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Cytotoxicity of Metallic Complexes of Furan Oximes in Murine and Human Tissue Cultured Cell Lines

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The copper complexes of furan oxime derivatives were found to be potent cytotoxic agents in both murine and human tissue cultured cell lines which were suspended as well as solid tumors. Mode of action studies in murine L1210 lymphoid leukemia cells showed that the compounds suppressed DNA, RNA and protein synthesis after 60 min at 100 μM. Inhibition of purine and pyrimidine *de novo* syntheses, as well as inhibition of ribonucleoside reductase and nucleoside kinase activities with DNA strand scission occurred. All of these effects of the drug probably added to its ability to cause cell death but most important was the inhibition of DNA topoisomerase II activity with IC_{50} values lower than those afforded by VP-16, the standard, which should cause apoptosis. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

A series of metallic complexes of furan oximes have recently been synthesized. 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 pilot study of the metal complexes of furan oximes to determine their ability to retard tumor cell growth in tissue culture cells. The previous complexes were unlike *cis*-platinum derivatives in that the agents were inhibitors of metabolic enzyme activities, e.g. purine regulatory enzymes, and DNA topoisomerase II. Inhibition of DNA topoisomerase II activity in human cancer cells by antineoplastic agents, e.g. etoposide (VP-16), has led to agents which have specificity for cancer cells and have a higher log kill of the cancer cells.

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MATERIALS AND METHODS

Source of compounds

Synthesis of $[CuCl_2(5\text{-methyl-2-furaldehyde})_2]$ (**II**) and $[CuCl_2[3\text{-(2-furyl)prop-2-ene aldoxime}]_2]$ (**III**) were conducted as previously described.[5,6] $[CuCl_2(5\text{-nitro-2-furaldehyde})_2$ (**I**) and $[CuCl_2[3\text{-(2-furyl)prop-2-enal semicarbazone}]_2$ (**IV**) were obtained by reacting the ligand and copper chloride in stoichiometric amounts in refluxing anhydrous ethanol.[7]

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).

Pharmacological methods

Compounds **I–IV** (Table 1) were tested for cytotoxic activity by homogenizing drugs in a

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Table 1. Cytotoxicity of metallic complexes of furan oximes

	ED_{50} ($\mu g\ ml^{-1}$)											
Compound[a]	L1210 leukemia	Tmolt leukemia	HeLa-S_3 uterine	HeLa solid uterine	KB naso-pharynx	Skin A431	Ileum HCT-8	Colon SW480	Lung MB-9812	Lung A549	UMR-106	Glioma HS683
I	2.42	5.19	2.03	3.81	0.61	4.26	9.85	2.98	3.56	5.06	4.26	6.83
II	1.80	3.87	2.46	2.14	5.91	3.98	8.22	2.84	7.75	1.71	3.98	11.39
III	2.98	4.63	2.01	8.69	2.54	2.93	2.43	4.16	8.05	1.80	2.91	7.66
IV	1.53	3.61	1.55	9.27	4.53	8.44	3.45	3.31	8.25	2.50	8.44	8.40
6MP[a]	2.43	1.62	2.12	5.61	11.04	3.42	1.15	3.61	4.29	4.71	9.13	4.46
Ara-C[b]	2.41	2.67	2.13	4.74	2.84	0.92	2.54	3.42	6.16	6.28	0.86	1.88
Hydroxyurea	2.67	4.47	1.96	8.12	5.27	3.21	1.77	7.33	7.18	8.89	2.87	2.27
5-FU[c]	1.41	2.14	2.47	4.11	1.25	0.61	1.12	3.09	5.64	3.58	3.52	1.28
VP-16[d]	1.83		7.87	3.05	3.32	0.71	3.78	3.34	3.30	4.74	3.57	2.44

[a]Abbreviations: 6MP, 6-mercaptopurine; Ara-C, cytidine arabinoside; 5-FU, 5-fluorouracil; VP-16, etoposide.

Table 2. Effects of [$CuCl_2$(5-NO_2-2furaldehyde)$_2$ (**I**) on L1210 leukemia cell metabolism over 60 min

