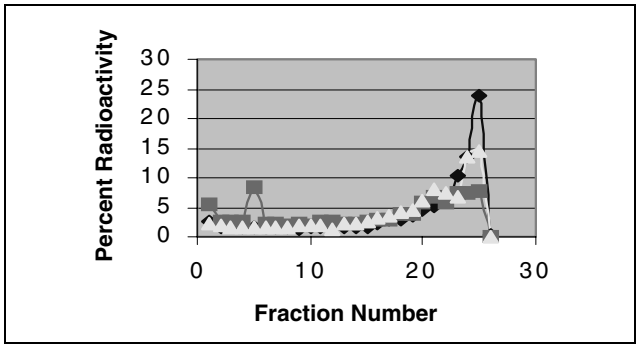


Fig.: L1210 DNA strand scission after 24 h incubation with drugs at 100 μM; —◆— Control, —■— #3, —▲— #4

Table 1: ***In vitro*** **cytotoxicity screen of 2-furfural metal complexes against murine or human tumor tissue culture cells**

Cell Line	L1210	P-388	Tmolt-3	Tmolt4	HeLa-S^3	HuT-78	HL-60	THP-1
Species	Mouse	Mouse	Human	Human	Human	Human	Human	Human
#1	1.64	1.47	1.90	6.01	1.31	4.30	2.23	2.34
#2	1.88	1.32	2.08	6.23	2.26	2.84	2.03	1.19
#3	1.64	1.32	3.45	4.79	2.22	3.78	0.20	3.40
#4	0.65	0.82	1.86	2.17	1.60	4.04	1.01	1.06
#5	0.24	1.16	2.80	3.66	2.47	2.49	0.61	2.53
#6	0.19	2.07	4.06	3.75	1.36	5.33	1.01	2.48
6-MP	2.43	2.04	1.62	2.67	2.12	1.63	3.35	3.03
Ara-C	2.07	0.79	2.67	2.36	2.13	2.50	4.00	2.54
Hydroxyurea	2.67	1.30	4.47	–	1.96	3.87	–	1.77
5-FU	1.41	1.41	2.14	2.75	2.47	5.81		1.12
6-Aza UMP	1.20	1.41	1.54	2.39	2.48	4.46		0.75
Etoposide	1.83	3.03	1.0	1.92	7.87	3.20	4.43	3.78
Antimycin A	1.79	1.08	2.15	2.5	5.83	4.13	3.35	4.64
Actinomycin D	1.98	1.41	–		5.88	4.88		3.71
Cycloheximide	1.44	1.84	–		3.57	6.31		0.81

(ED_{50} = μg/ml) N = 4

sarcoma and breast Mck-7 carcinoma growth. Glioma UM-86 growth was reduced by compounds **1** and **4.** Colon SW-480 growth was reduced by compounds **1**–**5**. Ovarian carcinoma growth was inhibited by compounds **2**–**6**. None of the complexes had any effects on the growth of human lung A549, KB nasopharynx, skin epidermoid A431 or ileum adenocarcinoma HCT-8 growth.

The mode of action study with compounds **3** and **4** showed that DNA synthesis was reduced from 66% to 76% at 100 μM over 60 min, RNA synthesis was reduced 40%–49% and protein synthesis was suppressed 20% to 34% under the same conditions. The effects of the complexes on these synthetic pathways were concentration dependent. Since nucleic acid metabolism appeared to be affected the most, a number of pathways were examined for the effects of the complexes. De novo purine synthesis was suppressed 25%–26% and de novo pyrimidine synthesis was reduced 44% to 48% at 100 μM over 60 min. Examination of the activities of the regulatory enzymes of these pathways showed that IMP dehydrogenase activity was suppressed 29% and 40% while PRPP-amido transferase activity was unaffected. Carbamyl phosphate synthetase activity was reduced 30% and 45% but aspartate transcarbamylase and thymidylate synthase activity were unchanged. Dihydrofolate reductase activity was suppressed 60% and 84% in a concentration dependent manner. Ribonucleotide reductase activity was suppressed by 44% and 57%. Thymidine kinase was suppressed by 38% and 55% while TMP-kinase activity was reduced 44% only by compound **3** and TDP kinase activity was inhibited 55% and 56% at 100 μM over 60 min. The inhibition of these enzymes by the complexes led to a 16% to 22% reduction of d[ATP] pool levels, a 21% and 28% reduction of d[GTP] pool levels, a 18% reduction of d[CTP] pool levels by both complexes and a 23% and 27% reduction of d[TTP] pool levels over 60 min at 100 μM. Examination of the polymerase activities showed that DNA polymerase α activity was reduced 16% and 31%, m-RNA polymerase activity 35% and 42%, r-RNA polymerase activity 35% and 42% and t-RNA polymerase activity 48% and 50%.

