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Multiple Mechanisms for Cytotoxicity Induced by Copper(II) Complexes of 2-Acetylpyrazine-*N*-substituted Thiosemicarbazones

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The purpose of this study was to evaluate the mechanism by which 2-acetylpyrazine-^{4}*N*-substituted thiosemicarbazone copper II complexes mediate their cytotoxicity. These compounds were shown to be cytotoxic to a variety of human and rodent tumors in cell culture and are potent cytocidal agents as determined by dilute agar colony assays. They demonstrated the ability to inhibit several enzymes *in vitro* including DNA topoisomerase II activity. The data presented suggest that cytotoxicity may be mediated by the cumulative effect of several enzymes being inhibited by the agents. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: 2-acetylpyrazine-^{4}*N*-substituted thiosemicarbazone copper II complexes; cytotoxicity; topoisomerase II; DNA synthesis; enzyme inhibition

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INTRODUCTION

Metal complexes of heterocyclic thiosemicarbazones, thioureas and 2-substituted pyridines have previously been shown to be potent antineoplastic agents in the Ehrlich ascites carcinoma screen.[1,2] Cytotoxicity was demonstrated against L1210 and $Tmolt_3$ leukemias, as well as human solid tumor growth, e.g. HeLa, KB, skin, bronchogenic lung, bone osteosarcoma and glioma. A mode-of-action study in L1210 leukemia cells showed that DNA synthesis followed by RNA synthesis was inhibited. The major enzymatic sites of inhibition by the agents include inosine-5′-monophosphate (IMP) dehydrogenase, dihydrofolate reductase, DNA polymerase α, ribonucleoside reductase and nucleoside kinase. More important was the observation that DNA strand scission occurred after 24 h of incubation with the agents. There did not appear to be any evidence of crosslinking of the DNA strands or of intercalation of the agents between nucleic bases in the DNA molecule.[1,2] The present investigation is a further study of the reason why the agents are causing L1210 DNA strand breaks. Recently it has been demonstrated that these types of copper(II) complexes of thiosemicarbazones inhibit the activity of L1210 DNA topoisomerase II activity *in vitro*[3] but the analogous nickel complexes do not.[4]

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MATERIALS AND METHODS

All three of the compounds (Table 1) were previously synthesized and the chemical and physical characteristics reported.[5] 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).

Antineoplastic activity in vivo

CF_1 male mice ($\sim$28 g) were innoculated with 2×10^6 Ehrlich ascites carcinoma cells on day 0. Drugs prepared in 0.05% Tween 80/water were administered for nine days at 8 mg kg^{-1} day^{-1} intraperitoneally (i.p.). On day 10 the tumor was

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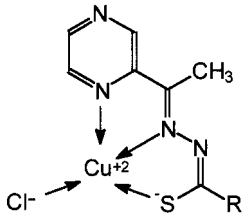
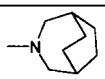
Table 1 Structures of 2-acetylpyrazine-^4N-substituted thiosemicarbazones and inhibition of L1210 lymphocytic leukemia DNA topoisomerase II activity



Compound	R	IC_{50} (μM)
1 Cu(Pzhexim)Cl Acetylpyrazine-3-hexamethyleneiminylthiosemicarbazonatochlorocopper(II)		6.25
2 Cu(Pzbnc)Cl Acetylpyrazine-3-azabicyclo[3.2.2]nonyl thiosemicarbazonatochloro copper(II)		12.2
3 Cu(Pz4DM)Cl Acetylpyrazine *N*(4)-dimethyl thiosemicarbazonatochloro copper(II)	$-N(CH_3)_2$	9.4

harvested and the volume and astrocrit were determined in order to calculate the percentage inhibition of tumor growth.[2] 6- Mercaptopurine (6-MP) and 5-fluorouracil (5FU) were used as the internal standards for the assay.

Cytotoxicity

Compounds **1**–**3** 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). The following cell lines were maintained by literature techniques:[1,2,6] murine L_{1210} lymphoid leukemia, rat UMR-106 osteosarcoma, human $Tmolt_3$ acute lymphoblastic T-cell leukemia, HeLa-S^3 suspended cervical carcinoma, HeLa solid cervical carcinoma, KB epidermoid nasopharynx, A431 epidermoid carcinoma, colorectal adenocarcinoma SW480, HCT-8 ileocecal adenocarcinoma, lung bronchogenic MB-9812, A549 lung carcinoma, TE418 osteosarcoma and glioma HS683. The protocol of Geran *et al.*[6] 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. Solid-tumor cytotoxicity was determined by the method of Leibovitz *et al.*[7] utilizing Crystal Violet/MeOH, and read at 562 nm (Molecular Devices). Values for cytotoxicity were expressed as ED_{50} (μg ml^{-1}), i.e. the concentration of the compound inhibiting 50% of cell growth. A value of less than 4 μg ml^{-1} was required for significant activity of growth inhibition.

