APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 12, 87–97 (1998)

The Synthesis and Antitumor Activity of Metal Complexes of Amine–Carboxyborane Adducts

M. C. Miller III[1], A. Sood[2] B. F. Spielvogel[2] and I. H. Hall[1†]
[1] Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7360, USA
[2] Boron Biologicals, Inc., 620 Hutton St., Raleigh, NC 27606, USA

The amine carboxyborane metal complexes, tetrakis - μ - (trimethylamine – boranecarbo - xylato)acetonitrile dicopper(II) and bis-μ-(morpholine–boranecarboxylato)zinc(II) dihydrate demonstrated cytotoxic activity against human Tmolt$_3$, HeLa-S^3 and MB-9812 cell growth. Tetrakis-μ-(trimethylamine–boranecarboxylato)-acetonitrile dicopper(II) and bis - μ - (morpholine – boranecarboxylato)zinc (II) dihydrate inhibited L$_{1210}$ DNA, RNA and protein syntheses, with greatest inhibitory effects on DNA synthesis. The reduction in DNA synthesis correlates well with inhibition of *de novo* purine synthesis and the key enzymes involved in this pathway, i.e. IMP dehydrogenase and PRPP amido transferase. These compounds were also observed to induce DNA strand scission but did not appear to intercalate between base pairs of DNA, alkylate bases or cause cross-linking of the strands of DNA. Tetrakis-μ-(trimethylamine – boranecarboxylato)acetonitrile dicopper(II) also demonstrated the ability to inhibit L$_{1210}$ DNA topoisomerase II activity. © 1997 John Wiley & Sons, Ltd.

Appl. Organometal. Chem. **11**, 87–97 (1998)

Keywords: antitumor, metal complexes, amine–carboxyborane

Received 8 January 1997; accepted 9 June 1997

† Correspondence to: I. H. Hall, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7360, USA.
Contract grant sponsor: The Elsa U. Purdee Foundation.
Contract grant sponsor: North Carolina Biotechnology.

INTRODUCTION

Previously several amine–carboxyborane analogs of α-amino-acids were shown to be effective antineoplastic agents.[1] However, when metal ions (e.g. Cu^{2+}, Co^{2+}, Co^{3+}, Cr^{3+}, Fe^{3+} and Zn^{2+}) were coupled with these amine–carboxyboranes to form metal complexes, their antineoplastic activity was markedly improved *in vivo* and in tissue culture.[2] A related compound, tetrakis - μ - (trimethylamine – borane - carboxylato)acetonitrile dicopper(II) (**I**) has previously been synthesized,[3] although this compound's antitumor activity was never investigated. Recent ly a series of heterocyclic aminecarboxyboranes were shown to have similar antineoplastic/cytotoxic action to trimethylamine carboxyborane.[4] A zinc complex of a heterocyclic aminecarboxyborane has previously been synthesized,[5] but studies evaluating the biological activity of these types of compounds have not been performed to date. The purpose of this study is to compare the cytotoxic activity and mode of action of two amine–carboxyborane metal complexes with those of the corresponding parent compounds tetrakis-μ-(trimethylamine–boranecarboxylato) - bis(tri - methylamine – car - boxyborane)dicopper(II) and morpholine–carboxyborane, respectively.

MATERIALS AND METHODS

Chemistry

Reagents and apparatus

All chemicals were used as received from the manufacturer. Solvents were distilled prior to use. Trimethylamine carboxyborane was provided by Boron Biologicals, Inc. (Raleigh, NC, USA). All other chemicals used in syntheses were purchased from Aldrich Chemical Com-

CCC 0268–2605/98/020087–11 $17.50

pany (Milwaukee, WI, USA). A Perkin–Elmer 1320 Infrared Spectrophotometer was used for infrared (IR) analyses. IR spectra were obtained as KBr disks or as Nujol mulls using sodium chloride plates. A Varian 300 MHz NMR spectrometer was used to generate ^{1}H NMR spectra. Chemical shifts are relative to the external standard tetramethylsilane (δ=0 ppm). A Thomas–Hoover capillary melting-point apparatus was used to determine melting points, which were uncorrected. Elemental analyses were performed by M–H–W Laboratories (Phoenix, AZ, USA). Silica-gel 60F 254 plates (silica gel on aluminum; Aldrich Chemical Company) were used for thin-layer chromatography (TLC).

Synthesis of tetrakis-μ-(trimethylamine–boranecarboxylato)acetonitrile dicopper(II) (1)

Tetrakis-μ-(trimethylamine–boranecarboxylato) acetonitrile dicopper(II) (**1**) was prepared by the method of Silvey.[3] Tetrakis-μ-(trimethylamine – boranecarboxylato) - bis(trimethylamine – carboxyborane) dicopper(II) (0.147 g, 0.178 mmol) was dissolved in anhydrous acetonitrile (25 ml) to give a green–blue solution. This solution was slowly concentrated under nitrogen over 24 h to a final volume of 2 ml, resulting in small, dark-green crystals of compound **1**. Yield: 68.7 mg (62%); m.p. 155–165 °C (dec.). IR $(cm^{-1})_{KBr}$: 2370 ν_{BH}, 2220 ν_{CN}, 1480 ν_{COO}. Calcd: C, 34.21; H, 7.50; N, 11.08; Found: C, 34.21; H, 7.60; N, 10.90%.