In order to examine if the DNA molecule itself is a target of the complexes ct-DNA studies were performed. Neither complex **3** nor **4** caused any hyperchromic shift in the UV

Table 2: ***In vitro*** **cytotoxicity screen of 2-furfural analogs against murine or human tumor tissue culture cells**

Cell Line	Lung A549	Lung MB9812	KB	Melanom a-Sk-2	A-431	Bone Saos-2	Glioma UM-86	Ileum HCT-8	Colon SW-480	Ovary 1-A9	Breast\ MCf-7	HeLa Uterine
Species	Human	Human	Human	Human	Human	Human	Human	Human	Human	Human	Human	Human
#1	10.69	1.58	10.56	8.91	9.11	6.39	3.48	6.51	1.94	4.47	5.69	6.85
#2	7.74	1.84	10.46	10.04	4.48	5.98	5.59	8.11	2.36	3.96	5.81	5.14
#3	9.52	1.53	9.38	11.92	5.12	6.82	4.32	7.91	2.15	2.63	6.11	6.44
#4	7.20	1.55	6.14	12.68	5.59	1.52	3.60	4.42	2.03	1.95	3.01	8.28
#5	6.11	4.16	6.59	13.84	11.39	4.88	4.26	7.57	2.62	2.01	16.38	8.14
#6	8.57	3.92	11.38	11.51	5.39	5.68	7.16	6.25	4.75	1.84	11.62	6.95
6-MP	4.71	4.29	11.04	6.86	3.42	5.07	4.46	1.15	3.61	6.64	8.84	5.61
Ara-C	5.62	6.16	2.84	10.53	0.92	8.90	1.88	2.54	3.42	5.39	12.45	4.34
Hydroxyurea	8.89	7.18	5.27	–	3.21	–	2.27	1.77	7.33	–	–	8.12
5-FU	3.54	5.64	1.25	5.93	0.61	8.73	1.28	1.30	2.47	5.25	6.82	4.11
6-Aza UMP	2.63	2.39	3.57	2.06	1.09	7.33	1.93	0.75	2.04	2.88	7.63	4.69
Etoposide	4.74	3.50	3.32	3.53	0.71	8.61	2.44	1.13	0.93	6.24	11.0	3.05
Antimycin A	6.01	2.96	5.40	4.53	2.28	7.62	3.990	4.46	2.76	8.18	9.0	4.29
Actinomycin D	0.90	1.28	0.93	–	0.30	–	1.15	3.71	3.18	–	–	–
Cyclohexi-mide	1.34	1.18	0.57	–	0.61	–	2.04	1.70	10.10	7.58	–	3.39

(ED_{50} = μg/ml) N = 4

Table 3: Effects of analog #3 on L1210 leukemia cell metabolism after 60 min incubation (percent of control)