Incorporation studies

The effects of drugs at 25, 50 and 100 μM on the incorporation of labeled precursors into [^{3}H]-DNA, [^{3}H]-RNA and [^{3}H]-protein for 10^6 L1210 cells were determined.[1,8] The incorporation of [^{14}C]glycine (53.0 mCi $mmol^{-1}$) into purines was obtained by the method of Cadman *et al.*[9] Incorporation of [^{14}C]formate (53.0 mCi $mmol^{-1}$) into pyrimidines was determined by the method of Christopherson *et al.*[10]

Enzyme assays

Inhibition of various enzyme activities was per-

formed by first preparing the appropriate L1210 cell homogenates or subcellular fractions, then adding the drug to be tested after optimizing the enzyme assay. For the concentration response studies, inhibition of enzyme activity was determined at 25, 50 and 100 μM of compounds **1**–**3** after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by the method of Eichler *et al.*[11,12] The DNA polymerase assay was described by Sawada *et al.*[13] with 2-deoxy[^{3}H]ribothymidine-5′-triphosphate (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]uridine-5′-triphosphate (UTP).[14,15] Ribonucleoside reductase activity was measured using [^{14}C]cytidine-5′-diphosphate (CDP) with dithioerythritol. 2′-Deoxy[^{14}C][16] ribocytidine- 5′-diphosphate was separated from the [^{14}C]CDP by thin-layer chromatography (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 mC mmol^{-1}) in the medium of Maley and Ochoa.[17] Carbamyl phosphate synthetase activity was determined by the method of Kalman *et al.*[18] and citrulline was determined colorimetrically.[19] Aspartate transcarbamylase activity was measured using incubation medium of Kalman *et al.*[18]; carbamyl aspartate was determined colorimetrically by the method of Koritz and Cohen.[20] Thymidylate synthetase activity was analyzed by the method of Kampf *et al.*[21] The ^3H_2O measured was proportional to the amount of TMP formed from 2′-deoxy[^{3}H]ribouridine-5′-monophosphate (UMP). Dihydrofolate reductase activity was determined by the spectrophotometric method of Ho *et al.*[22] Phosphoribosyl pyrophosphate (PRPP)- amidotransferase activity was determined by the method of Spassova *et al*[23] inosine-5′-monophosphate (IMP) dehydrogenase activity was analyzed with 8-[^{14}C]IMP (54 mC mmol^{-1}) (Amersham, Arlington Heights, IL, USA) after separating xanthosine-5′-monophosphate (XMP) on [PEI] plates (Fisher Scientific) by TLC.[24] Protein content was determined for the enzymic assays by the Lowry technique.[25]

DNA studies

After deoxyribonucleoside triphosphates (d[NTP]) had been extracted,[26] their levels were determined by the method of Hunting and Henderson[27] with calf thymus DNA, *Escherichia coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of [*methyl*-^{3}H]TTP 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 **1–3** on DNA strand scission were determined by the methods of Suzuki *et al.*,[28] Pera *et al.*[29] and Woynarowski *et al.*[30] L1210 lymphoid leukemia cells were incubated with 10 μCi [*methyl*-^{3}H]-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 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 sodium ethylenediamine tetra-acetic acid (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 0.0 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 12 000 $g \times 17$ 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 from 220 to 340 nm and DNA viscosity studies were conducted after incubation of compounds **1–3** at 100 μM at 37 °C for 24 h.[31]

L1210 DNA topoisomerase II was isolated by literature methods.[32,33] The 170 kDa topoisomerase II is present in the final preparation obtained using the following procedures. All steps were carried out at 0–4 °C. L1210 cells (2×10^8) were collected by centrifugation (500 g × 5 min.), washed twice with PBS and resuspended in buffer solution containing 0.25 M sucrose, 20 mM potassium phosphate buffer (pH 7.5), 2 mM $MgCl_2$, 1 mM spermidine, 0.1 mM EDTA 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM NaS_2O_3 at 4 °C. Cell membranes were lysed using a Potter's tissue grinder following the addition of Triton X-100 at a volume equivalent to 1/100th of the total volume of the cell suspension. Trypan Blue staining of nuclei was used to determine complete cell lysis microscopically. An equal volume of buffer containing 1.75 M sucrose was added to the cell suspension and mixed by gentle swirling. After mixing, the total volume was loaded on a sucrose cushion [1.4 M sucrose,

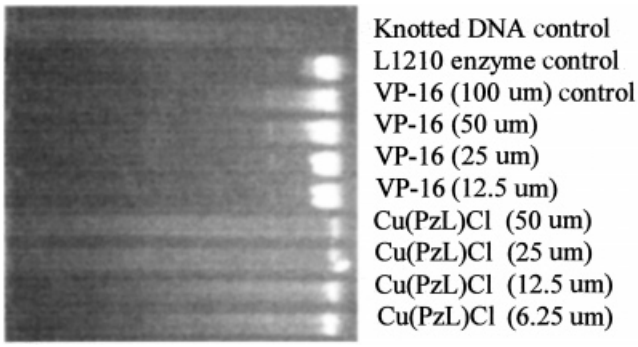


Figure 1 Effects of various concentrations of compound **3** on isolated L1210 topoisomerase II activity.