Synthesis of bis-μ-(morpholine–boranecarboxylato)zinc(II) dihydrate (2)

Morpholine–carboxyborane (1.03 g, 7.1 mmol) was dissolved in water (deionized and distilled, 20 ml) at 54 °C and then cooled to 40 °C. A solution of sodium carbonate (0.753 g, 7.1 mmol) in water (deionized and distilled, 5 ml) was heated to 40 °C and combined with the aqueous solution of the amine–carboxyborane. The temperature of this solution was held between 40 and 45 °C for 35 min. The solvent was removed under reduced pressure, leaving a white solid. The solid was extracted and dried (four times with anhydrous methanol), and the remaining insoluble white solid was removed by vacuum filtration. The filtrate was evaporated to dryness on a rotary evaporator and the residue was then dried *in vacuo* for 3 h, leaving the sodium salt of morpholine–carboxyborane. Yield: 1.119 g (94%); m.p. 274–276 °C (dec.).

A column (300 mm×20 mm) was packed with IR-120 cation-exchange resin to a bed volume of 77 ml. The packing was washed with water (deionized and distilled, 4×100 ml) then charged with hydrochloric acid (1 M, 4×100 ml). The column was washed with water (deionized and distilled, 100 ml) to remove the excess acid from the packing. A solution of zinc chloride (1 M, 4×100 ml) was allowed to pass through the column. This was followed by washing the excess zinc solution off the column with water (deionized and distilled) until the sample gave a negative chloride test result using 1% $AgNO_3$. The solvent in the column was changed from water to methanol by using a gradient, increasing methanol content by 10% each 100 ml, until pure methanol was eluted. Sodium morpholine–borylcarboxylate (600 mg) was dissolved in dry methanol (8 ml) and eluted through the column. After elution of approximately 75 ml of methanol, white crystals appeared in the collection flask yield: 372.7 mg (59%); m.p. 195–200 °C (dec.). ^{1}H NMR (CD_3OD): δ 3.85 (m, 4H, $2CH_2$); δ 3.68 (m, 4H, $2CH_2$); δ 3.15 (m, 4H, $2CH_2$); δ 2.71 (m, 4H, $2CH_2$); δ 1.84 (m, 4H, $2BH_2$); IR $(cm^{-1})_{Nujol}$: 3300 ν_{NH}, 2360 ν_{BH}. Calcd: C, 30.85; H, 6.73; N, 7.20. Found: C, 31.24; H, 6.54; N, 7.15%.

In vivo antineoplastic activity

CF_1 male mice (25–30 g) were inoculated with 2×10^6 cells intraperitoneally in isotonic saline (pH 7.0) a day before the administration of the drugs. Drugs were suspended and homogenized in 0.05% Tween-80/H_2O and administered to the mice intraperitoneally at 8 mg kg^{-1} day^{-1} from day 1 to day 9. On day 10, animals were sacrificed by cervical dislocation. A transverse incision was made across the abdomen and the ascitic fluid was drained and the volume measured. Samples of ascitic fluid were collected and centrifuged, and astrocrits were calculated. Percentage inhibition values were calculated for all compounds according to the literature procedures.[1, 2, 4] The antineoplastic agents 5-FU and 6-MP were used as standards in this screen.

Cytotoxicity

Compounds **1** and **2** were tested for cytotoxic activity by homogenizing drugs in 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:[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 and glioma HS683. The protocol of Geran *et al*.[7] 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*.[6] utilizing crystal violet/MeOH and read at 562 nm (Molecular Devices). 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.

Incorporation studies

Incorporation of labeled precursors into [^{3}H]DNA, [^{3}H]RNA and [^{3}H]protein for 10^6 L_{1210} cells was obtained.[8] The concentration response at 25, 50 and 100 μM for inhibition of DNA, RNA and protein synthesis was determined for 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*.[9] Incorporation of [^{14}C]formate (53.0 mCi/mmol) into pyrimidines was determined by the method of Christopherson *et al*.[10]

Enzyme assays

Inhibition of various enzyme activities was performed by first preparing the appropriate L_{1210} cell homogenates or subcellular fractions, then adding the drug to be tested during the enzyme assay. For the concentration response studies, inhibition of enzyme activity was determined at 25, 50 and 100 μM of compounds **1** and **2** after 60-min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by the method of Eichler *et al*.[11] The assay for DNA polymerase α was described by Sawada *et al*.[12] 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.[13, 14] Ribonucleoside reductase activity was measured using [^{14}C]CDP with dithioerythritol.[15] The deoxyribonucleotides [^{14}C]dCDP were separated from the ribonucleotides by TLC on polyethyleneamine (PEI) plates. Thymidine, TMP and TDP kinase activities were determined using [^{3}H]thymidine (58.3 mCi mmol) in the medium of Maley and Ochoa.[16] Carbamyl phosphate synthetase activity was determined with the method of Kalman *et al*.;[17] citrulline was determined colorimetrically.[18] Aspartate transcarbamylase activity was measured using the incubation medium of Kalman *et al*.;[17] carbamyl aspartate was determined colorimetrically by the method of Koritz and Cohen.[19] Thymidylate synthetase activity was analyzed by the Kampf *et al*. method.[20] 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*.[21] PRPP amidotransferase activity was determined by the method of Spassova *et al*.;[22] 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.[23] Protein content was determined for the enzymic assays by the Lowry technique.[24] After deoxyribonucleoside triphosphates were extracted,[25] levels were determined by the method of Hunting and Henderson[26] 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 [*methyl*-^{3}H]dTTP or [5-^{3}H]dCTP.