	Percentage of Control (x+SD)			
Assay (n=6)	Control	25 μM	50 μM	100 μM
DNA synthesis	100±5[a]	88±5	78±4*	52±3*
RNA synthesis	100±6[b]	47±4*	35±3*	24±3*
Protein synthesis	100±5[c]	93±5	72±4*	46±3*
DNA polymerase α	100±6[d]	96±6	93±5	88±6
mRNA polymerase	100±7[e]	153±5*	127±6*	104±5
rRNA polymerase	100±4[f]	70±4*	61±3*	59±4*
tRNA polymerase	100±7[g]	83±6	71±4*	61±4*
Ribonucleoside reductase	100±5[h]	53±4*	26±4*	25±3*
Dihydrofolate reductase	100±5[i]	106±7	103±5	67±4*
Purine *de novo* synthesis	100±5[j]	114±6	86±5	6±2*
PRPP amido transferase	100±6[k]	63±4*	48±3*	46±3*
IMP dehydrogenase	100±5[l]	43±4*	25±4*	19±4*
Carbamoyl phosphate synthetase	100±7[m]	88±6	71±5*	58±5*
Aspartate transcarbamoylase	100±6[n]	104±5	80±5*	72±4*
Thymidylate synthase	100±5[o]	44±5*	93±5	92±5
Thymidine kinase	100±6[p]	118±6	114±6	117±6
Thymidine monophosphate kinase	100±7[q]	48±5*	46±5*	56±5*
Thymidine diphosphate kinase	100±6[r]	44±3*	33±3*	52±4*
d(ATP)	100±5[s]			67±4*
d(GTP)	100±6[t]			91±5
d(CTP)	100±5[u]			139±6*
d(TTP)	100±4[v]			96±5

Control values for 10^6 cells/h:
[a]7719 dpm, [b]1014 dpm, [c]17492 dpm, [d]9019 dpm, [e]1343 dpm, [f]325 dpm, [g]400 dpm, [h]48780 dpm, [i]0.144 O.D. units, [j]28624 dpm, [k]0.0878 O.D. units, [l]19575 dpm, [m]0.807 mol *N*-carbamoyl aspartate, [n]0.273 mmol citrulline, [o]77616 dpm, [p]1371 dpm, [q]1179 dpm, [r]1891 dpm, [s]17.07 pmol, [t]13.58 pmol, [u]33.60 pmol, [v]31.04 pmol.
*$P \leqslant 0.001$ (Student's t test).

1 mM solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passing them through an acrodisc filter (0.45 μm). The following cell lines were obtained from American Type Culture Collection, Rockville, MD, USA, and maintained by literature techniques cited by the ATCC catalog: murine L1210 lymphoid leukemia, human $Tmolt_3$ acute lymphoblastic T cell leukemia, colorectal adenocarcinoma SW480, HCT-8 ileocecal adenocarcinoma, lung A549 carcinoma, lung bronchogenic MB-9812, rat osteosarcoma UMR-106, human KB epidermoid nasopharynx, epidermoid skin A431, HeLa-S^3 suspended and solid cervical carcinoma and glioma EH 118 MG. The protocol of Geran *et al*.[8] was used to assess the cytotoxicity of the compounds and standards in each cell line. Values for cytotoxicity were expressed as ED_{50} ($\mu g\ ml^{-1}$), i.e. the concentration of the compound inhibiting 50% of cell growth. ED_{50} values were determined by the Trypan Blue exclusion technique. A value of less than 4 $\mu g\ ml^{-1}$ was required for significant activity of growth inhibition. Solid tumor cytotoxicity was determined by the method of Liebovitz *et al*.[9] utilizing Crystal Violet/MeOH, and read at 580 nm (Molecular Devices).

Incorporation studies

Incorporation of labeled precursors into [^{3}H]DNA, [^{3}H]RNA and [^{3}H]protein for 10^6 L1210 cells was determined.[10] The concentration response at 25, 50 and 100 μM required for inhibition of DNA, RNA and protein syntheses was determined after 60 min incubations. The incorporation of [^{14}C]glycine (53.0 mCi $mmol^{-1}$) into purines was obtained by the method of Cadman *et al*.[11] Incorporation of [^{14}C]formate (53.0 mCi $mmol^{-1}$) into pyrimidines was determined by the method of Christopherson *et al*.[12]

Enzyme assays

Inhibition of various enzyme activities was performed by first preparing the appropriate L1210 cell homogenates or subcellular fractions, then adding the drug to be tested during the

Table 3. The effects of [$CuCl_2$(5-methyl-2-furaldehyde)$_2$] (**II**) on L1210 cell metabolism over 60 min

	Percentage of control (x+SD)			
Assay (n=6)	Control	25 μM	50 μM	100 μM
DNA synthesis	100±5	84±5	82±4*	52±3*
RNA synthesis	100±6	30±3*	30±4*	30±3*
Protein synthesis	100±5	66±4*	45±4*	35±3*
DNA polymerase α	100±6	123±5*	74±5*	73±3*
mRNA polymerase	100±7	120±6	116±5	93±5
rRNA polymerase	100±4	44±3*	38±3*	34±3*
tRNA polymerase	100±7	127±6	124±5*	83±6
Ribonucleoside reductase	100±5	79±6*	57±5*	52±5*
Dihydrofolate reductase	100±5	62±4*	44±3*	16±3*
Purine *de novo* synthesis	100±5	52±5*	30±4*	9±3*
PRPP amidotransferase	100±6	51±4*	48±4*	25±3*
IMP dehydrogenase	100±5	62±5*	48±6*	43±4*
Carbamoyl phosphate synthetase	100±7	86±6	70±4*	57±4*
Aspartate transcarbamoylase	100±6	89±5	73±5*	61±4*
Thymidylate synthase	100±5	80±6	93±6	137±7*
Thymidine kinase	100±6	119±6	88±6	42±4*
Thymidine monophosphate kinase	100±7	94±6	110±7	86±5
Thymidine diphosphate kinase	100±6	118±6	49±6*	51±4*
d(ATP)	100±5			88±5
d(GTP)	100±6			110±5
d(CTP)	100±5			172±7*
d(TTP)	100±4			131±6*