20 mM potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 1 mM PMSF] and centrifuged at 25 000 $g \times$ 45 min at 4 °C. The sucrose cushion was removed by vacuum aspiration and the remaining pellet was resuspended in buffer containing 20 mM potassium phosphate buffer (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 1 mM PMSF, 1 mM-mercaptoethanol, 10% glycerol, and 100 mM NaCl. The suspension was incubated at 4 °C for 30 min. The process was repeated with buffers containing increasing concentrations of NaCl up to 400 mM. The supernatants containing enzyme activity were used for assays.

The effects of compounds **1–3** on isolated DNA topoisomerase II activity were determined by the method of Miller *et al.*[33] (Fig. 1). Reactions containing 0.05 M Tris (pH 7.5), 0.1 M KCl, 0.01 M $MgCl_2$, 30 μg ml^{-1} bovine serum albumin, 0.5 mM EDTA, 1.0 mM DTT, 1.0 mM ATP, 0.1 μg knotted DNA (isolated by the method of Liu *et al.*[32]), 1 unit L1210 topoisomerase II and the drugs were prepared. The samples were allowed to incubate at 37 °C for 1 h and then stopped by the addition of stop buffer [50% w/v sucrose, 0.5% w/v sodium dodecylsulfate (SDS), and 0.25% w/v Bromophenyl Blue]. Each sample was run for 18 h using a 0.7% agarose gel in electrophoresis buffer (pH 8.0) (90 mM Tris, 2 mM EDTA, 90 mM boric acid) on a Gibco BRL Horizon 11 × 14 electrophoresis apparatus at 23 V. VP-16 (etoposide) was used as an internal standard inhibitor for DNA topoisomerase II assay. Photographs of gels were made by illumination of gels on a UV light table using Polaroid 667 film. Densitometric analysis was performed by the method of Hofmann *et al.*[35] using a GS 300 transmittance reflectance scanning densitometer and the *GS 365 Densitometry Program* (version 2) for personal computers (Hoefer Scientific Instruments, San Francisco, CA, USA). Photographs of agarose gels were scanned by the densitometer, in reflectance mode, perpendicularly to the direction of DNA migration, aligned with the unknotted DNA bands. To standardize the quantification of unknotted DNA, known amounts of completely unknotted P4 DNA were subjected to electrophoresis and photographed. The areas under the curves corresponding to unknotted DNA bands were calculated using the densitometry software package. Data were plotted as percentages of enzyme control. IC_{50} values were calculated by non-linear regression analysis of plotted data using *Prism*®, version 2 (GraphPad Software Inc., San Diego, CA, USA).

DNA protein-linked breaks

DNA protein-linked breaks were evaluated by the method of Rowe *et al.*[34] using approximately 10^7 L1210 cells growing in growth medium with 50 μCi [6-^{3}H]thymidine (15.0 Ci $mmol^{-1}$) for 24 h. The cell suspension was then centrifuged at 2000 g × 10 min at 4 °C to pellet the cells. The cells were resuspended in fresh medium at 37 °C for 2–3 h to wash out any unincorporated [*methyl*-^{3}H]thymidine.

Interference assay

The interference assay followed the procedures described for DNA protein-linked breaks, only differing in that after the 1–24 h incubation with sample compound, an equal volume of etoposide (VP-16) was added to each sample.[35] The cells were allowed to incubate at 37 °C for an additional hour after the addition of VP-16 solution.

Table 2 The cytotoxicity of 2-acetylpyrazine-^{4}*N*-substituted thiosemicarbazones

	ED_{50} (μg/mol^{-1})												
Compound	L1210	$Tmolt_3$	HeLa-S^3	HeLa	UMR-106	Bone TE418	Nasoph. KB	Skin A431	Colon SW480	Ileum HCT-8	Lung A549	Lung MB9812	Glioma Hs-683
1	1.76	0.72	2.49	0.11	0.32	1.44	0.27	0.01	0.45	0.39	2.74		1.28
2	0.33	2.75	2.89	0.80	1.66	—	1.11		1.02	0.55	1.79	0.89	0.75
3	2.00	1.84	3.11	0.12	0.23	0.62	0.48	0.02	0.47	0.29	3.29		1.98
5-Fluorouracil	1.41	2.14	2.47	4.11	3.52		1.25	0.61	3.09	1.12	3.58	5.64	1.28
Cytosine arabinoside	2.43	2.67	2.13	4.74	0.86		2.84	0.92	3.42	2.54	6.28	6.16	1.88
Etoposide (VP-16)	1.83		7.87	3.05	3.57		3.32	0.71	3.34	3.78	4.74	3.50	2.44
Actinomycin D	1.98		5.88	2.46	0.33	0.65	0.93	0.30	3.18	3.71	0.89	1.28	1.15
6-Mercaptopurine	2.43	1.62	2.12	5.61	9.13	7.46	11.04	3.42	3.61	1.15	4.78	4.29	4.46

Cleavage-interference in vitro

The method used to assess interference with drug-induced topoisomerase II-mediated DNA cleavage *in vitro* was based on the procedure of Harker *et al.*[36] The Hind III-cut PBR322 DNA was end-labeled with [o-^{32}P]dCTP (3000 Ci mmol^{-1}, ICN) using a commercial T4 polymerase labeling system with a 30 min incubation at 37 °C.

