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The effects of boron containing peptides on L_{1210} lymphoid leukemia metabolism

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Summary. The purpose of this study was to establish the efficacy and mode of action of peptide boron derivatives as antineoplastic agents and to evaluate their safety in vivo. Boron-containing phenylalanine and tyrosine methyl esters were found to be potent cytotoxic agents in a number of murine and human cancer cell lines. DNA, RNA and protein syntheses were inhibited by selected agents, e.g. [(trimethylamine boryl)carbonyl]-phenylalanine-acetyl ester (**9**) and *N*-acetyl-p-boron-phenyl-alanyl-phenlalanine-methyl ester (**10**), in L_{1210} lymphoid leukemia cells. IMP dehydrogenase, OMP decarboxylase, m-RNA, t-RNA, r-RNA polymerase and ribonucleoside reductase activities were inhibited. d(CTP) levels were reduced. DNA strand scission occurred after 24 hr incubation. Acute toxicity studies in mice demonstrated that the key derivative was safe at therapeutic levels with no effects on histology of major organs, hematopoietic parameters and clinical values.

Keywords: Amino acids – Antineoplastic agents – Boron peptide methyl esters – Cytotoxicity – L_{1210} leukemia

Introduction

Our initial studies involved the synthesis of a series of boron analogues of amino acids, e.g. glycine, alanine and betaine (Spielvogel et al., 1991). These compounds were extended to di- and tri- peptides of boron derivatives (Sood et al., 1990). These derivatives included a tyrosine and a phenylalanine dipeptide. By redesigning the boron derivatives so that the boron atom is moved from the α carboxylic acid group and incorporated in lieu of a carbon atom of the alkyl chain, the compound resembles more of an antimetabolite. The phenylalanine moiety has been used as a carrier for a number of alkylating moieties with increased uptake by cancer cells. The original peptides with aromatic amino

acids at 8 mg/kg/day demonstrated significant in vivo antineoplastic activity against Ehrlich ascites carcinoma growth. For example, the [(trimethylamine-boryl)carbonyl]phenylalanine methyl ester afforded 96% inhibition, while the corresponding tyrosine methyl ester caused 81% (Sood et al., 1990). The studies reported earlier were preliminary and limited in scope. We have now examined the activity of boron-containing peptides against the growth of a number of human and murine tumor cell lines, and studied their mechanism of action as cytotoxic agents, and examined their acute toxicity in mice.

Materials

Source of compounds

Compounds **1–9** and **12–18** (Fig. 1) were synthesized by methods previously reported in the literature (Sood et al., 1990). The physical and chemical characteristics have previously been published (Sood et al., 1990). The structures may be found in Fig. 1 with their IUPAC nomenclature.

N-Acetyl-p-boronophenylalanine was prepared as described previously (Snyder et al., 1958). Other reagents were obtained commercially and were used as obtained or dried by routine methods. ^{1}H-NMR spectra were obtained on a Varian XL 300 spectrometer (Varian Associates, Inc., Instrument Group, 611 Hansen Way, Palo Alto, CA 94303, USA) while ^{11}B-NMR spectra were recorded on a JEOL FX90Q spectrometer (JEOL, 11 Dearborn Road, Peabody, MA 01960, USA). Elemental analysis were performed by Oneida Research Services, Inc. (1 Halsey Road, Whitesboro, NY 13492).

Synthesis of boron containing peptides

N-Acetyl-*p*-boronophenylalanylphenylalanine methyl ester **10**

N-Acetyl-*p*-boronophenylalanine (1.00 g, 3.70 mmol), *L*-phenylalanine methyl ester hydrochloride (0.79 g, 3.70 mmol) and triphenylphosphine (1.16 g, 4.44 mmol) were taken up in anhydrous CH_3CN (30 ml) and anhydrous dimethylformamide (4 ml). To the reaction mixture triethylamine (1.10 ml, 7.86 mmol) and carbon tetrachloride (0.6 ml, 6.20 mmol) were added and the mixture was stirred under N_2 at room temperature overnight. The solvents were removed under reduced pressure and the residue was washed with water (2 × 50 ml) and filtered. The filtrate was allowed to evaporate and the residue was dissolved in 5% methanol in chloroform, dried over anhydrous Na_2SO_4 and concentrated to afford a yellow semi-solid (2.58 g). The crude product was purified by flash chromatography on silica gel using $CHCl_3$: MeOH (20 : 1) and then recrystallized from ethylacetate and dichloromethane mixture. Yield = 0.43 g, 28.2% ^{1}H-NMR (acetone-d^6): δ 1.93 ppm, two singlets (for two diastereomers), $OC(O)CH_3$; 3.02 ppm, m, CH_2's; 3.74 ppm, *s*, OCH_3; 4.65–4.83, two multiplets, 2 CH's; 6.80–7.88 ppm, aromatic and NH's. ^{11}B-NMR (acetone-d^6): ~32 ppm, very broad peak. Analysis calculated: %C, 61.18; %H, 6.11; %N, 6.80. Found: %C, 60.10; %H, 5.85; %N, 6.51.