Control values are given in Table 2.

enzyme assay. For the concentration response studies, inhibition of enzyme activity was determined at 25, 50 and 100 μM of compounds **I–IV** after 60 min incubations. DNA polymerase α-activity was determined by cytoplasmic extracts isolated by the method of Eichler *et al*.[13] Nuclear DNA polymerase β was determined by isolating nuclei.[14] The polymerase assay for both α and β was described by Sawada *et al*.[15] with [^{3}H]TTP. Messenger-, ribosomal- and transfer-RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using [^{3}H]UTP.[16,17] Ribonucleoside reductase activity was measured using [^{14}C]CDP with and without dithiothreitol.[18] The deoxyribonucleotides [^{14}C]dCDP were separated from the ribonucleotides by TLC on polyethyleneimine (PEI) plates. Thymidine, TMP and TDP kinase activities were determined using [^{3}H]thymidine (58.3 mCi mmol^{-1}) in the medium of Maley and Ochoa.[19] Carbamoyl phosphate synthetase activity was determined with the method of Kalman *et al*.;[20] citrulline was determined colorimetrically.[21] Asparate transcarbamoylase activity was measured by the method of Kalman *et al*.;[20] carbamoyl aspartate was determined colorimetrically.[22] Thymidylate synthetase activity was analyzed by the method of Kampf *et al*.[23] 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*.[24] PRPP amidotransferase activity was determined by the method of Spassova *et al*.;[25] IMP dehydrogenase activity was analyzed with 8-[^{14}C]IMP (54 mCi mmol^{-1}) (Amersham, Arlington Heights, IL, USA) after separating XMP on PEI plates (Fisher Scientific) by TLC.[26] Protein content was determined for the enzymic assays by the Lowry technique.[27]

After deoxyribonucleoside triphosphates were extracted,[28] levels were determined by the method of Hunting and Henderson[29] with calf thymus DNA, *E. coli* DNA polymerase I, nonlimiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of [^{3}H-*methyl*]dTTP or [5-^{3}H]dCTP.

Table 4. The effects of [$CuCl_2$(3-2-furyl) prop-2-ene aldoxime)$_2$] (**III**) on L1210 cell metabolism over 60 min

	Percentage of control (x + SD)			
Assay (n = 6)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	125 ± 6	95 ± 4	45 ± 3*
RNA synthesis	100 ± 6	34 ± 3*	31 ± 3*	27 ± 3*
Protein synthesis	100 ± 5	39 ± 4*	35 ± 4*	26 ± 4*
DNA polymerase α	100 ± 6	93 ± 5	93 ± 6	71 ± 5*
mRNA polymerase	100 ± 7	70 ± 5*	44 ± 3*	39 ± 3*
rRNA polymerase	100 ± 4	115 ± 6	65 ± 4*	37 ± 4*
tRNA polymerase	100 ± 7	126 ± 7	97 ± 4	77 ± 5*
Ribonucleoside reductase	100 ± 5	102 ± 7	81 ± 5	33 ± 4*
Dihydrofolate reductase	100 ± 5	108 ± 6	107 ± 5	98 ± 6
Purine *de novo* synthesis	100 ± 5	95 ± 7	38 ± 5*	22 ± 3*
PRPP amidotransferase	100 ± 6	106 ± 5	97 ± 6	80 ± 4*
IMP dehydrogenase	100 ± 5	148 ± 6*	11 ± 4*	11 ± 2*
Carbamoyl phosphate synthetase	100 ± 8	64 ± 4*	56 ± 5*	45 ± 3*
Aspartate transcarbamoylase	100 ± 6	88 ± 5	71 ± 5*	64 ± 4*
Thymidylate synthase	100 ± 5	99 ± 6	137 ± 6*	168 ± 9*
Thymidine kinase	100 ± 6	86 ± 5	108 ± 6	111 ± 6
Thymidine monophosphate kinase	100 ± 7	90 ± 6	68 ± 5*	54 ± 4*
Thymidine diphosphate kinase	100 ± 6	77 ± 6*	69 ± 5*	73 ± 5*
d(ATP)	100 ± 5			98 + 5
d(GTP)	100 ± 6			107 ± 7
d(CTP)	100 ± 5			172 ± 9*
d(TTP)	100 ± 4			110 ± 6

Control values are given in Table 2.