The effects of compounds **1** and **2** on DNA strand scission were determined by the methods of Suzuki *et al*.,[27] Pera *et al*.[28] and Woynarowski *et al*.[29] L_{1210} lymphoid leukemia cells were incubated with 10 μCi [*methyl*-^{3}H]thymidine (84.0 Ci $mmol^{-1}$) for 24 h at 37 °C. L_{1210} cells (10^7) were harvested and then centrifuged at 600 $\boldsymbol{g} \times 10$ 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 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 was incubated for 2.5 h at room temperature, it was centrifuged at 12,000 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 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** and **2** at 100 μM at 37 °C for 24 h.[30]

L_{1210} DNA topoisomerase II was isolated by the method of Miller *et al.*[31] The 170 kDa topoisomerase II is present in the final preparation using the following procedures. All steps were carried out at 0–4 °C. L_{1210} cells (2×10^8) were collected by centrifugation (500 $g\times5$ min), washed twice with PBS and resuspended in buffer solution containing 0.25 M sucrose, 20 mM potassium phosphate (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_5 at 4 °C. Cell membranes were lysed using a Dounce tissue grinder following the addition of Triton X-100 at a volume equivalent to 1/100th of that of the total 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 gently swirling. After mixing, the total volume was loaded on a sucrose cushion [1.4 M sucrose, 20 mM potassium phosphate (pH .7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 1 mM PMSF] and centrifuged at 18 000 rpm for 45 min at 4 °C. The sucrose cushion was removed via vacuum aspiration and the remaining pellet was resuspended in buffer containing 20 mM potassium phosphate (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 1 mM β-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.

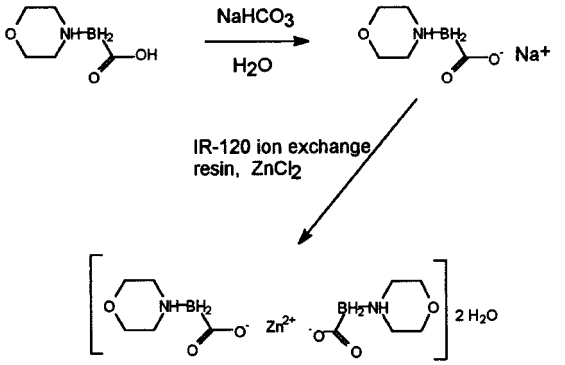
The effects of compounds **1** and **2** on isolated DNA topoisomerase II activity was determined by the method of Miller *et al.*[31] Reaction mixtures containing 0.05 M Tris (pH 7.5), 0.1 M KCl, 0.01 M $MgCl_2$, 30 μg ml^{-1} bovine serum albumin, 0.5 mM EDTA, 1.0 mM DTT, 1.0 mM ATP, 0.1 μg knotted DNA (isolated by the method of Liu and Davis[32]), 1 U L_{1210} topoisomerase II and 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 UV light table using Polaroid 667 film. Densitometric analysis was performed by the method of Hofmann *et al.*[33] 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 percentage 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).

Acute toxicity study

Acute toxicity studies were carried out in CF_1 male mice (approx. 28 g) using the method of Litchfield and Wilcoxon.[34] A single dose of experimental drug, beginning at a dose of 100 mg kg^{-1} intraperitoneally (i.p.), was administered to each mouse on day 1. The number of deaths occurring over the next seven days were then documented. Depending on the resulting number of deaths from the 50 mg kg^{-1} dose, the experiment was then repeated with dosage adjustments made accordingly to provide at least three data points from which to estimate LD_{50} values.

Statistical analysis

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

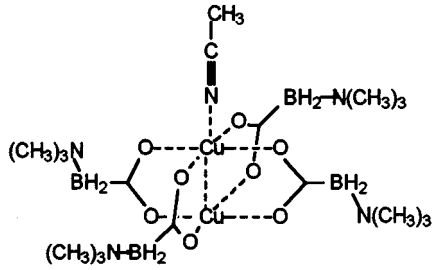


Scheme 1 The synthesis of bis-μ-(morpholine–boranecarboxylato)zinc(II) dihydrate (**2**).

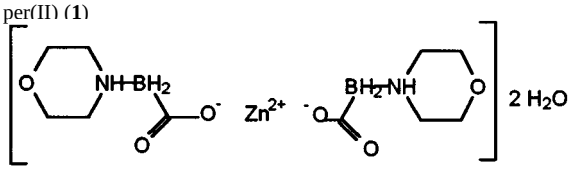
RESULTS

Synthesis

The metal complexes were successfully synthesized by ligand exchange in acetonitrile or by using IR-120 cation-exchange resin charged with the desired cation (Scheme 1). Ion exchange proceeded by elution of sodium salt solutions of amine–carboxyboranes through a column of ion-exchange resin. The structures (Fig. 1) and purity of the compound were confirmed by elemental analysis, melting points and ^{1}H-NMR and infrared spectra; the NMR and IR values were within the acceptable limits. Attempts to form complexes by mixing methanolic solutions of carboxyborane sodium salts with methanolic solutions of metal chloride salts were unsuccessful.



Tetrakis-μ-[(trimethylamine-boranecarboxylato)acetonitrile dicopper(II) (**1**)



Bis-μ-(morpholine-boranecarboxylato) zinc(II) dihydrate (**2**)

Figure 1 Molecular structures of the metal complexes of amine–carboxyborane adducts.