N-Acetyl-*p*-boronophenylalanyltyrosine methyl ester **11**

N-Acetyl-*p*-boronophenylalanine (2.60 g, 9.50 mmol), tyrosine methyl ester hydrochloride (2.19g, 9.50 mmol) and triphenylphosphine (2.98 g, 11.40 mmol) were taken in anhydrous CH_3CN under N_2. To this mixture, triethylamine (2.80 ml, 20.00 mmol) and carbon tetrachloride (1.50 ml, 15.5 mmol) were added. The mixture was stirred under N_2 at room temperature for 24 hr. The solvent was removed under reduced pressure. The residue was washed with water (2 × 60 ml) and filtered. The filtrate was concentrated by evaporation of

water at room temperature. The residue was extracted with ethyl acetate (3 × 50 ml) and the extracts were concentrated. Addition of hot chloroform to this residue followed by filtration gave a glassy yellow solid (1.40 g). This glassy solid and the residue from chloroform washing (which still contained some product) were separately purified by flash chromatography on silica gel using CH_3Cl/MeOH (50 : 1 followed by 20 : 1). Attempted crystallization of the partially purified product from chloroform failed, so the product was further purified twice by flash chromatography using CH_3Cl/MeOH (20 : 1) for the first column and CH_3Cl/MeOH (10 : 1) for the second column to give pure product in a very low yield. Yield = 0.21 g, 5.16%. ^{1}H-NMR (acetone-d^6 + D_20): δ = 1.82 ppm, 2 singlets, $OC(O)CH_3$ (for two diastereomers); 2.67–3.09 ppm, m, CH_2's; 3.57 and 3.62, 2 singlets, OCH_3 (for two diastereomers); 4.45–4.59 ppm, multiplet, 2 CH's; 6.98–7.63, aromatic ^{11}B-NMR (acetone-d^6 + D_2O): δ = ~29 ppm, broad peak. Analysis calculated: %C, 58.90; %H, 5.88; %N, 6.54. Found: %C, 56.94; %H, 5.87; %N, 6.30.

Cytotoxic activity

Compounds **1–18** were tested for cytotoxic activity by preparing a 1 mM solution of each of the drugs in 0.05% Tween 80/H_2O by homogenization. The drug solutions were sterilized by passing them through an Acrodisc 45 μM. The following cell lines, obtained from the American Tissue Cultured Collection (Rockville, MD), were maintained by literature techniques (Geran et al., 1972): murine L_{1210} lymphoid leukemia (Geran et al., 1972), P_{388} lymphocytic leukemia (Geran et al., 1972), human $Tmolt_3$ acute lymphoblastic T cell leukemia (Minowada et al., 1972), colorectal adenocarcinoma SW480 (Leibovitz et al., 1976), lung bronchogenic MB-9812 (Aaronson et al., 1970), osteosarcoma TE418 (Smith et al., 1976), KB epidermoid nasopharynx (Eagle, 1955), HeLa-S^3 suspended cervical carcinoma (Puck et al., 1956) and glioma EH 118 MG (Nelson-Rees et al., 1975). The protocol used to assess cytotoxicity was that of Geran et al., 1972. Standards were determined for each cell line. Values are expressed for the drug's cytotoxicity as ED_{50} = μg/ml, i.e., the concentration which inhibits 50% of the cell growth determined by the trypan blue exclusion technique. A value of <4 μg/ml indicates significant activity. Solid tumor cytotoxicity was determined by the method of Leibovitz et al. (1976), using 0.2% crystal violet in 20% EtOH. Cytotoxicity was determined at 580 nm with a Molecular Devices (Menlo Park, CA) SOFTmax programmable spectrophotometer.

Incorporation studies

Compounds **9** and **10** were selected as being typical of the peptide boron compounds demonstrating good cytotoxicity. The L_{1210} tissue culture system was selected because it was representative of a single cell cancer growth system used previously to establish mode of action of new antineoplastic agents. Moreover, the characteristics of this cell line are well known. Incorporation of labeled precursors into ^{3}H DNA, ^{3}H RNA and ^{3}H protein for 10^6 L_{1210} cells was determined by the method of Liao et al., 1976. The inhibition of DNA, RNA and protein synthesis was determined at 25, 50 and 100 μM of **9** and **10** at 60 min. ^{14}C-Glycine (53.0 mCi/mol) incorporation into purines was determined by the method of Cadman et al. (1981), ^{14}C-Formate (53.0 mCi/mol) incorporation into pyrimidines was determined by the method of Christopherson et al., 1981.