Dilute agar colony method

Following the modified method of Chu and Fischer,[37] murine L1210 lymphoid leukemia cells were grown as colonies suspended in agar. Cells (1×10^5), in RPMI-1640 with 10% fetal bovine serum, and penicillin / streptomycin were exposed to experimental drugs, or vehicle (dimethyl sulfoxide, DMSO) as the control for 1 h. After centrifugation, the cell pellet was resuspended in fresh medium, diluted and suspended in a 0.13% agar solution of growth medium, heated to 45 °C. Following five days of incubation at 37 °C, 0.01% Neutral Red was gently layered over each sample. On day 7, samples were decanted into six-well plates and colonies were counted using a colony counter over a 2 mm × 2 mm grid. Results were expressed as percentages of the colonies in the untreated control.

Statistical analysis

Data are displayed in tables and figures as the means ± standard deviations or standard errors of the mean. *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

Antineoplastic Activity in vivo

Compounds **1** and **3** were toxic to the mice when administered from 2 to 8 mg kg^{-1} day^{-1} i.p. for nine days. Compound **2** at 1 mg kg^{-1} day^{-1} afforded 99.8% inhibition of Erhlich ascites carcinoma growth. This value was competitive with 6-mercaptopurine, which afforded 99% inhibition of tumor growth, and 5-fluorouracil, which caused 95% inhibition of growth at 12 mg kg^{-1} day^{-1} i.p.

Cytotoxicity

All three compounds demonstrated potent cytotoxicity against the growth of all of the tumor cell lines tested, with ED_{50} values of less than 4 μg ml^{-1}, the required value for significant activity (Table 2). Most of the ED_{50} values were superior to the clinical standards tested in each tumor cell line. Compound **2** was very effective in suppressing growth of murine L1210 leukemia. Compound **1** inhibited human $Tmolt_3$ leukemia growth. HeLa solid uterine carcinoma growth was effectively inhibited by all three compounds, but in the HeLa-S^3 suspended screen, whereas the ED_{50} values were below 4 μg ml^{-1}, the compounds were less active. Compounds **1** and **3** were effective in suppressing rat UMR-106 osteosarcoma growth and compound **3** afforded an ED_{50} values of less than 1 μg ml^{-1} in the human bone screen. Compounds **1** and **3** were more effective in reducing the growth of KB nasopharynx and epidermoid skin A431 cancer growth as well as colon SW480 and ileum HCT-8 growth. Compound **2** demonstrated potent activity against the growth of lung A549, lung MB-9812 and glioma cells.

Mode-of-action study

In the L1210 leukemia cell culture, all three compounds effectively reduced DNA synthesis by 90% at 100 μM but RNA synthesis was inhibited ~34% by compound **1,** ~44% by compound **3** and ~56% by compound **2** at 100 μM (Tables 3–5). Compound **2** markedly reduced protein synthesis by 63% at 100 μM, but it was only marginally reduced by compounds **1** and **3** after 60 min.

DNA polymerase α activity was reduced more than 56% at 100 μM by all three agents. m-RNA polymerase activity was significantly reduced by the agents from 36% to 61% and r-RNA polymerase activity was also suppressed to a lesser magnitude, from 24% to 52%. t-RNA polymerase activity was suppressed marginally by compounds **2** and **3** but was actually stimulated by compound **1.** Ribonucleoside reductase and dihydrofolate reductase activities were reduced by all three agents. Compounds **1** and **3** achieved greater than 70% inhibition of ribonucleoside reductase activity at 100 μM. It should be noted that compound **2** had only marginal effects on dihydrofolate reductase activity, compared with the 79% and 84% inhibition of compounds **3** and **1,** respectively.