Antitumor activity

Both metal complexes demonstrated inhibition of Ehrlich ascites carcinoma growth *in vivo* in male CF_1 male mice at 8 mg kg^{-1} day^{-1}, i.p. Tetrakis - μ - (trimethylamine – boranecarboxy - lato)acetonitrile dicopper(II) (**1**) afforded 78% inhibition of growth and bis-μ-(morpholine–boranecarboxylato) zinc (II) dihydrate (**2**) resulted in 95% inhibition of Ehrlich ascites growth *in vivo*, 6-MP and 5-FU afforded 99% and 95% inhibition of tumor growth, respectively, *in vivo* in mice.

Cytotoxicity

The metal complexes demonstrated *in vitro* cytotoxicity primarily in suspended tumor cell lines with variable activity in human solid tumor cell cultures (Table 1). Murine L_{1210} lymphoid and P-388 lympholytic leukemias as well as human $Tmolt_3$ T-cell leukemia cell growth were effectively reduced by compounds **1** and **2**. Both compounds were active against human HeLa-S[3] suspended cervical carcinoma growth. Only compound **1** effectively reduced SW480 colorectal adenocarcinoma and HCT-8 ileocecal adenocarcinoma cell growth. MB-9812 bronchogenic lung growth was reduced by compounds **1** and **2** (ED_{50}<4 μg ml^{-1}) but both compounds were inactive against lung A549 growth. Neither of the compounds tested was effective in reducing the growth of rat UMR-106 osteosarcoma, or human lung A549, glioma HS-683, KB nasopharynx and epidermoid A431 cells.

Mode-of-action study

Tetrakis-μ-(trimethylamine–boranecarboxylato) acetonitrile dicopper(II) (**1**) (Table 2) and bis-μ-(morpholine–boranecarboxylato) zinc(II) dihydrate (**2**) (Table 3) caused significant reductions in DNA synthesis at 100 μM, but demonstrated more potent activity in reducing RNA synthesis, causing reductions of 45 and 39%, respectively, at 25 μM. L_{1210} protein synthesis was not significantly altered by either compound. Reductions in DNA polymerase α activity were caused by both agents, with a 44 and 28% reduction in activity by compounds **1** and **2**, respectively, at 100 μM. Purine *de novo* synthesis was reduced by compounds **1** and **2** at 100 μM by 58 and 52%, respectively. Com-

Table 1 *In vitro* cytotoxicity of boronated metal complexes of carboxyborane adducts (*N*=6)

	ED_{50} ($\mu g\ ml^{-1}$)[a]												
	Murine		Rat	Human									
Cpd	L_{1210}	P-388	UMR-106	$Tmolt_3$	HeLa-S^3	HeLa solid	KB	SW480	HCT-8	MB-9812	A549	A431	HS-683
1	2.81	2.27	4.56	1.68	2.61	4.98	7.57	2.57	2.42	3.38	8.57	4.05	6.16
2	2.95	1.51	9.22	1.47	3.09	6.38	6.33	6.28	6.53	3.37	7.82	4.71	6.92
Standard agents for comparison													
6-MP	2.43	2.16	9.13	1.62	21.2	56.1	11.04	3.61	1.15	4.29	4.71	3.42	4.46
Ara-C	2.43	2.38	0.86	2.67	2.13	4.74	2.84	3.42	2.54	6.16	6.28	0.92	1.88
Hydroxyurea	2.67	1.30	2.87	4.47	1.96	8.12	5.27	7.33	1.77	7.18	8.89	3.21	2.27
5-FU	1.41	1.41	3.52	2.14	2.47	4.11	1.25	3.09	1.12	5.64	3.58	0.61	1.28
VP-16	1.83	3.03	3.57	—	7.87	3.05	3.32	3.34	3.78	3.50	4.74	0.71	2.44

[a] Significant ED_{50} values $< 4\ \mu g\ ml^{-1}$.

Table 2 Effects of tetrakis-μ-(trimethylamine–boranecarboxylato)acetonitrile dicopper(II) (**1**) on L_{1210} leukemia cell metabolism *in vitro* over 60 min (N=4)

	Percentage of control (mean ± SD)			
Assay	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	88 ± 7	81 ± 6	53 ± 4*
RNA synthesis	100 ± 6[b]	55 ± 6*	29 ± 4*	15 ± 3*
Protein synthesis	100 ± 5[c]	104 ± 6	110 ± 6	115 ± 6
DNA polymerase α	100 ± 6[d]	74 ± 5*	73 ± 5*	56 ± 5*
mRNA polymerase	100 ± 7[c]	102 ± 6	84 ± 5	92 ± 6
rRNA polymerase	100 ± 4[f]	89 ± 7	88 ± 5	83 ± 6
tRNA polymerase	100 ± 7[g]	87 ± 6	85 ± 5	81 ± 4*
Ribonucleoside reductase	100 ± 5[h]	92 ± 6	86 ± 6	81 ± 6
Dihydrofolate reductase	100 ± 5[i]	77 ± 6*	51 ± 5*	46 ± 3*
De novo purine synthesis	100 ± 5[i]	99 ± 5	55 ± 5*	42 ± 4*
PRPP amido transferase	100 ± 6[k]	56 ± 5*	41 ± 4*	36 ± 4*
IMP dehydrogenase	100 ± 5[l]	56 ± 4*	45 ± 4*	38 ± 4*
Carbamyl phosphate synthetase	100 ± 7[m]	100 ± 5	107 ± 6	114 ± 7
Aspartate transcarbamylase	100 ± 6[n]	102 ± 6	97 ± 6	95 ± 6
Thymidylate synthetase	100 ± 5[o]	125 ± 7*	123 ± 5*	99 ± 5
Thymidine kinase	100 ± 6[p]	99 ± 5	77 ± 6*	44 ± 4*
Thymidine monophosphate kinase	100 ± 7[q]	72 ± 6*	20 ± 4*	17 ± 4*
Thymidine diphosphate kinase	100 ± 6[r]	92 ± 7	91 ± 5	91 ± 4
d(ATP)	100 ± 5[s]	—	—	106 ± 5
d(GTP)	100 ± 6[t]	—	—	119 ± 6
d(CTP)	100 ± 5[u]	—	—	107 ± 5
d(TTP)	100 ± 4[v]	—	—	99 ± 5