Enzyme assays

Inhibition of various enzyme activities were carried out by first preparing the appropriate L_{1210} cell homogenate or subcellular fraction, then adding the drug to be tested during the enzyme assay. For the concentration response studies, the inhibition of enzyme activity was determined at 25, 50 and 100 μM for 60 min. Kinetic studies of select enzyme activities were conducted at 100 μM of **9** and **10** for 15, 30, 45 and 60 min incubations. DNA polymerase

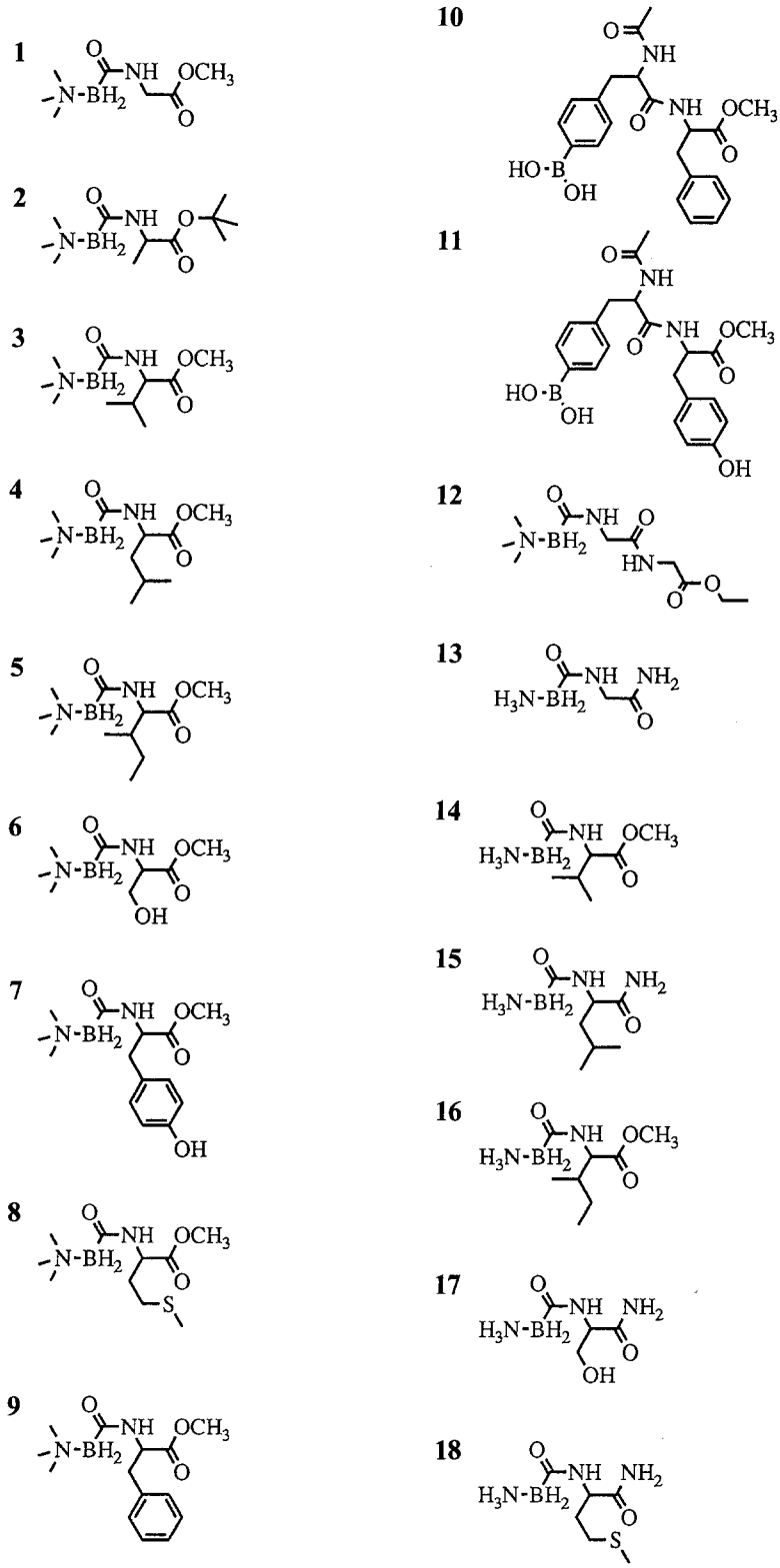

Fig. 1. Structure of compounds

Compound #	Name
1	(N,N-dimethylmethanamine)dihydro[[(2-methoxy-2-oxoethyl)-amino]carbonyl]boron
2	[[[2-(1,1-dimethylethoxy)-1-methyl-2-oxoethyl]amino]-carbonyl](N-N-dimethylmethanamine)dihydroboron
3	(N,N-dimethylmethanamine)dihydro[[[l-(methoxycarbonyl)-2-methylpropyl]amino]carbonyl]boron
4	(N,N-dimethylmethanamine)dihydro[[[l-(methoxycarbonyl)-3-methylbutyl]amino]carbonyl]boron
5	(N,N-dimethylmethanamine)dihydro[[[l-(methoxycarbonyl)-2-methylbutyl]amino]carbonyl]boron
6	(N,N-dimethylmethanamine)dihydro[[[l-(hydroxymethyl)2-methoxy-2-oxoethyl]amino]carbonyl]boron
7	(N,N-dimethylmethanamine)dihydro[[[1-[(4-hydroxyphenyl)-methyl]-2-methoxy-2-oxoethyl]amino]carbonyl]boron
8	(N,N-dimethylmethanamine)dihydro[[[l-(methoxycarbonyl)-3-(methylthio)propyl]amino]carbonyl]boron
9	(N,N-dimethylmethanamine)dihydro[[[2-methoxy-2-oxo-1-(phenylmethyl)ethyl]amino]carbonyl]boron
10	N-Acetyl-4-boronophenylalanylphenylalanine methyl ester
11	N-Acetyl-4-boronophenylalanyltyrosine methyl ester
12	(N,N-dimethylmethanamine)[[[2-[(2-ethoxy-2-oxoethyl)amino]-2-oxoethyl]amino]carbonyl]dihydroboron
13	[[(2-amino-2-oxoethyl)amino]carbonyl]ammine-dihydroboron
14	Amminedihydro[[[l-(methoxycarbonyl)-2-methylpropyl]amino]-carbonyl]boron
15	[[[l-(aminocarbonyl)-3-methylbutyl]amino]carbonyl]ammine-dihydroboron
16	Ammine[[[l-(methoxycarbonyl)-2-methylbutyl]amino]carbonyl]-dihydroboron
17	[[[2-amino-1-(hydroxymethyl)-2-oxoethyl]amino]carbonyl]-amminedihydroboron
18	[[[l-(aminocarbonyl)-3-(methylthio)propyl]amino]carbonyl]-amminedihydroboron

α activity was determined in a cytoplasmic extract isolated by the method of Eichler et al., (1977). Nuclear DNA polymerase was determined by isolating nuclei (Mamaril et al., 1970). The polymerase assay for both α and β was that of Sawada et al., 1974 with (^{3}H)-TTP. Messenger, ribosomal and transfer RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate (Anderson et al., 1975; Hall et al., 1974) and the individual RNA polymerase activities were determined using ^{3}H-UTP. Ribonucleoside reductase activity was measured with ^{14}C-CDP with and without dithioerythritol (Moore and Hurlbert, 1966). The