Assay (N = 6)	Control	25 μM	50 μM	100 μM
DNA Synthesis	100 ± 6 [a]	74 ± 5*	65 ± 4*	34 ± 3*
RNA Synthesis	100 ± 5 [b]	65 ± 4*	62 ± 3*	51 ± 3*
Protein Synthesis	100 ± 6 [c]	76 ± 5*	72 ± 4*	66 ± 4*
DNA Polymerase α	100 ± 5 [d]	108 ± 6	90 ± 5	84 ± 5
mRNA Polymerase	100 ± 4 [e]	69 ± 5*	69 ± 4*	58 ± 4*
rRNA Polymerase	100 ± 6 [f]	114 ± 5	98 ± 5	57 ± 3*
tRNA Polymerase	100 ± 6 [g]	88 ± 5	87 ± 4	52 ± 4*
Ribonucleotide Reductase	100 ± 6 [h]	66 ± 5*	62 ± 4*	56 ± 4*
De Novo Purine Synthesis	100 ± 6 [i]	91 ± 5	89 ± 4	74 ± 4*
PRPP Amido Transferase	100 ± 6 [j]	103 ± 4	106 ± 5	109 ± 5
IMP Dehydrogenase	100 ± 7 [k]	93 ± 5	71 ± 4*	60 ± 4*
De Novo PyrimidineSynthesis	100 ± 6 [l]	91 ± 6	82 ± 5*	56 ± 4*
Carbamyl Phosphate Synthetase	100 ± 6 [m]	96 ± 6	78 ± 5*	70 ± 4*
Aspartate Transcarbamylase	100 ± 7 [n]	104 ± 5	103 ± 6	99 ± 5
Thymidylate Synthase	100 ± 6 [o]	117 ± 6	100 ± 6	99 ± 5
Thymidine Kinase	100 ± 5 [p]	79 ± 5*	58 ± 4*	45 ± 3*
TDP kinase	100 ± 4 [q]	129 ± 6	84 ± 4*	56 ± 4*
TTP Kinase	100 ± 4 [r]	89 ± 5	69 ± 4	45 ± 4*
Dihydrofolate Reductase	100 ± 6 [s]	38 ± 4*	35 ± 3*	16 ± 2*
d(ATP)	100 ± 4 [t]			84 + 6
d(GTP)	100 ± 6 [u]			79 + 5*
d(CTP)	100 ± 6 [v]			82 + 5
d(TTP)	100 ± 6 [w]			73 + 4*

* P ≤ 0.001; [a] 12349 dpm, [b] 2569 dpm, [c] 17492 dpm, [d] 9019 dpm, [e] 1343 dpm, [f] 325 dpm, [g] 400 dpm, [h] 48780 dpm, [i] 24500 dpm, [j] 0.087 OD units, [k] 1487 dpm, [l] 19758 dpm, [m] 0.850 μmoles citrulline, [n] 0.807 mol N-carbamyl aspartate, [o] 14260 dpm, [p] 1317 dpm, [q] 1179 dpm, [r] 1891 dpm, [s] 0.144 OD units, [t] 17.07 pmoles, [u] 13.58 pmoles, [v] 33.60 pmoles, [w] 31.40 pmoles

absorption of ct-DNA from 220 to 340 nm. DNA denaturation Tm values did not differ the control values and the complexes did not change the ct-DNA viscosity values after incubation for at 100 μM over 60 min. Incubation of whole L1210 cells at 100 μM of complexes **3** and **4** over 60 min led to DNA strand scission with higher radioactivity in the lower molecular weight fractions of the gradient (Fig.). Human DNA topoisomerase II studies demonstrated that complexes **1**–**5** had the ability to inhibit the activity completely at 150 μM. Compound **6** caused only 10% reduction in DNA topoisomerase II inhibition at 150 μM. Complexes **1** and **4** caused 50% of the DNA to be nicked at 100 μM and 90% at 150 μM. None of the remaining complexes had the ability to cause DNA nicks.

3. Discussion

Previous studies with copper and cobalt complexes of furan and 2-furaldehyde oximes had shown good cytotoxicity activity [1–3]. One previous related derivative was the copper complex of 2-furaldehyde semicarbazone [2]. When this derivatives was compared to the copper complex **1** (5-methyl 2-furural semicarbazone), the latter compound demonstrated better cytotoxic activity in the murine

Table 4: Effects of analog #4 onL1210 cell metabolism after 60 min incubation (percent of control)