The effects of compounds **I–IV** on DNA strand scission were determined by the methods of Suzuki *et al*.,[30] Pera *et al*.[31] and Woynarowski *et al*.[32] L1210 leukemia cells were incubated with 10 μCi [^{3}H-methyl]thymidine (84.0 Ci mmol^{-1}) for 24 h at 37 °C. L1210 cells (10^7) were harvested and then centrifuged at 600 $g \times 10$ min in 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 17 500 rpm at 20 °C for 60 min (Beckman rotor SW60). 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 DNA radioactivity. Thermal calf thymus DNA denaturation studies, UV absorption studies and DNA viscosity studies were conducted after incubation of compounds **I–IV** at 100 μM at 37 °C for 24 h.[33]

Isolation of L-1210 topoisomerase II enzyme

The procedure for isolating L1210 DNA topoisomerase II enzyme has been published previously.[34] The isolated enzyme was used for the P4 phage DNA unknotting assay. Inhibition of the activity was noted as a smear of the DNA, e.g. unknotted DNA fragments in the gel, as opposed to DNA knotting at the top of the gel.

DNA topoisomerase II activity inhibition

L1210 cells were pelleted and the DNA topoisomerase II enzyme was isolated from the nuclei as outlined by the published method of Liu *et al*.[35,36] The inhibition by compounds **I–IV** from 1 to 100 μM of DNA strand-passing activity was determined by a standard relaxation and unknotting assay for DNA topoisomerases.[36] Briefly, the P4 knotted DNA was prepared from the phage heads of infected *E. coli* (C117) cells and was used as the substrate. The enzyme reaction contained a unit of DNA topoisomerase II [that

Table 5. The effects of [$CuCl_2$(3-(2-furyl)prop-2-enal semicarbazone]$_2$] (**IV**) on L1210 nucleic acid metabolism after 60 min

	Percentage of control (x+SD)			
Assay (N=6)	Control	25 μM	50 μM	100 μM
DNA synthesis	100±5	104±6	113±6	104±5
RNA synthesis	100±6	49±4*	47±4*	20±3*
Protein synthesis	100±5	128±6	89±5	73±4*
DNA polymerase α	100±6	88±6	87±6	86±5
mRNA polymerase	200±7	88±6	64±4*	57±4*
rRNA polymerase	100±4	125±5*	68±5*	52±3*
tRNA polymerase	100±7	79±5*	51±3*	23±3*
Ribonucleoside reductase	100±5	78±4*	69±5*	44±4*
Dihydrofolate reductase	100±5	107±6	118±5	102±6
Purine *de novo* synthesis	100±5	93±5	36±3*	6±2*
PRPP amidotransferase	100±6	115±5	114±6	118±6
IMP dehydrogenase	100±5	114±5	52±3*	40±4*
Carbamoyl phosphate synthetase	100±7	92±5	79±6*	71±5*
Aspartate transcarbamoylase	100±6	83±6	74±4*	65±3*
Thymidylate synthase	100±5	79±6*	89±7	127±5*
Thymidine kinase	100±6	70±6*	61±5*	65±6*
Thymidine monophosphate kinase	100±7	140±6	81±7	68±5*
Thymidine diphosphate kinase	100±6	82±5	62±5*	51±5*
d(ATP)	100±5			88±6
d(GTP)	100±6			109±5
d(CTP)	100±5			178±7*
d(TTP)	100±4			100±4

Control values are given in Table 2.

amount which catalyzed the unknotting of 0.2 μg of P4 DNA] in 20 μl of 50 mM Tris-HCl pH 7.5 buffer, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM Na_3EDTA, BSA (30 μg ml^{-1}) and 1 mM ATP. The reaction was incubated for 60 min at 37 °C and then was stopped by adding 5 μl of 5% SDS (w/v), 50 mM Na_3EDTA, 20% sucrose (w/v), and 0.05 mg ml^{-1} Bromophenol Blue. The reaction product was analyzed on 0.7% (w/v) agarose gel in 89 mM Tris–borate buffer, pH 8.2 + 2 mM Na_3EDTA, electrophoresed at 23 V for 18 h, and then stained with ethidium bromide. The IC_{50} values for inhibition by the compounds were determined using a densitometer, and a photograph of the gel [Fig. 2] with UV at 300 nm illumination was taken, as permanent records. Photographs were obtained with Polaroid Type 667 film. IC_{50} values were determined by plotting the log of the concentration of the compounds versus the percentage of enzyme activity.

Cleavage–interference in L1210 cells

Whole L1210 cells or prepared nuclei from cells in log growth were pre-labeled with [^{3}H]thymidine (60–90 Ci $mmol^{-1}$, NEN) for 24 h. The reaction medium (250 μl) contained whole cells or isolated nuclei in buffer supplemented with 3 mM ATP and compounds **I–IV** at 100 μM for 60 min. Additional tubes were incubated with VP-16 at 40 μM for an additional 60 min with and without the compounds. After incubation at 37 °C, cells or nuclei were collected by centrifugation [500 $g \times 2$ min] and analyzed for protein-linked DNA breaks by the method detailed by Rowe *et al.*[37] and counted.