deoxyribonucleotides (^{14}C)-dCDP were separated from the ribonucleotides (^{14}C-CDP) by TLC on PEI plates. Thymidine, TMP and TDP kinase activities were measured with ^{3}H-thymidine (58.3 mCi/mol) and the medium of Maley and Ochoa (1958). PRPP amidotransferase activity was determined by the method of Spassova et al. (1976) and IMP dehydrogenase activity was determined with ^{14}C-IMP (Amersham, Arlington Heights, IL) where XMP was separated on PEI plates (Fisher Scientific, Pittsburgh, PA) by TLC (Becker and Lohr, 1979). Carbamyl phosphate synthetase activity was determined by the method of Kalman et al. (1986) and citrulline was determined colorimetrically (Archibald, 1944). Aspartate transcarbamylase activity was determined by the method of Kalman et al. (1966) and carbamyl aspartate was determined colorimetrically (Koritz and Gohen, 1968). OMP decarboxylase activity was determined by the method of Appel (Appel, 1968). Thymidylate synthetase activity was analyzed by the method of Kampf et al., 1976. 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., 1971. Protein was determined for all of the enzymatic assays by the Lowry technique (Lowry et al., 1951).

Deoxyribonucleoside triphosphates were extracted by the method of Bagnara and Finch, 1972. Deoxyribonucleoside triphosphates were determined by the method of Hunting and Henderson, 1981 with calf thymus DNA, *E. coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 μCi of ^{3}H-methyl-dTTP or 5-^{3}H-dCTP.

The effects of the agents on isolated DNA topoisomerase II activity was determined by the method of Miller et al., 1981. Knotted DNA was prepared from bacteriophage P4 as outlined in the literature (Liu and Davis, 1981). The enzyme, DNA topoisomerase II, was isolated from HeLa uterine carcinoma cells. The reaction medium contained 0.20 M Tris, pH 7.5, 0.4 M KCl, 0.04 M MgCl2, 120 μg/ml BSA, 2.0 M EDTA, 4.0 mM DDT, 4 mM ATP. For the enzyme assay 2.5 μl of the reaction medium and 0.25 μl knotted DNA were added and diluted to 8 μl with distilled water. The agents were added with 1.0 μl of enzyme for a final volume of 10 μl which was incubated for 60 min at 37°C. 2.5 μl buffer (50% w/v sucrose, 0.5% w/v sodium dodecyl sulfate, 0.25% w/v bromophenyl blue and 0.25% xylene cyanol) was added to stop the reaction. Samples of the reaction, pure DNA and pure enzyme were placed on an agrose electrophoresis gel at 23V. VP-16, etoposide, was used as an internal standard. Inhibition of the activity is noted in the gel when topoisomerase II the ability to unknot the knotted DNA is reduced. The inhibition of the activity appears as a smear of DNA in the gel as opposed to distinct separation of bands.