* $P \leqslant 0.001$.

[a] 26 152 dpm.
[b] 4851 dpm.
[c] c 7461 dpm.
[d] 47 804 dpm.
[e] 1502 dpm.
[f] 4239 dpm.
[g] 6400 dpm.
[h] 2744 dpm.
[i] 0.868 OD units
[j] 92 551 dpm.
[k] 0.121 OD units
[l] 76 058 dpm.
[m] 0.392 mol citrulline
[n] 1.064 mol *N*-carbamyl aspartate
[o] 18 463 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

pounds **1** and **2**, at 100 μM, caused reductions in dihydrofolate reductase activity of 54 and 87%, respectively. PRPP amido transferase activity was reduced 36 and 68% by compounds **1** and **2** at 100 μM, respectively. Compounds **1** and **2**, at 100 μM, reduced IMP dehydrogenase activity by 62 and 82%, respectively. Decreases in thymidine kinase activity were significant at 100 μM for both compounds, while decreases in thymidine monophosphate kinase activity were significant at all drug concentrations, with more than 50% reduction being induced by both compounds at 50 μM. No significant alterations in deoxynucleotide pool levels were induced by compound **1**. Compound **2**, however, caused significant increases in levels of d(GTP), d(CTP), and d(TTP) at 100 μM.

Compounds **1** (Fig. 2) and **2** (Fig. 3) also demonstrated the ability to cause L_{1210} DNA strand scission as indicated by the appearance of low-molecular-weight DNA throughout the alkaline sucrose gradient and a reduction in the relative amounts of higher-molecular-weight double-stranded DNA compared with control. ct-DNA UV absorption did not change after incubation for 24 h with compounds **1** or **2**, so the agents did not chemically interact with or alkylate the DNA bases. There were no effects on UV absorption from 220 to 340 nm after 24-h incubations with **1** or **2**. There was no change in the ct-DNA thermal denaturation, T_m, values after 24-h incubation with the drugs, so no

intercalation between DNA base pairs occurred. In the ct-DNA viscosity studies less time was required to move through the reservoirs, indicating reduced DNA viscosity or DNA fragmentation.

Compound **2** was not an inhibitor of L_{1210} DNA topoisomerase II activity at 100 μM (Fig. 4). Compound **1**, however, demonstrated a concentration-dependent inhibitory effect on L_{1210} topoisomerase II activity with an IC_{50} value of 25 μM. The prototypical topoisomerase II inhibitor VP-16, in comparison, exhibited an IC_{50} value of 22 μM by this method. Acute toxicity studies in CF_1 male mice resulted in LD_{50} values of 90 mg kg^{-1} and >100 mg kg^{-1} for compounds **1** and **2**, respectively.

DISCUSSION

Initial attempts to synthesize metal complexes of carboxyborane adducts followed the method of Norwood *et al*.[36, 37] in which methanolic solutions of sodium salts were added to methanolic solutions of metal chlorides. When applied in this study, a mixture of impurities was present at each stage of Norwood's procedure and purification was not possible. However, the use of IR-120 cation-exchange resin provided a more successful means to synthesize metal complexes of amine–carboxyborane adducts without the need for further purification. The synthesis of other metal complexes using ion-exchange resin was not pursued due to the limited amounts of

Table 3 Effects of bis-μ-(morpholine–boranecarboxylato) zinc(II) dihydrate (**2**) on L_{1210} cell metabolism *in vitro* over 60 min (*N*=4)