Assay (N = 6)	Control	25 μM	50 μM	100 μM
DNA Synthesis	100 ± 6 [a]	31 ± 4*	30 ± 3*	24 ± 2*
RNA Synthesis	100 ± 5 [b]	66 ± 5*	64 ± 3*	60 ± 3*
Protein Synthesis	100 ± 6 [c]	85 ± 6	82 ± 5	80 ± 4*
DNA Polymerase α	100 ± 5 [d]	76 ± 5*	74 ± 4*	69 ± 4*
MRNA Polymerase	100 ± 4 [e]	76 ± 6*	68 ± 5*	65 ± 5*
RRNA Polymerase	100 ± 6 [f]	86 ± 6	75 ± 4*	62 ± 4*
TRNA Polymerase	100 ± 6 [g]	57 ± 5*	52 ± 4*	50 ± 3*
Ribonucleotide Reductase	100 ± 6 [h]	52 ± 4*	45 ± 5*	43 ± 4*
De Novo Purine Synthesis	100 ± 6 [i]	110 ± 6	99 ± 5	75 ± 4*
PRPP Amido Transferase	100 ± 6 [j]	120 ± 5	116 ± 6	118 ± 6
IMP Dehydrogenase	100 ± 7 [k]	91 ± 5	74 ± 4*	71 ± 3*
De Novo Pyrimidine Synthesis	100 ± 6 [l]	73 ± 5*	62 ± 4*	52 ± 3*
Carbamyl Phosphate Synthetase	100 ± 6 [m]	74 ± 5*	62 ± 4*	55 ± 4*
Aspartate Transcarbamylase	100 ± 7 [n]	105 ± 6	104 ± 5	101 ± 6
Thymidylate Synthase	100 ± 6 [o]	94 ± 6	94 ± 5	94 ± 6
Thymidine Kinase	100 ± 5 [p]	97 ± 5	78 ± 5*	62 ± 5
TDP Kinase	100 ± 4 [q]	234 ± 7*	210 ± 8*	196 ± 7*
TTP Kinase	100 ± 4 [r]	52 ± 4*	51 ± 5*	44 ± 5*
Dihydrofolate Reductase	100 ± 6 [s]	106 ± 7	82 ± 4	40 ± 3*
d(ATP)	100 ± 4 [t]			78 ± 5*
d(GTP)	100 ± 6 [u]			72 ± 5*
d(CTP)	100 ± 6 [v]			82 ± 6*
d(TTP)	100 ± 6 [w]			77 ± 6*

* P ≤ 0.001; Control values are listed as above

L1210, P388 leukemia, and human colon SW480, and lung MB9812 but the copper complex of 2-furaldehyde semicarbazone demonstrated better activity in the KB nasopharynx, skin A431 and lung A549 screens than reported previously [2]. The $CoCl_2$ and $CoBr_2$ complexes of 2-furaldehyde semicarbazone were more active in the murine L1210 and P388 leukemia and HL-60 leukemia and colon SW480 than the copper complex of 2-furaldehyde semicarbazone which was more active in the KB nasopharynx, skin A431 and lung A549 screens [3]. The cobalt complexes **2** and **3** afforded almost the same cytotoxicity in most of the screens but the Br_2 complex **2** demonstrated more activity in the HL-60 leukemia and the Cl_2 complex **3** was more active in the THP-1 acute monocytic leukemia screen. Comparing the copper and cobalt complex of 5-methyl 2 furfural thiosemicarbazone showed that the two complexes were approximately equal in their effects on suspended cell cytotoxicity; nevertheless, the cobalt complex **4** was more effective in the $Tmolt_3$ and $Tmolt_4$ leukemia, HeLa-S^3 uterine carcinoma, THP-1 acute monocytic leukemia, lung MB9812, Saos-2 osteosarcoma, breast MCK-7 and both Co and Cu complexes were effective against ovary carcinoma growth. In most of the screen the unique copper complex **6** was equal to or less active than the other complexes, although it was active in the human ovary and murine L1210 leukemia screens.

Mode of action studies in L1210 leukemia cells showed that the copper complex **4** (5-methyl 2-furfural thiosemicarbazone) was more effective in reducing DNA synthesis after 60 min than the previously reported copper complex of 2-furaldehyde semicarbazone [2]. It reduced mRNA and rRNA polymerase, ribonucleotide reductase, de novo pyrimidine synthesis, carbamyl phosphate synthetase more than copper complex of 2-furaldehyde semicarbazone which was more effective in reducing t-RNA polymerase, de novo purine synthesis, PRPP-amido transferase, IMP dehydrogenase, dihydrofolate reductase and the nucleoside kinases activities [2].