The effects of compound **9** and **10** on DNA strand scission was determined by the in vitro method of Suzuki et al., 1978; Pera et al., 1981 and Woynarowski et al., 1981. L_{1210} lymphoid leukemia cells were incubated with 10 μCi thymidine (methyl-^{3}H, 84.0 Ci/mmol) for 24 hr at 37°C. After harvesting the L_{1210} cells (10^7), the cells were centrifuged at 600 g × 10 min in PBS, washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml; 0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100 and 2.5% sucrose) was layered onto a 5–20% alkaline-sucrose gradient (5 ml; 0.3 M NaOH, 0.7 KCl and 0.01 M EDTA) followed by 0.2 ml cell preparation. After incubating 2.5 hr at room temperature, the gradient was centrifuged at 12,000 RPM at 20°C for 60 min (Beckman rotor SW60). Fractions (0.2 ml) were collected from the top of the gradient, neutralized with 0.2 ml of 0.3 N HCl, and radioactivity measured. Thermal calf thymus DNA denaturation studies and DNA viscosity studies were conducted after incubation of **9** and **10** at 100 μM at 37°C for 24 hr (Zhao et al., 1987).

*Acute toxicity studies of compounds **9** and **10***

LD_{50} acute toxicity was determined in CF_1 male mice (~28 g) i.p. using doses of 5 mg/kg to 1 g/kg as a single dose. The number of deaths in each group was noted over the next 7 days.

Acute toxicity studies of Compounds **9** and **10** were conducted in CF_1 male mice (~28 g), which were dosed at 8, 16 or 40 mg/kg/day i.p. for 7 days. Daily food consumption was determined and water was given ad libitum. The animals were maintained in 12 hr light and dark cycles at 72°F.

Clinical chemistry

At the time of the sacrifice, the major organs were excised, trimmed of fat and weighed. Blood was obtained from the carotid artery and centrifuged at 3500 g × 10 min to obtain the serum. Chemical or enzymatic assays were performed with Sigma Chemical kits: urea nitrogen (BUN, No. 640), alanine amino transferase (SGPT, No. 505), alkaline phosphatase (AP, No. 104), glucose (No. 510), lactic dehydrogenase (LDH, No. 500), creatine phosphokinase (CP-kinase, No. 661), and total and direct bilirubin (No. 605). Serum triglycerides were determined with a diagnostic kit from Boehringer Mannheim; serum cholesterol was determined by the method of Ness et al., 1984. Albumin and total protein was determined by the method of Lowry et al., 1951. Cholic acid and uric acid were determined as outlined by Tietz, 1976.

Hematopoietic parameters

After blood was obtained from the carotid artery, a drop was placed on glass slides and fixed in Wright's stain. Differential white blood cell counts, platelet counts and hematocrits were obtained for each mouse group sacrificed at the specified times (Brown, 1984).

Histological sections

The animals were killed by carbon dioxide asphyxiation. After all vital signs had ceased, a midline incision was made from the lower jaw to the inguinal area. Thymus, spleen, liver, and kidney were excised and weighed, and representative tissue samples were fixed in 10% buffered formalin, trimmed and sectioned at 6 μ in thickness and stained with hematoxylin and eosin.

Statistics

The mean and standard deviation are designated by "X + SD." N is the number of animals per group. Significant differences between treated and control samples (i.e., the probability level (p) ≤ 0.001) was determined by the Student's "t" test using the raw data.

Results

Compounds **10** and **11** were prepared by a method similar to the one described previously (Sood et al., 1990) for other boron containing dipeptides. A major difficulty encountered during their preparation was the low solubility of *N*-acetyl-*p*-boronophenylalanine in acetonitrile. Due to this low solubility, a very low yield (5.16%) of **11** was obtained. Use of a small amount of dimethylformamide to enhance solubility of the starting materials did give a better yield (28.2%) of **10**, although the yield was still less than the average yield observed for the other peptides used in this study. Another possible reason for lower yield may be the side reactions of the boronic acid moiety which could reversibly complex

with the amino function of the free amino acid and slow down the condensation. In the case of **11**, yet another possibility is the side reaction of the phenolic OH. No attempts were made to optimize this method for the preparation of compounds **10** and **11** because sufficient quantities of materials were available for the planned studies. The new compounds have been characterized by ^{1}H, ^{11}B NMR spectroscopy and elemental analysis. The data are presented in the Experimental section.

In the murine tissue culture cell lines, the boron containing peptides demonstrated select cytotoxic activity (Table 1). Only compounds **6**, **9**, **10**, **13**, **14** and **15** demonstrated potent activity; i.e. <4 μg/ml in the L_{1210} screen and compound **14** in the P_{388} lymphoid leukemia screen. Improved activity was observed against $Tmolt_3$ leukemic growth for all of the compounds tested with **17** resulting in an ED_{50} value of 0.67 μg/ml. Activity against adenocarcinoma colon growth was shown by **2**, **3**, **5**, **6**, **9**, **12** and **18**. Only **18** was active against lung bronchogenic tumor growth. All of the compounds tested in the HeLa-S^3 uterine screen demonstrated good activity. Only **15**, **16**, **17** and **18** demonstrated activity against glioma growth. KB nasopharanx tumor growth was significantly inhibited by **6**, **9**, **10** and **14**. Osteosarcoma growth was inhibited by **2**, **3**, **6**, **12**, **15**, **16** and **17**.