De novo purine synthesis was reduced from 45% to 90% by each of the three compounds. PRPP

Table 3 Effects of compound **1** on L1210 leukemia cell metabolism over 60 min (% of control ± SD)

		Concentration of **1**		
Assay ($N = 6$)	Control†	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^{a}	57 ± 5*	46 ± 4*	2 ± 1*
RNA synthesis	100 ± 6^{b}	80 ± 5*	76 ± 5*	66 ± 4*
Protein synthesis	100 ± 5^{c}	132 ± 6*	91 ± 7	81 ± 5
DNA polymerase α	100 ± 6^{d}	76 ± 5*	72 ± 4*	43 ± 4*
mRNA polymerase	100 ± 7^{e}	78 ± 5*	70 ± 4*	64 ± 4*
rRNA polymerase	100 ± 4^{f}	64 ± 6*	60 ± 5*	55 ± 4*
tRNA polymerase	100 ± 7^{g}	101 ± 5	127 ± 5	193 ± 9*
Ribonucleoside reductase	100 ± 5^{h}	65 ± 4*	57 ± 5*	27 ± 4*
Dihydrofolate reductase	100 ± 5^{i}	87 ± 4*	67 ± 5*	16 ± 2*
Purine synthesis *de novo*	100 ± 5^{j}	40 ± 4*	40 ± 3*	10 ± 3*
PRPP amidotransferase	100 ± 6^{k}	124 ± 6	174 ± 9*	243 ± 9*
IMP dehydrogenase	100 ± 5^{l}	47 ± 6*	41 ± 5*	24 ± 3*
Pyrimidine synthesis *de novo*	100 ± 6^{m}	56 ± 4*	47 ± 5*	39 ± 5*
Carbamyl phosphate synthetase	100 ± 8^{n}	168 ± 8*	138 ± 5*	132 ± 6*
Aspartate transcarbamylase	100 ± 6^{o}	101 ± 6	100 ± 5	98 ± 5
Thymidylate synthetase	100 ± 5^{p}	72 ± 4*	59 ± 4*	56 ± 4*
Thymidine kinase	100 ± 6^{q}	101 ± 5	97 ± 4	85 ± 4
Thymidine monophosphate kinase	100 ± 7^{r}	93 ± 4	91 ± 5	73 ± 4*
Thymidine diphosphate kinase	100 ± 6^{s}	94 ± 5	85 ± 4	72 ± 5*
d[ATP]	100 ± 5^{t}			53 ± 5*
d[GTP]	100 ± 6^{u}			83 ± 5
d[CTP]	100 ± 5^{v}			224 ± 6*
d[TTP]	100 ± 4^{w}			182 ± 7*

* $P \leq 0.001$.

† The measured control values were as follows (dpm, disintegrations per minute; OD, optical density):

[a] 26152 dpm
[b] 4851 dpm
[c] 7461 dpm
[d] 47804 dpm
[e] 4239 dpm
[f] 1502 dpm
[g] 6400 dpm
[h] 2744 dpm
[i] 0.868 OD units
[j] 92551 dpm
k 0.121 OD units
[l] 76058 dpm
[m] 0.392 mol citrulline
[n] 1.064 mol *N*-carbamyl aspartate
[o] 18463 dpm
[p] 1317 dpm
[q] 1179 dpm
[r] 1891 dpm
[s] 6.17 pmol
[t] 5.27 pmol
[u] 6.87 pmol
[v] 6.94 pmol
[w] $P \leq 0.001$

amido transferase activity was suppressed only by compound **3,** by 32% at 100 μM but the regulatory site of IMP dehydrogenase activity was suppressed by agents from 49–76% at 100 μM. *De novo* pyrimidine synthesis was also markedly reduced by all three compounds but the site of inhibition by the agents was not early in the pathway since carbamyl phosphate synthetase and aspartate transcarbamylase activities were unaffected by the three agents. On the other hand, thymidylate synthetase activity was reduced significantly, from 38% to 44% at 100 μM by the compounds. Thymidine kinase activity was inhibited 40% by compound **2** but the activity was inhibited (15% to 26%) only moderately by compounds **1** and **2.** TMP kinase activity was inhibited 26% to 28% by compounds **3** and **1** and TDP kinase activity was inhibited 28% to 44% by all three compounds. d[GTP] pool levels were reduced by compounds **1** and **2** whereas d[ATP] pool levels were reduced by all three compounds, yet d[CTP] and d[TTP] pools were elevated by all three agents after 60 min incubations.

Calf thymus DNA (ct-DNA) studies showed that after incubation with the agents at 100 μM, the thermal denaturation value T_m rose from the control value of 87.5 °C to 92.5 °C for compounds **2** and **3.** DNA viscosity after 24 h of incubation for the control was 323 s (time to pass through the reservoirs). Compound **3** afforded a time of 275 s, compound **1** 291.6 s and compound **2** 287 s. The UV absorption of ct-DNA from 220 to 340 nm indicated no hyperchromic shift with the drugs. L1210 DNA strand scission after incubation with the agents at 100 μM demonstrated DNA fragmentation (Fig. 2).