	Percentage of control (mean ± SD)			
Assay	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	100 ± 6	99 ± 6	45 ± 4*
RNA synthesis	100 ± 6[b]	61 ± 5*	58 ± 7*	52 ± 6*
Protein synthesis	100 ± 5[c]	104 ± 5	120 ± 6	105 ± 5
DNA polymerase α	100 ± 6[d]	73 ± 5*	73 ± 6*	72 ± 6*
mRNA polymerase	100 ± 7[e]	105 ± 6	100 ± 5	84 ± 5
rRNA polymerase	100 ± 4[f]	92 ± 6	111 ± 6	80 ± 4*
tRNA polymerase	100 ± 7[g]	86 ± 7	81 ± 5*	78 ± 5*
Ribonucleoside reductase	100 ± 5[h]	137 ± 6*	122 ± 6	101 ± 5
Dihydrofolate reductase	100 ± 5[i]	66 ± 5*	53 ± 4*	13 ± 3*
De novo purine synthesis	100 ± 5[j]	62 ± 5*	59 ± 4*	48 ± 4*
PRPP amido transferase	100 ± 6[k]	54 ± 4*	36 ± 3*	32 ± 3*
IMP dehydrogenase	100 ± 5[l]	73 ± 5*	59 ± 5*	18 ± 4*
Carbamyl phosphate synthetase	100 ± 7[m]	103 ± 5	107 ± 5	98 ± 6
Aspartate transcarbamylase	100 ± 6[n]	115 ± 5	102 ± 6	102 ± 5
Thymidylate synthetase	100 ± 5[o]	127 ± 6*	121 ± 5	113 ± 6
Thymidine kinase	100 ± 6[p]	100 ± 5	117 ± 6	62 ± 5*
Thymidine monophosphate kinase	100 ± 7[q]	54 ± 5*	28 ± 4*	11 ± 3*
Thymidine diphosphate kinase	100 ± 6[r]	99 ± 6	90 ± 5	72 ± 5*
d(ATP)	100 ± 5[s]	—	—	96 ± 5
d(GTP)	100 ± 6[t]	—	—	151 ± 6*
d(CTP)	100 ± 5[u]	—	—	122 ± 5*
d(TTP)	100 ± 4[v]	—	—	121 ± 4*

* $p \leq 0.001$.

[a] 26 152 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
[j] 92 551 dpm.
[k] 0.121 OD units
[l] 76 058 dpm.
[m] 0.392 molcitrulline
[n] 1.064 mol *N*-carbamyl aspartate
[o] 18 463 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

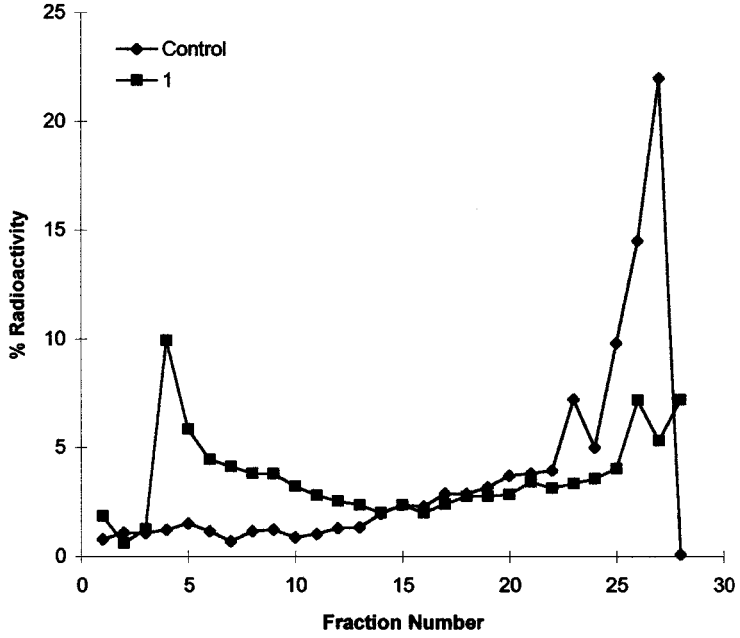


Figure 2 L_{1210} DNA strand scission induced by 24-h incubation with compound **1** at 100 μM concentration.

starting materials remaining after multiple attempts at metal complexation using Norwood's method.

In L_{1210} cells, the parent of compound **1**, tetrakis-μ-(trimethylamine–boranecarboxylato)-bis(trimethylamine–carboxyborane) dicopper(II)

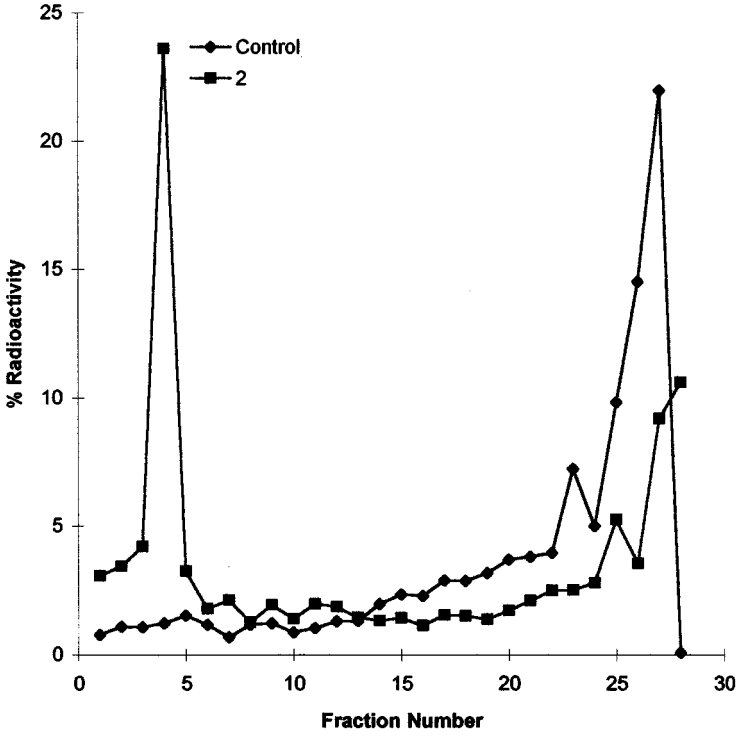


Figure 3 L_{1210} DNA strand scission induced by 24-h incubation with compound **2** at 100 μM concentration.