When **9** and **10** were examined for their effects on L_{1210} cell metabolism, compound **9** effectively blocked DNA, RNA and protein synthesis in a concentration dependent manner achieving greater than 50% inhibition at 100 μM (Table 2). Compound **10** also inhibited DNA, RNA and protein synthesis, but at 100 μM only DNA and protein was inhibited greater than 50%. Compound **9** was more effective in blocking these macromolecular syntheses. ^{14}C-Glycine incorporation into purines was moderately inhibited by **9** by 19%, but **10** was more effective in this pathway with a concentration dependent inhibition that achieved 76% inhibition at 100 μM. Formate incorporation into pyrimidines was inhibited effectively with greater than 50% inhibition at 100 μM by **9**. Compound **10** only caused 34% inhibition at 100 μM. Activity of the regulatory enzyme of the purine pathway, IMP dehydrogenase, was only marginally inhibited by **9** by 21% but **10** was more effective with 44% inhibition. Neither agent was effective against PRPP amido transferase activity. Carbamyl phosphate synthetase activity was inhibited 22% by **9** but only 10% by **10**. Aspartate transcarbamylase activity was inhibited 14% by **10**. OMP decarboxylase activity was inhibited 39% by **10** and 26% by **9**. Thymidylate synthetase and thymidine kinase activities were not inhibited by either agent. TMP and TDP kinase activities were only marginally inhibited 11–18% by both drugs. DNA polymerase α activity was not inhibited by either compound (data not shown). DNA polymerase β activity was actually stimulated by both agents. m-RNA, r-RNA and t-RNA polymerase activities were inhibited by both agents in a concentration dependent manner. Dihydrofolate reductase activity was inhibited marginally by **10**, i.e., 18%, but ribonucleoside reductase activity was significantly inhibited by **9** and **10** with greater than 50% inhibition at 10 μM. dATP and dGTP levels were not affected by either drug. dCTP levels were reduced by both agents; dTTP was reduced by **10** but not **9**. HeLa-S^3 Topoisomerase II activity was not inhibited by either agent at 100 μM.

Table 1. The effects of boronated peptides on murine and human tissue cell growth

($N = 5$)	ED_{50} Value uG/mL								
	Murine		Human						
Compound	L_{1210}	P_{388}	$Tmolt_3$	Colon adenocarcinoma	Lung bronchogenic	HeLa-S[3] uterine	Glioma	KB nasopharyngeal	Osteosarcoma
1	7.00	12.11	2.90*	4.50	5.21	1.97*	4.48	6.46	6.09
2	7.21	11.43	3.34*	1.25*	6.37	1.70*	6.02	4.54	3.61*
3	12.62	15.19	2.68*	1.24*	6.18	2.12*	6.83	8.27	1.68*
4	6.80	11.63	2.55*	5.50	6.05	2.16*	6.18	4.64	7.25
5	10.30	10.82	2.42*	2.01*	6.56	2.49*	6.29	7.23	4.49
6	1.98*	9.21	2.17*	2.37*	4.77	2.07*	4.64	3.29*	3.84*
7	4.03	9.36	—	4.36	4.17	—	3.45*	1.07*	3.06*
8	6.62	12.86	3.45*	7.12	4.82	2.02*	4.82	6.59	4.56
9	2.93*	8.89	1.94*	2.78*	5.78	—	—	3.43*	5.96
10	3.72*	—	1.14*	7.46	4.74	—	—	1.76*	4.01
11	5.05	—	2.05*	7.16	5.93	—	—	4.11	8.24
12	4.97	10.38	2.02*	2.75*	5.71	2.07*	5.00	7.02	3.30*
13	1.40*	7.62	3.18*	4.37	5.96	1.83*	4.61	5.83	5.69
14	3.44*	3.98*	2.08*	5.84	8.41	2.25*	4.50	3.36*	6.07
15	3.60*	11.61	2.81*	4.29	7.23	1.35*	1.26*	7.55	2.47*
16	14.70	9.80	1.05*	6.16	6.99	2.87*	2.48*	4.39	2.71*
17	10.60	8.73	0.67*	6.00	6.53	3.50*	3.69*	4.05	1.12*
18	4.17	8.57	2.35*	2.25*	3.42*	2.49*	1.46*	4.21	7.21

* $p \leq 0.001$ by Student's t test at less than 4 ug/ml (NCI protocol)

Table 2. The effects of compounds **9** and **10** on L_{1210} DNA, RNA and protein syntheses and enzyme activities