Phosphorylation of the isolated L1210 DNA topoisomerase II enzyme was measured by the method of DeVore *et al.*[38] using [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) (Dupont) for 30 to 60 min. The reaction was inactivated by 10% trichloroacetic acid (TCA) and filtered on GF/A glass-fiber filters and counted. Standard protein kinase C inhibitors, A_3 and bisindolylmaleimide (Calbiochem–Novabiochem) were also used.

Statistics

The mean and standard deviation are designated by $x \pm$ SD. The probable level of significance (P) between test and control samples were deter-

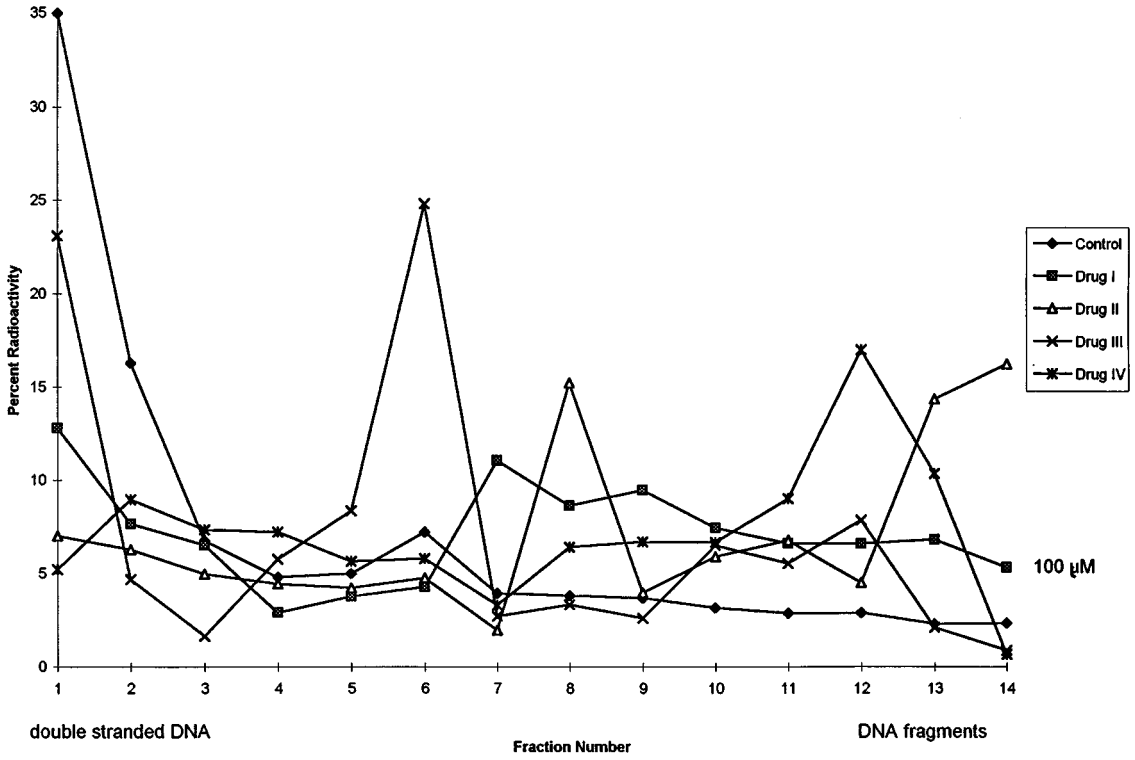

Figure 1 L1210 DNA strand scission after 24 h incubation with compounds **I–IV** or no drug (control).

mined by Student's *t* test or Dunnett's test with the raw data.

RESULTS

The metallic complexes of furan oximes proved to be potent cytotoxic agents (Table 1). In mouse L1210 lymphoid leukemia cells, compounds **I–IV** afforded ED_{50} values less than 3 $\mu g\ ml^{-1}$ (Table 1) with compounds **II** and **IV** exhibiting the best activity with ED_{50} values of 1.8 and 1.53 $\mu g\ ml^{-1}$, respectively. $Tmolt_3$ leukemia growth was inhibited only by compound **IV** with an ED_{50} value of 3.61 $\mu g\ ml^{-1}$. HeLa-S^3 suspended uterine cell growth was inhibited by compounds **I–IV** with ED_{50} values from 2.46 to 1.55 $\mu g\ ml^{-1}$. Compounds **I** and **II** were active against solid HeLa uterine carcinoma growth with ED_{50} values of 3.81 and 2.14 $\mu g\ ml^{-1}$, respectively. The colon adenocarcinoma growth was reduced by compounds **I, II** and **IV** with ED_{50} values from 3.31 to 2.84 $\mu g\ ml^{-1}$. HCT-8 ileum carcinoma growth was inhibited by compounds **II** and **IV** with ED_{50} values of 2.43 and 3.45 $\mu g\ ml^{-1}$. KB nasopharynx carcinoma growth was markedly reduced by compound **I** with an ED_{50} value of 0.61 $\mu g\ ml^{-1}$ and moderately by compound **III** with an ED_{50} value of 2.54 $\mu g\ ml^{-1}$. Skin A431 epidermoid growth was reduced by compounds **II** and **III** with ED_{50} values 3.98 and 2.93 $\mu g\ ml^{-1}$, respectively. Lung A549 growth was markedly reduced by compounds **II, III** and **IV** with ED_{50} values of