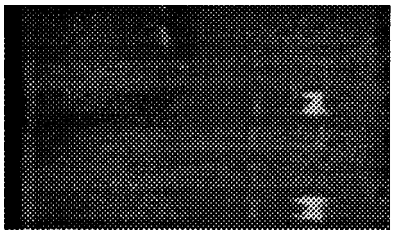


Figure 4 The inhibition of L_{1210} topoisomerase II activity by various derivatives of the dipeptide N-[(trimethylamine-boryl)carbonyl]-L-phenylalanine methyl ester and metal complexes of amine carboxyborane adducts at 100 μM.

($ED_{50} = 3.14\ \mu g\ ml^{-1}$) was a potent cytotoxic agent, and chemical transformation to compound **1** maintained cytotoxic activity.[2] In $Tmolt_3$ cells, transformation of the parent compound to compound **1** led to an improvement in cytotoxic activity compared with the parent compound. Against HeLa-S^3 and MB-9812 cell growth, there was little difference between the cytotoxic activity of compound **1** and its parent compound. In $Tmolt_3$ cells there was very little difference between the cytotoxic ED_{50} values of compound **2** and its parent compound, morpholine–carboxyborane.[4] In HeLa-S^3 cells, compound **2** was more potent than its parent compound. Whereas the parent compound was inactive in reducing MB-9812 cell growth, compound **2** was marginally active. Thus, it is apparent that metal complexation produces variable effects on cytotoxicity specific to a given tumor cell line.

Mode-of-action studies indicated the major effects of compounds **1** and **2** were inhibition of L_{1210} DNA and RNA syntheses. The primary sites by which these inhibitory effects are mediated appear to be IMP dehydrogenase, PRPP amido transferase, and TMP kinase activities. L_{1210} DNA strand scission induced by compounds **1** and **2** would also contribute to the observed inhibition of DNA synthesis and cell death. Reductions in DNA viscosity were also consistent with the observed DNA fragmentation observed in the DNA strand scission studies for both compounds. The results obtained by examination of the effect of the compounds on ct-DNA UV absorbance, thermal denaturation and viscosity suggest that DNA cross-linking and/or intercalation between base pairs are not a major mode of action mediating L_{1210} cell killing by compounds **1** and **2**. Comparing compound **1** with its parent compound, the most striking difference in their modes of action is evident in these compounds' effects on tRNA polymerase activity. The parent compound demonstrated a 77% reduction in tRNA polymerase activity whereas compound **1** inhibited this enzyme by 21%.[2] The mode-of-action studies for the parent of compound **2** have not been published. Piperidine–carboxyborane, another related heterocyclic amine–carboxyborane, primarily inhibited DNA synthesis by reducing *de novo* purine synthesis and reducing dihydrofolate reductase, and nucleoside and nucleotide kinase activities.[4] L_{1210} DNA topoisomerase II activity was inhibited by compound **1** but compound **2** was less active. Previous studies have demonstrated that copper(II), iron(III), cobalt(III) and chromium(III) complexes of trimethylamine–carboxyborane were potent L_{1210} DNA topoisomerase II inhibitors [$IC_{50} = 10–42\ \mu M$], but complexes of sodium, calcium and iron(II) were not active, nor was trimethylamine–carboxyborane itself at $>100\ \mu M$.[2] These new compounds, as well as compound **1**, compared favorably with VP-16 with an IC_{50} of 22 μM,[2] but they do not function like VP-16 to produce cleavable products. It has been shown that this enzyme activity was sensitive to calcium and copper inorganic salts in our studies.[38] Furthermore, it has been shown that $ZnCl_2$ and $CuCl_2$ inhibit shrimp DNA topoisomerase activity at millimolar concentrations.[38] Nevertheless, there is no evidence that the copper(II) complexes of trimethylamine–carboxyboranes dissociate in aqueous mediums to release free cations. The complexation of trimethylamine–carboxyborane with copper may form the correct three-dimensional or ternary structure that fits best in the receptor active site of the enzyme and affords the best inhibition of DNA topoisomerase activity. The inability of the zinc bis-morpholine–carboxyborane complex to inhibit DNA topoisomerase II activity *in vitro* might indicate that inhibitor activity requires the 'tetrakis' arrangement of ligands, as in the copper(II) complex, as opposed to zinc's 'bis' arrangement. However, neither of the metal complexes induced DNA protein-linked breaks

when whole cells were incubated with these drugs alone. Yet, the metal complexes as well as the free ligand were synergistic with VP-16 in inducing DNA protein-linked breaks, suggesting that the synergistic effects with VP-16 are characteristic of the amine–carboxyborane moiety. Complexation with the metal appears to increase the cytotoxic potency of the compound. Whether this action is via some non-specific interaction with *in vivo* enzyme is unclear at present. Anthraquinone–copper(II) complexes have been shown to interact non-specifically with DNA to induce DNA topoisomerase I- and II-mediated strand breaks.[39] The present studies suggest that more than one mode of action is afforded by these metal complexes in inducing cell death and the inhibition of L1210 DNA topoisomerase activity is only one of these mechanisms.

Acknowledgement The authors thank the Elsa U. Pardee Foundation and North Carolina Biotechnology for sponsoring this research and Dr Ken Bastow for technical assistance with the topoisomerase II assays.