The copper(II) complexes of 2-acetylpyrazine-^{4}*N*-substituted thiosemicarbazones were

Table 4 Effects of compound **2** on L1210 leukemia cell metabolism over 60 min (% of control ± SD)

		Concentration of **2**		
Assay ($N = 6$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^{a}	102 ± 6	21 ± 4*	2 ± 1*
RNA synthesis	100 ± 6^{b}	66 ± 4*	61 ± 5*	44 ± 3*
Protein synthesis	100 ± 5^{c}	63 ± 7*	52 ± 5*	37 ± 3*
DNA polymerase α	100 ± 6^{d}	57 ± 5*	54 ± 5*	41 ± 5*
mRNA polymerase	100 ± 7^{e}	91 ± 5	61 ± 5*	55 ± 4*
rRNA polymerase	100 ± 4^{f}	199 ± 8*	130 ± 6*	76 ± 5*
tRNA polymerase	100 ± 7^{g}	128 ± 5	85 ± 5	81 ± 4
Ribonucleoside reductase	100 ± 5^{h}	99 ± 7	89 ± 6	52 ± 5*
Dihydrofolate reductase	100 ± 5^{i}	110 ± 6	85 ± 5	82 ± 4
Purine synthesis *de novo*	100 ± 5^{j}	61 ± 5*	55 ± 5*	45 ± 4*
PRPP amido transferase	100 ± 6^{k}	166 ± 8*	134 ± 6*	215 ± 7*
IMP dehydrogenase	100 ± 5^{l}	73 ± 7*	61 ± 6*	51 ± 5*
Pyrimidine synthesis *de novo*	100 ± 6^{m}	29 ± 4*	27 ± 5*	15 ± 3*
Carbamyl phosphate synthetase	100 ± 7^{n}	102 ± 5	103 ± 5	92 ± 6
Aspartate transcarbamylase	100 ± 6^{o}	102 ± 5	99 ± 6	98 ± 6
Thymidylate synthetase	100 ± 5^{p}	69 ± 4*	65 ± 4*	62 ± 3*
Thymidine kinase	100 ± 6^{q}	137 ± 6*	82 ± 5	60 ± 4*
Thymidine monophosphate kinase	100 ± 7^{r}	129 ± 5*	124 ± 5	88 ± 5
Thymidine diphosphate kinase	100 ± 6^{s}	142 ± 7*	106 ± 5	72 ± 3*
d(ATP)	100 ± 5^{t}			10 ± 2*
d(GTP)	100 ± 6^{u}			78 ± 5*
d(CTP)	100 ± 5^{v}			248 ± 7*
d(TTP)	100 ± 4^{w}			243 ± 8*

* $P \leq 0.001$.
† See Table 3 footnote.

potent inhibitors of L1210 (Fig. 3) topoisomerase II, with IC_{50} values of 6.25–12.2 μM, compared with etoposide (VP-16) with an IC_{50} value of 22.5 μM (Table 1). VP-16 induces DNA protein-linked breaks but the copper pyrazine thiosemicarbazones at concentrations from 25–100 μM did not cause breaks when incubated alone over 24 h. However, when incubated with VP-16, the compounds reduced the ability of VP-16 to cause DNA protein-linked breaks in a concentration-dependent manner (Fig. 4). A more sensitive assay, the end-labeled PBR322 DNA interference assay, confirmed that the agents blocked VP-16-induced breaks in a concentration-dependent manner over 30 min. To determine whether these effects were reversible, cells were exposed to various concentrations of compound **1** for 1 h, then resuspended in drug-free dilute agar. Compound **1** demonstrated an ED_{50} value of ~100 nM in its ability to inhibit colony growth. These results suggest that at concentrations that affect DNA topoisomerase II activity in cell culture, i.e. 5–10 μM, compound **1** causes irreversible damage to cells following short exposure (Fig. 5).

DISCUSSION

The three copper complexes of 2-acetylpyrazine ^{4}N-substituted thiosemicarbazone demonstrated similar antineoplastic activity and modes of action to the copper(II) and nickel(II) complexes of heterocyclic thiosemicarbazones and 2-substituted pyridine *N*-oxides. DNA synthesis appears to be the major site of action of the agents and was reduced by more than 90% over 60 min. RNA synthesis was moderately reduced as observed for other thiosemicarbazone complexes, and protein synthesis was selectively reduced by certain compounds only. DNA synthesis was reduced because *de novo* purine synthesis and pyrimidine synthesis were markedly reduced. Purine synthesis appeared to be blocked at the IMP dehydrogenase site but not at the other regular site in the pathway, i.e. that of PRPP amidotransferase. The reduction of the IMP dehydrogenase activity by the agents is sufficient to explain the observed reduction in purine synthesis. Dihydrofolate reductase activity, when inhibited by compounds **1** and **2,** should be additive with the overall inhibition of purine synthesis, in that one

Table 5 Effects of compound **3** on L1210 leukemia cell metabolism over 60 min (% of control ± SD)