The mode of action study in L1210 cells of $CoBr_2$ complex of 2-furaldehyde thiosemicarbazone **3** when compared to the $CoCl_2$ complex of 3-(2-furyl)prop-2-ene aldoxime, previously published [3], showed that similar activities were found in the ability of the two complexes to inhibit DNA and RNA syntheses. The thiosemicarbazone did not produce as much inhibition of DNA polymerase α activity or de novo purine synthesis as well as its regulatory enzymes but did cause more inhibition of the RNA polymerase activities, ribonucleotide reductase, dihydrofolate reductase, and nucleoside kinases and de novo pyrimidine synthesis including carbamyl phosphate synthetase activity.

The thiosemicarbazone complexes of copper **4** and cobalt **3** did not have their effects on a single enzyme in nucleic acid metabolism; rather they affected multiple sites or enzymes in this pathway. When the magnitude of their effects on different enzymes are added, they would more than account for the observed inhibition of overall DNA synthesis in 60 min as well as the reduction of dNTP pools for the incorporation into the new strand of DNA. The DNA molecule itself did not appear to be a target of the complexes indicating no SN1 or SN2 alkylation of the nucleotide bases of DNA, intercalation of the complexes between the base pairs of DNA or crosslinking of the strands of DNA. However, upon incubation of the L1210 cells with the complexes at 100 μM for 24 h did cause DNA fragmentation, which should cause apoptosis or cell death. All of the copper and cobalt 2-furaldehyde complexes as well as metal complexes of thiosemicarbazones caused leukemia cell DNA fragmentation at 100 μM.

4. Experimental

4.1. Source of compounds

Complex **1** [$CuCl_2$(5-methyl 2 furfural semicarbazone)] [5] and complex **6** [Cu(= *N*,*N*′-bis(2-hydroxyacetophenyl)-1,2 diaminoethane)$(H_2O)_2$]$Cl_2 \cdot H_2O$ [6] were synthesized previously and reported. Synthesis of complex **2** [$CoCl_2$(2-furfural thiosemicarbazone)$_2$], complex **3** [$CoBr_2$(2-furfural thiosemicarbazone)$_2$], complex **4** [$CuCl_2$(5-methyl 2 furfural thiosemicarbazone] and complex **5** [$CoCl_2$(5-methyl 2 furfural thiosemicarbazone)] are currently being reported. Briefly ligands 2-furfural thiosemicarbazone and 5-methyl-2-furfural thioemicarbazone were prepared with equimolar amounts of aldehyde and thiosemicarbazidium chloride with some acetic acid mixed in absolute ethanol and heated up to refluxing [4]. After 2 h, the volume of the solution is reduced until the thiosemicarbazone precipitates which was filtered and recrystallized in absolute alcohol. The complexes were prepared with stoichiometric amounts of ligand and metallic salt which was allowed to react in absolute alcohol for 24 h for 2-furfural thiosemicarbzone and 4 h for 5- methyl-2-furfural thioemicarbazone under refluxing conditions. The complexes precipitated upon cooling and were filtered and washed with ethanol and n-pentane.

4.2. Cytotoxicity

Compounds **1**–**6** were tested for cytotoxic activity by homogenizing drugs as a 1 mg/ml 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 μg/ml against each cell line. The following cell lines were maintained by literature techniques [7] and the growth mediums and growth conditions are according to American Type Culture Collection protocols: murine L_{1210} lymphoid leukemia and P388 lymphocytic leukemia, 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, Saos-2 osteosarcoma 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 [8] using crystal violet/MeOH and read at 562 nm (Molecular Devices) after 4–5 days incubation when the controls have converged. Values for cytotoxicity were expressed as ED_{50} (μg/ml), i.e. the concentration of the compound inhibiting 50% of cell growth. A value of less than 4 μg/ml was required for significant activity of growth inhibition [7].

4.3. Incorporation studies

The effects of drugs on the incorporation of radiolabeled precursors thymine, uracil or leucine into ^{3}H-DNA, ^{3}H-RNA or ^{3}H-protein for 10^6 L1210 cells at 25, 50 and 100 μM was determined for 60 min incubations [9]. The acid insoluble labeled DNA, RNA or protein was collected on discs which were counted in a Packard beta counter. The incorporation of ^{14}C-glycine (53.0 mCi/mmol) into purines was obtained by the method of Cadman et al. [10]. Incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines was determined by the method of Christopherson et al. [11]. The final purines or pyrimidines were separated by TLC from starting components using the appropriate standard nucleoside bases and counted.