($N = 6$)	Compound **9** Percent of control ($\bar{X} \pm$ SD)					Compound **10** Percent of control ($\bar{X} \pm$ SD)			
	Control	10 μM	25 μM	50 μM	100 μM	10 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 6[a]	62 ± 5*	56 ± 4*	39 ± 4*	29 ± 3*	152 ± 6*	113 ± 5*	93 ± 7	41 ± 5*
RNA synthesis	100 ± 5[b]	44 ± 5*	30 ± 3*	30 ± 3*	24 ± 3*	95 ± 7	76 ± 6*	67 ± 5*	65 ± 6*
Protein synthesis	100 ± 6[c]	60 ± 6*	59 ± 4*	52 ± 3*	46 ± 4*	203 ± 8*	178 ± 5*	68 ± 5*	48 ± 4*
Purine synthesis	100 ± 8[d]	102 ± 7	89 ± 5	88 ± 6	81 ± 5	99 ± 6	72 ± 4*	56 ± 5*	24 ± 4*
Pyrimidine synthesis	100 ± 7[e]	148 ± 8*	51 ± 6*	50 ± 5*	47 ± 5*	95 ± 6	83 ± 5*	72 ± 6*	66 ± 5*
PRPP amido-transferase	100 ± 6[f]	107 ± 6	103 ± 6	101 ± 7	93 ± 5	115 ± 6	109 ± 7	108 ± 7	103 ± 7
IMP dehydrogenase	100 ± 7[g]	95 ± 6	84 ± 5	80 ± 6*	79 ± 5*	73 ± 5*	71 ± 5*	59 ± 5*	56 ± 5*
Carbamyl phosphate synthetase	100 ± 7[h]	89 ± 7	83 ± 6	82 ± 6	78 ± 7*	102 ± 6	99 ± 6	94 ± 7	90 ± 5
Aspartate transcarbamylase	100 ± 5[i]	85 ± 6	106 ± 7	120 ± 8	109 ± 7	111 ± 7	99 ± 7	90 ± 6	86 ± 6
OMP decarboxylase	100 ± 6[j]	95 ± 5	92 ± 6	82 ± 5	74 ± 5*	101 ± 4	98 ± 5	91 ± 4	61 ± 5*
Thymidylate synthetase	100 ± 6[k]	103 ± 6	108 ± 7	113 ± 6	124 ± 8	104 ± 6	113 ± 5	115 ± 6	116 ± 7
Thymidine kinase	100 ± 7[l]	108 ± 6	116 ± 6	118 ± 7	128 ± 7*	145 ± 6*	130 ± 7*	134 ± 6*	138 ± 7*
TMP kinase	100 ± 6[m]	265 ± 9*	153 ± 8*	102 ± 6	83 ± 5	67 ± 5*	64 ± 5*	62 ± 4*	42 ± 4*
TDP kinase	100 ± 4[n]	124 ± 6*	106 ± 7	87 ± 6	86 ± 6	84 ± 5	28 ± 4*	15 ± 3*	12 ± 3*
DNA polymerase beta	100 ± 5[o]	112 ± 7	123 ± 7*	126 ± 6*	160 ± 7*	59 ± 4*	53 ± 4*	51 ± 4*	46 ± 4*
m-RNA polymerase	100 ± 5[p]	86 ± 7	78 ± 6*	76 ± 5*	61 ± 5*	113 ± 5	144 ± 6*	126 ± 7*	97 ± 6
r-RNA polymerase	100 ± 4[q]	72 ± 6*	36 ± 5*	25 ± 5*	17 ± 3*	220 ± 7*	101 ± 6	84 ± 5	82 ± 5
t-RNA polymerase	100 ± 6[r]	87 ± 7	80 ± 8	69 ± 7*	36 ± 5*	122 ± 6*	93 ± 7	90 ± 5	89 ± 6
Ribonucleoside reductase	100 ± 7[s]	42 ± 5*	38 ± 4*	24 ± 3*	22 ± 4*	20 ± 3*	23 ± 3*	22 ± 4*	15 ± 2*
Dihydrofolate reductase	100 ± 6[t]	99 ± 7	99 ± 6	96 ± 6	97 ± 5	97 ± 7	90 ± 6	97 ± 7	82 ± 6
dATP levels	100 ± 4[u]	—	—	—	127 ± 6*	—	—	—	94 ± 6
dGTP levels	100 ± 6[v]	—	—	—	101 ± 7	—	—	—	91 ± 5
dCTP levels	100 ± 5[w]	—	—	—	69 ± 5*	—	—	—	60 ± 4*
dTTP levels	100 ± 6[x]	—	—	—	74 ± 7*	—	—	—	26 ± 4*

[a] 7719 dpm
[b] 1014 dpm
[c] 17492 dpm
[d] 28614 dpm
[e] 19758 dpm
[f] 0.0878 O.D. Units
[g] 19575 dpm
[h] 0.273 mol citrulline
[i] 0.807 mol N-carbamyl aspartate
[j] 57387 dpm
[k] 77616 dpm
[l] 400 dpm
[m] 1371 dpm
[n] 1179 dpm
[o] 1891 dpm
[p] 9019 dpm
[q] 1343 dpm
[r] 325 dpm
[s] 48780 dpm
[t] 0.114 O.D. units
[u] 17.07 pmoles
[v] 13.58 pmoles
[w] 33.60 pmoles
[x] 31.04 pmoles

Studies with calf thymus DNA for 24 hr at 37°C showed that neither drug affected UV absorption or caused a hyperchromic shift of DNA's absorption. Thermal denaturation (TM values) of calf thymus DNA was not affected by drug incubation. DNA viscosity was altered. The control time to pass through the reservoirs was 274.5 sec; for **9** and **10**, it was 294.1 sec and 352 sec, respectively.