		Concentration of **3**		
Assay ($N = 6$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^{a}	90 ± 4	30 ± 4*	2 ± 2*
RNA synthesis	100 ± 6^{b}	80 ± 4	66 ± 4*	56 ± 4*
Protein synthesis	100 ± 5^{c}	97 ± 6	96 ± 5	87 ± 5
DNA polymerase α	100 ± 6^{d}	50 ± 4*	47 ± 3*	40 ± 3*
mRNA polymerase	100 ± 7^{e}	71 ± 5*	70 ± 6*	39 ± 4*
rRNA polymerase	100 ± 4^{f}	66 ± 5*	64 ± 4*	48 ± 4*
tRNA polymerase	100 ± 7^{g}	99 ± 7	96 ± 5*	88 ± 5
Ribonucleoside reductase	100 ± 5^{h}	79 ± 5*	25 ± 3*	24 ± 2*
Dihydrofolate reductase	100 ± 5^{i}	100 ± 6	30 ± 3*	21 ± 3*
Purine synthesis *de novo*	100 ± 5^{j}	80 ± 5*	63 ± 5*	55 ± 4*
PRPP amido transferase	100 ± 6^{k}	107 ± 6	102 ± 5	68 ± 5*
IMP dehydrogenase	100 ± 5^{l}	70 ± 5*	50 ± 5*	39 ± 4*
Pyrimidine synthesis *de novo*	100 ± 6^{m}	33 ± 4*	28 ± 3*	13 ± 3*
Carbamyl phosphate synthetase	100 ± 7^{n}	134 ± 5*	123 ± 5	119 ± 6
Aspartate transcarbamylase	100 ± 6^{o}	102 ± 4	101 ± 4	99 ± 5
Thymidylate synthetase	100 ± 5^{p}	82 ± 4	81 ± 3*	59 ± 4*
Thymidine kinase	100 ± 6^{q}	99 ± 5	76 ± 4*	74 ± 3*
Thymidine monophosphate kinase	100 ± 7^{r}	92 ± 5	80 ± 5	74 ± 4*
Thymidine diphosphate kinase	100 ± 6^{s}	125 ± 5	69 ± 4*	56 ± 3*
d[ATP]	100 ± 5^{t}			117 ± 5
d[GTP]	100 ± 6^{u}			78 ± 3*
d[CTP]	100 ± 5^{v}			294 ± 7*
d[TTP]	100 ± 4^{w}			259 ± 6*

* $P \leq 0.001$.
† See Table 3 footnote.

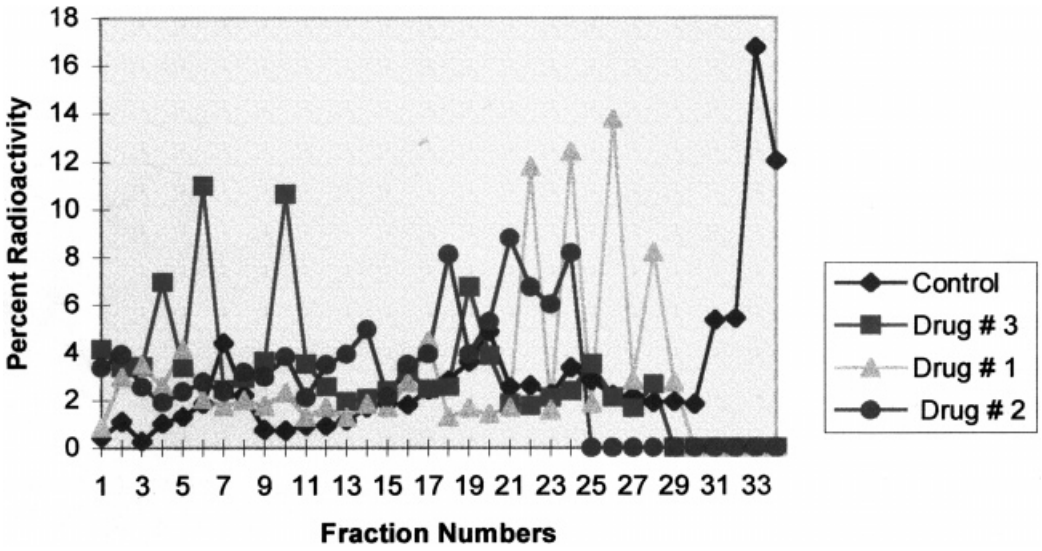


Figure 2 L1210 strand scission due to 24 h of incubation with **1, 2** and **3** at 100 μM.

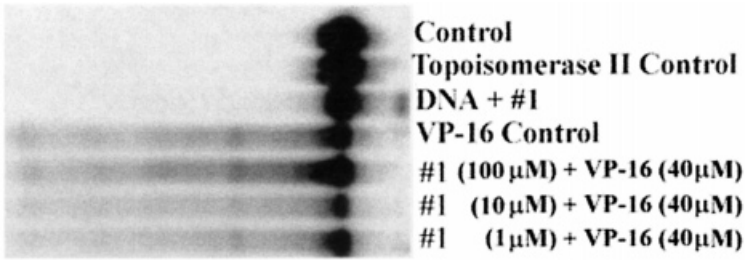


Figure 3 Effects of compound **1** on VP-16-induced breaks in [^{32}P]-end-labeled PBR322 DNA. The K^+/SDS-precipitated counts for each sample are as follows: enzyme control, 193 cpm; VP-16, 1293 cpm; **1** (100 μM) = 982 cpm; **1** (10 μM) = 1110 cpm; **1** (1 μM) = 1212 cpm. Note that the deposit at the origin is similar in size to the DNA topoisomerase II control