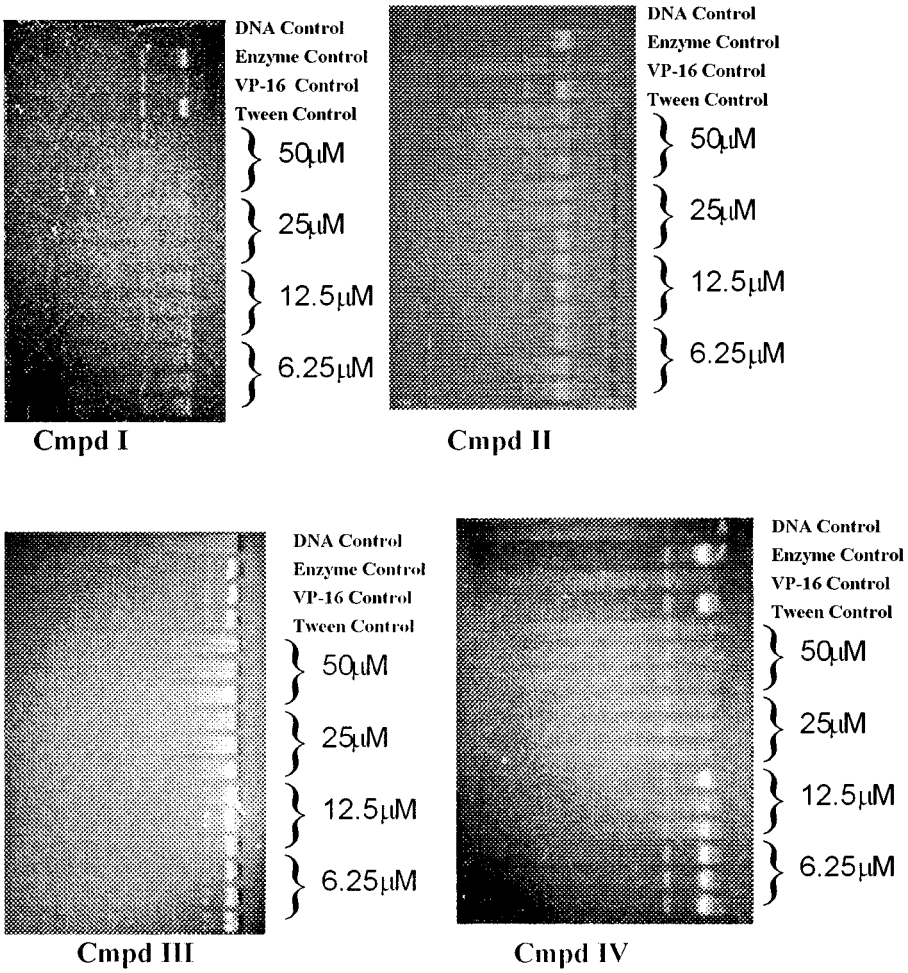

Figure 2

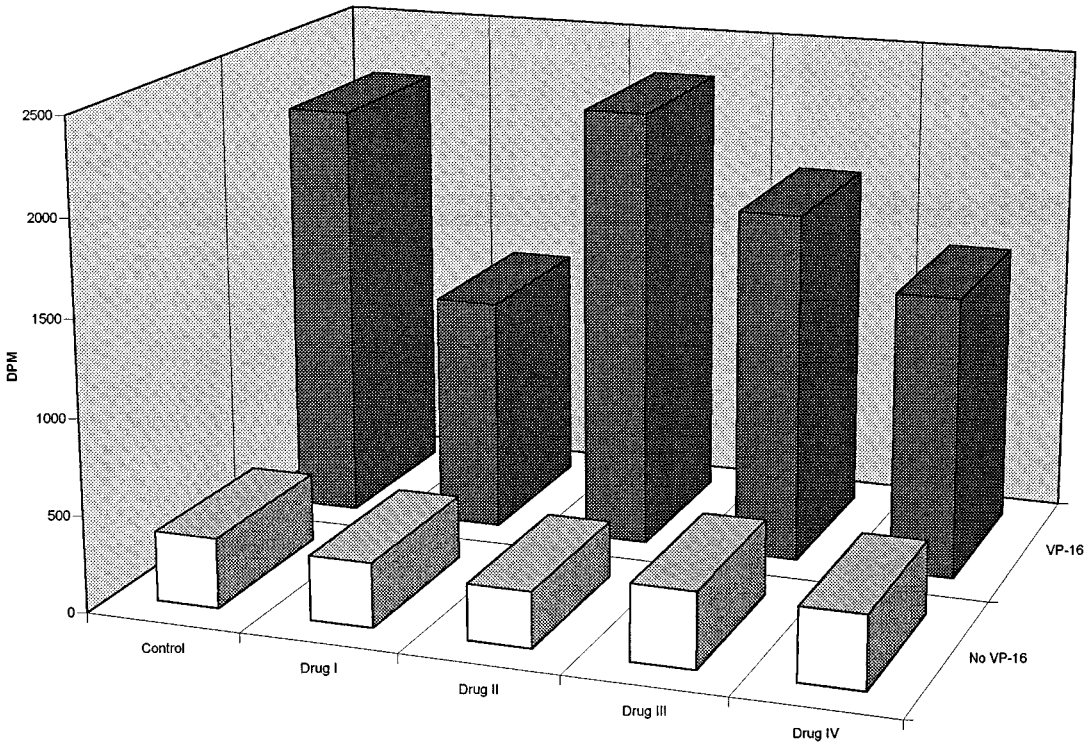

Figure 3 DNA protein-linked breaks

1.71, 1.80 and 2.50 $\mu g\,ml^{-1}$, respectively, but lung MB-9812 growth was only reduced by compound **I** with an ED_{50} value of 3.56 $\mu g\,ml^{-1}$. Human brain glioma solid cell growth was not inhibited significantly by any of the compounds, with ED_{50} values greater than 4 $\mu g\,ml^{-1}$. Rat osteosarcoma growth was affected only by compounds **II** and **III** with ED_{50} values of 3.98 and 2.91 $\mu g\,ml^{-1}$, respectively.

A mode-of-action study was conducted with the four compounds in the L1210 lymphoid leukemia tissue culture model (Tables 2–5). Compounds **I–III** inhibited DNA synthesis in a concentration-dependent manner (approximately 50% at 100 μM). RNA synthesis was actually reduced more significantly (approximately 70% at 100 μM after 60 min) and protein synthesis was reduced 54%, 65%, 74% and 27% respectively by compounds **I–IV**. DNA polymerase alpha activity was marginally inhibited 12–29% by the four compounds at 100 μM. mRNA polymerase activity was reduced only by compounds **III** and **IV**, affording 61% and 43% reduction at 100 μM after 60 min, whereas rRNA polymerase activity was reduced by all four compounds in a concentration-dependent manner with 41–66% reduction at 100 μM. t-RNA polymerase activity was reduced significantly by compounds **I** and **IV** with 39% and 77% reduction at 100 μM. Ribonucleoside reductase activities were moderately