REFERENCES

1. I. H. Hall, B. F. Spielvogel and A. Sood, *Anti-Cancer Drugs* **1**, 133 (1990).
2. I. H. Hall, K. W. Morse, B. F. Spielvogel and A. Sood, *Anti-Cancer Drugs* **2**, 389 (1991).
3. G. L. Silvey, Ph.D. Dissertation, Duke University, pp. 1–255 (1984).
4. C. K. Sood, A. Sood, B. F. Spielvogel, J. A. Yousef, B. S. Burnham and I. H. Hall, *J. Pharm. Sci.* **80**, 1133 (1991).
5. J. T. McCubbins, Ph.D. Dissertation, Duke University, pp. 1–402 (1990).
6. A. Leibovitz, J. C. Stinson, W. B. McCombs III, C. E. McCoy, K. C. Mazur and N. D. Mabry, *Cancer Res.* **36**, 4562 (1976).
7. R. I. Geran, N. H. Greengerg, M. M. MacDonald, A. M. Schumacher and B. J. Abbott, *Cancer Chemo. Rep.* **3**, 7 (1972).
8. L. L. Liao, S. M. Kupchan and S. B. Horwitz, *Mol. Pharmacol.* **12**, 167 (1976).
9. E. Cadman, R. Heimer and C. Benz, *J. Biol. Chem.* **252**, 1695 (1981).
10. R. I. Christopherson, M. L. Yu and M. E. Jones, *Anal. Biochem.* **11**, 240 (1981).
11. D. C. Eichler, P. A. Fisher and D. Korn, *J. Biol. Chem.* **252**, 4011 (1977).
12. H. Sawada, J. Tatsumi, M. Sadada, S. Shirakawa, R. I. Nakamura and G. Wakisaka, *Cancer Res.* **34**, 3341 (1974).
13. K. M. Anderson, I. S. Mendelson and G. Guzik, *Biochem., Biophys. Acta* **383**, 56 (1975).
14. I. H. Hall, G. L. Carlson, G. S. Abernathy and C. Piantadosi, *J. Med. Chem.* **17**, 1253 (1974).
15. E. C. Moore and R. B. Hurlbert, *J. Biol. Chem.* **241**, 4802 (1966).
16. R. Maley and S. Ochoa, *J. Biol. Chem.* **233**, 1538 (1958).
17. S. M. Kalman, P. H. Duffield and T. J. Brzozuski, *J. Biol. Chem.* **241**, 1871 (1966).
18. R. M. Archibald, *J. Biol. Chem.* **156**, 121 (1944).
19. S. B. Koritz and P. P. Cohen, *J. Biol. Chem.* **209**, 145 (1954).
20. A. Kampf, R. L. Barfknecht, P. J. Schaffer, S. Osaki and M. P. Mertes, *J. Med. Chem.* **19**, 903 (1976).
21. Y. K. Ho, T. Hakala and S. F. Zakrzewski, *Cancer Res.* **32**, 1023 (1972).
22. M. K. Spassova, G. C. Russev and E. V. Goovinsky, *Biochem. Pharmacol.* **25**, 923 (1976).
23. J. H. Becker and G. W. Lohr, *Klin. Wochenschr.* **57**, 1109 (1979).
24. O. H. Lowry, J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
25. A. S. Bagnara and L. R. Finch, *Anal. Biochem.* **45**, 24 (1971).
26. D. Hunting and J. F. Henderson, *Can. J. Biochem.* **59**, 723 (1982).
27. H. Suzuki, T. Nishimura, S. K. Muto and N. Tanaka, *J. Antibacteriol.* **32**, 875 (1978).
28. J. F. Pera, Sr., C. J. Rawlings, J. Shackleton and J. J. Roberts, *Biochem. Biophys. Acta* **655**, 152 (1981).
29. J. W. Woynarowski, T. A. Beerman and J. Konopa, *J. Biochem. Pharmacol.* **30**, 3005 (1981).
30. Y. Zhao, I. H. Hall, C. B. Oswald, T. Yokoi and K. H. Lee, *Chem. Pharm. Bull.* **35**, 2052 (1987).
31. K. G. Miller, L. F. Liu and P. T. Englund, *J. Biol. Chem.* **256**, 9334 (1981).
32. L. F. Liu and J. L. Davis, *Nucleic Acid Res.* **9**, 3979 (1981).
33. G. A. Hofmann, C. K. Mirabelli and F. H. Drake, *Anti-Cancer Drug Design* **5**, 273 (1990).
34. J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Ther.* **96**, 99 (1949).
35. A. Sood, C. K. Sood, B. F. Spielvogel, I. H. Hall and O. T. Wong, *J. Pharm. Sci.* **81**, 458 (1992).
36. V. M. Norwood III and K. W. Morse, *Inorg. Chem.* **25**, 3690 (1986).
37. V. M. Norwood III and K. W. Morse, *Inorg. Chem.* **26**, 284 (1987).
38. M. C. Miller III, A. Sood, B. F. Spievogel and I. H. Hall, *Res. Commun. Pharm. Toxicol.* **1**, 245 (1996).
39. N. N. Chung, C. L. Lin and H. K. Chen, Comp. Biochem. Phys. Pt. B, *Mol. Biol.* **114**, 145 (1996).
40. S. Routier, N. Cotelle, J. P. Catteau, J. L. Bernier, M. J. Waring, J. F. Riou and C. Bailey, *Biorg. Med. Chem.* **4**, 1185 (1996).