carbon transfer is blocked for nucleic acid synthesis. Pyrimidine synthesis was blocked by the agents but this was not at the early steps in the pathway. Thymidylate synthetase activity was moderately blocked by all three agents but the effect of the drugs on sites such as OMP decarboxylase were not determined in this study. The compounds were able to reduce the activity of DNA polymerase (α) and ribonucleoside reductase activities, which should again lead to reduced DNA synthesis. The reduction of the activity of RNA polymerases would lead to moderate reductions in RNA synthesis being observed. The RNA pool in mammalian cells is greater than the DNA pools; therefore, blockage of the purine and pyrimidine pathways would take longer than 60 min to be reflected in overall RNA levels. As can be observed from the deoxyribonucleotide (d[NTP]) pool levels, the purine deoxynucleosides were markedly affected by the agents but the deoxypyrimidine pools were not. If DNA polymerase (α) activity was inhibited, one would expect the d[NTP] pools to accumulate since they are not being incorporated into the new strand of DNA. This elevation was certainly observed for the deoxypyrimidine pools but not for the deoxypurine pools, which suggests a major metabolic block by the agents suppressing their synthesis in the purine pathway. Since IMP dehydrogenase activity was markedly affected by the drugs, this should lower purine nucleosides, which should be reflected in a lowering of d[GTP] and d[ATP] pool levels. L1210 DNA strand scission was evident after incubating the agents at 100 μM for 24 h. This may be due to the potent blockage of DNA topoisomerase II activity, which should lead directly to apoptosis. The decrease in viscosity evident in the ct-DNA studies would be

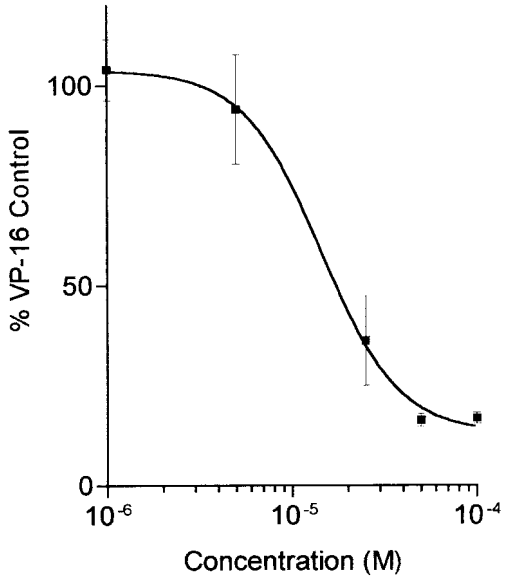


Figure 4 Interference with VP-16-induced L1210 cell protein-linked DNA breaks in the presence of various concentrations of compound **1.**

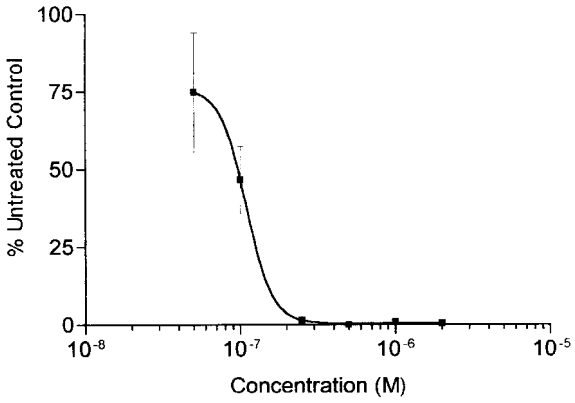


Figure 5 Colony formation of L1210 cells in dilute agar in response to various concentrations of compound **1.**

consistent with the observed fragmentation of the DNA by the agents. ct-DNA studies also indicated that the agents caused changes in DNA thermal denaturation and UV absorption of the DNA indicative of some type of interaction between the agents and DNA which was not intercalation of the drug between base pairs of the DNA. Like the 2-acetyl-(6-picolyl)-^4N-substituted thiosemicarbazones, these pyrazine derivatives were potent inhibitors of DNA topoisomerase II activity without forming cleavage products. The mode of action was not similar to VP-16, which does give rise to cleavage products. Nevertheless, the agents did block the ability of VP-16 to form DNA protein-linked breaks in a concentration-dependent manner. This would suggest that these derivatives are DNA topoisomerase inhibitors which function similarly to fostriencin at high concentrations, which also does not form cleavage products. However, the ability of these compounds to inhibit colony outgrowth at nanomolar concentrations indicated that the inhibition of DNA topoisomerase II activity is probably not the only target of the drug involved in cytotoxicity. It is highly possible that the cell death results from a cumulative effect on DNA synthesis and DNA topoisomerase II activity, leading to strand scission. Therefore, these agents demonstrate multiple means of inhibiting DNA synthesis and affording tumor cell death.

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