reduced by 48–75%, with compounds **I** and **IV** affording the best activity. Dihydrofolate reductase activity was reduced 33% at 100 μM concentration by compound **I** and 84% by compound **II**. Purine *de novo* synthesis was significantly reduced by 78% by compound **III** whereas compounds **I**, **II** and **IV** caused greater than 90% reduction. Phosphoribosyl pyrophosphate (PRPP) amidotransferase activity was significantly inhibited by 54%, 75% and 20% at 100 μM, respectively for compounds **I, II** and **III**. IMP dehydrogenase activity was inhibited 57–89%, with compounds **I** and **IV** resulting in greater than 80% reduction. The pyrimidine *de novo* synthetic pathway was inhibited significantly: carbamoyl phosphate synthetase activity was reduced in a concentration-dependent manner with 29–55% reduction at 100 μM after 60 min, and aspartate transcarbamoylase activity was inhibited 28–39% at 100 μM after 60 min. Thymidylate synthetase activity was actually stimulated slightly by

compounds **II–IV**. Thymidine kinase activity was reduced 58% and 35% by compounds **II** and **IV**, respectively, but compounds **I** and **III** had no effect. TMP kinase activity was reduced 14–46% by the agents. TDP kinase activity was reduced 48–49% by compounds **I**, **II** and **IV** but only 27% by compound **III**. d(CTP) Pool levels were elevated 39–78% after 60 min incubation with all four compounds at 100 μM; however, d(TTP) and d(GTP) levels were not affected at 100 μM concentrations of the agents.