4.4. Enzyme assays

The effects of the cobalt complexes on nucleic acid metabolism were determined at 25, 50 and 100 μM of compounds **3** and **4** after 60 min incubation. DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler et al.'s method [12, 13]. The DNA polymerase α assay was described by Sawada et al. [14] 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) [15, 16]. The following enzyme activities were determined using L1210 homogenates. Ribonucleotide reductase activity was measured using ^{14}C-cytidine-5′-diphosphate [CDP] with dithioerythritol [17]. ^{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) in the medium of Maley and Ochoa [18] and separated by TLC. Carbamyl phosphate synthetase activity was determined by the

method of Kalman et al. [19] and the product citrulline was determined colorimetrically [20]. Aspartate transcarbamylase activity was measured using the incubation medium of Kalman et al. [19]; the product carbamyl aspartate was determined colorimetrically by the method of Koritz et al. [21]. Thymidylate synthase activity was analyzed by Kampf et al.'s method [22]. The ^3H_2O separated by charcoal was proportional to the amount of TMP formed from ^{3}H-2′-deoxyribouridine-5′-monophosphate [UMP]. Dihydrofolate reductase activity was determined by the NADH disappearance spectrophotometric method of Ho et al. [23] at 340 nm. Phosphoribosylpyrophosphate [PRPP]-amidotransferase activity was determined by Spassova et al.'s method as the generation of NADH [24]; inosine-5′-monophosphate [IMP] dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi/mmol) (Amersham, Arlington Heights, IL) after separating ^{14}C xanthosine-5′-monophosphate [XMP] on [PEI] plates (Fisher Scientific) by TLC [25] which was then counted. Protein content was determined for the enzymatic assays by the Lowry technique [26].

4.5. DNA studies

After deoxyribonucleoside triphosphates [d[NTP]] were extracted [27], d[NTP] levels were determined by the method of Hunting and Henderson [28] 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 mM.

The effects of compounds **3** and **4** on DNA strand scission were determined by the methods of Suzuki et al. [29], Pera et al. [30] and Woynarowski et al. [31]. L1210 lymphoid leukemia cells were incubated with 10 μCi [methyl-^{3}H]-thymidine (84.0 Ci/mmol) for 24 h at 37 °C. L1210 cells (10^7) were harvested and then centrifuged at 600 g X 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 KCl and 0.01 M EDTA); this was followed by 0.2 ml of the cell preparation. After the gradient was incubated for 2.5 h at room temperature, it was centrifuged at 12,000 g X 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 N HCl, and measured for radioactivity.

Thermal calf thymus DNA denaturation studies, changes in DNA U.V. absorption from 220–340 nm, and DNA viscosity studies were conducted after incubation of compounds **1**–**4** at 100 μM at 37 °C for 24 h [32].

4.6. Human DNA topoisomerase inhibition

Sample drugs were prepared in DMSO so that the stock final concentration was 5 mM [w/v]. The enzyme assay consisted of test drugs at 50 to 200 μM, 1 units of human topoisomerase II (p170 isoform) [TopoGen, Inc., Columbus, OH], ~ 0.5 μg of supercoiled PBR322 DNA in 50 mM Tris buffer, pH 7.5, 15 mM β-mercaptoethanol, 30 mg/ml bovine serum albumin, 1 mM ATP, 10 mM $MgCl_2$ and 150 mM KCl [4]. After 30 min incubation at 37 °C the reaction was terminated with 1% SDS and 1 mg/ml proteinase K (v/v). After an additional hour of incubation, aliquots were applied to a 0.8% agarose TBE gel (v/v) containing 0.5 mg/ml ethidium bromide and 1% SDS (w/v). Following overnight electrophoresis at 30 v (constant), the gel was destained and photographed using a UV-transilluminator and Polaroid film.

4.7. Statistical analysis

Data is displayed in Tables and the Fig. as the means ± standard deviations of the mean expressed as percentage of control. N is the number of samples per group. The Student's "t"-test was used to determine the probable level of significance (p) between test samples and control samples.

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