Incubation of compounds **I–IV** at 100 μM for 24 h with ctDNA showed that a maximum absorption of ctDNA shifted to a lower wavelength in the UV absorption range with an increased absorption occurring. T_m values for thermal denaturation of ctDNA demonstrated values less than the control ctDNA values. ctDNA viscosity studies indicated less time necessary to move ctDNA through the reservoirs than for the control ctDNA. However, incubation of compounds **I–IV** with L1210 cells for 24 h resulted in DNA fragmentation (Fig. 1) since more of the radioactivity appeared in fraction numbers higher in gradient rather than double-stranded DNA at the bottom of the gradient (fraction 1). L1210 topoisomerase II activity was significantly inhibited by compounds **I–IV** with IC_{50} values of 15.4, 23.5, 21.5 and 15.5 μM, respectively (Fig. 2). These IC_{50} values were better than for VP-16, which afforded an IC_{50} value of 25.5 μM. L1210 DNA protein-linked breaks were not produced by the agents when used alone for 1 h (front row), nor did they increase those DNA protein-linked breaks afforded by VP-16 at 40 μM/control (back row) after another 1 h incubation (Fig. 3). In fact compound **I** attenuated the DNA protein-linked breaks caused by VP-16 and compounds **II** and **IV** were moderately effective in reducing breaks produced by VP-16. These data suggest that the copper complexes do have the same mechanism of inhibiting DNA topoisomerase II as the standard VP-16 which produces a cleavable DNA product.

Protein kinase C phosphorylation of the isolated L1210 DNA topoisomerase II enzyme was reduced after 30 min by 93%, 97%, 99% and 97% and after 60 min by 88%, 89%, 83% and 89% by compounds **I, II, III** and **IV**, respectively, at 100 μM. The standards A_3 at 100 μM caused 23% reduction of phosphorylation and bisindolylmaleimide at 20 nM caused 85% reduction at 30 min. After 2 h incubation, there was a 34% reduction for A_3 and a 64% reduction for bisindolylmaleimide.

DISCUSSION

The metallic complexes of furan oximes proved to be potent cytotoxic agents in human and murine tissue culture cell lines. Not only were the four derivatives active against the growth of suspended cell lines but also produced ED_{50} values of < 4 μg ml^{-1} against the growth of solid human tumors. For example, compound **III** was active against the growth of human KB nasopharynx, skin A431 epidermoid, ileum HCT-8, and lung A549 carcinomas and rat UMR-106 osteosarcoma. On the other hand, compounds **I, II** and **IV** were active in the SW480 colon adenocarcinoma screen. These new compounds were as active as clinically useful standards. Mode-of-action studies in L1210 lymphoid leukemia cells demonstrated that the four compounds were metabolic inhibitors. The compounds preferentially inhibited DNA and RNA synthesis principally because the agents were very effective in blocking purine *de novo* synthesis. All of the agents inhibited IMP dehydrogenase activity significantly and two compounds also reduced PRPP amidotransferase activity. By blocking the two regulatory enzyme activities in the purine pathway, then, the reduction in nucleic acid synthesis would be expected. Other sites that the agents inhibited were ribonucleoside reductases which would lower d(NTP) pool levels converted from r(NTP)s. Two of the compounds reduced dihydrofolate reductase activity which would reduce one carbon transfer for purine and pyrimidine synthesis. Enzyme activities in the pyrimidine pathway, e.g. carbamoyl phosphate, aspartate transcarbamoylase and thymidylate synthetase, were only moderately effected by the agents, and certainly not by a magnitude to justify the observed reduction of nucleic acids in 60 min. The nucleic kinase enzyme activities were reduced by the agents. Since DNA polymerase α activity was moderately reduced by the agents, there was no observable reduction in nucleotide pools because the deoxynucleotides were not being incorporated into new strands of DNA, but were accumulating inside the cell. rRNA and tRNA polymerase activities were reduced by the compounds but mRNA polymerase activity was not

always inhibited. The reduction in these polymerase activities would add to the overall reduction of RNA synthesis.

ctDNA studies demonstrated that there was no increase in DNA viscosity after incubating drugs with ctDNA after 24 h, indicating no cross-linking. However, the hyperchromic shift of the UV-absorption of ctDNA to a lower wavelength and the alteration of T_m values in the thermal denaturation studies indicate some type of interaction with the DNA bases. L1210 studies in which the drug was incubated for 24 h demonstrated that DNA strand scission was evident with each of the agents at 100 μM. This finding is consistent with the observed reduction in ctDNA viscosity after 24 h incubation with the agents at 100 μM.

Most important was that these copper complexes inhibited L1210 DNA topoisomerase II activity better than the clinical agent etoposide (VP-16). Agents which are topoisomerase II inhibitors usually have high cytotoxic action. Some topoisomerase II inhibitors cause DNA protein-linked breaks, e.g. epipodophyllotoxins, amsacrine, doxorubicin, ellipticine[39] and metal complexes of trimethylamine carboxyborane,[40] whereas others kill the cell by inhibiting DNA topoisomerase II activity by another mechanism, e.g. merbarone,[41] aciarubicin, bis(2,6-dioxopiperazine)s,[42] fostriecin[43,44] and metal complexes of thiosemicarbazones.[45] Phosphorylation of DNA topoisomerase II by protein kinases maintains the enzyme in the highest catalytic state. Agents which block the phosphorylation of the topoisomerase enzyme are linked with more DNA fragmentation and cell death.[40,46]

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