Incubation of L_{1210} cells with **9** and **10** for 24 hr showed DNA strand scission with the DNA molecule shifting in the gradient (Fig. 2a and 2b) to smaller molecular weight fragments.

Acute toxicity studies in CF_1 male mice after treatment with compound **9** showed no significant alterations in total body weight increase over seven days or in daily food consumption (Table 3). All of the animals survived with hematocrit and platelet estimates within normal limits. There were no major changes

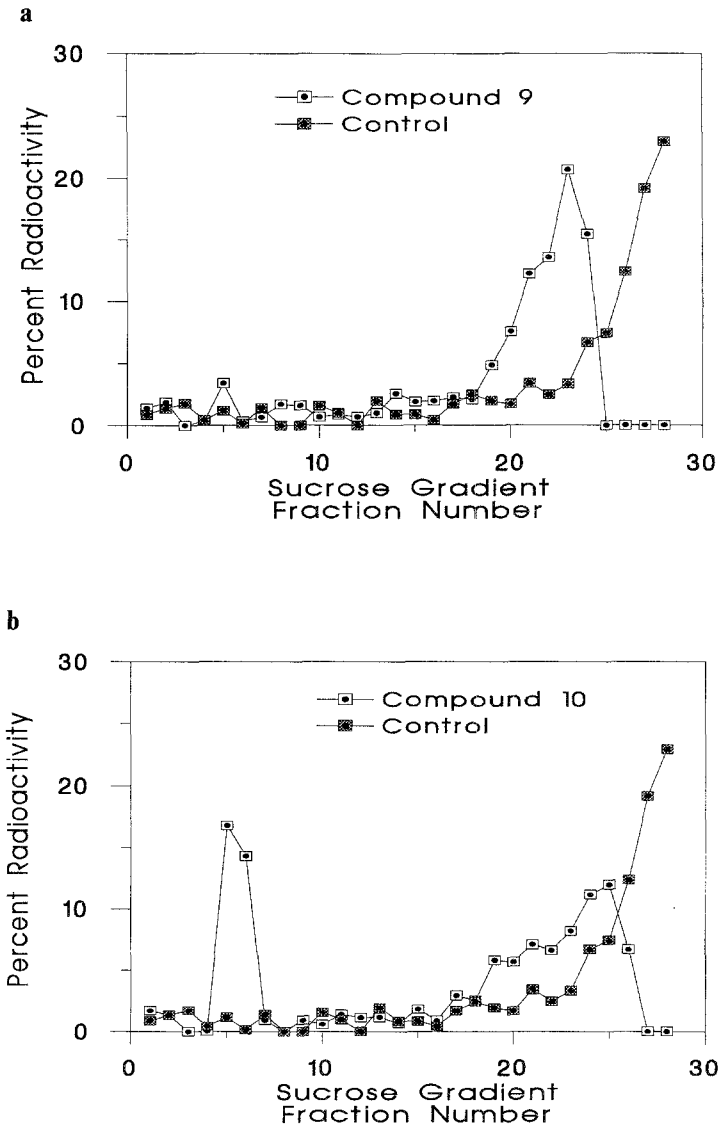

Fig. 2a and **b.** DNA strand scissier

Table 3. The acute toxicity in CF_1 male mice of compound **9**

($N = 5$)	Control	8 mg/kg	16 mg/kg	40 mg/kg
Body weight increase from day zero	104.3	99.5	97.5	97.8
Daily food consumption (g)	5.8	7.5	8.0	5.9
Survival	5/5	5/5	5/5	5/5
Hematocrit %	51.4	50.4	49.1	48.4
Platelet estimate $\times 10^4$	19.0	19.2	18.7	19.1
Differential white blood cell %				
Lymphocytes	100	100.8	100	104
PMNs	100	92	100	96
Eosinophils	100	100	200	—
Basophils	100	100	100	150
Monocytes	100	200	100	50
Red blood cells	OK*	OK	OK	OK
Organ weight (% body weight)				
Brain	1.0689	1.2508	1.2476	1.1989
Heart	0.4908	0.4997	0.6174	0.5631
Lung	0.7107	0.6442	0.6405	0.5837
Thymus	0.1221	0.1406	0.1547	0.1651
Liver	5.7264	4.9592	5.7145	5.6763
Kidney	1.8844	1.6401	1.8951	1.8462
Spleen	0.3513	0.2770	0.4551	0.4243
Stomach	2.9491	1.9731	1.7754	1.6952
Small intestine	5.9386	5.1414	4.6625	3.9538
Large intestine	2.9589	2.4481	2.7613	2.9027
Reproductive organs	3.1892	3.062	3.1036	3.4553
Clinical chemistry				
Cholesterol (mg/dL)	100	103	138	143
Triglycerides (mg/dL)	100	140	83	100
Total protein (g/100 mL)	100	107	102	97
Glucose (mg/dL)	100	114	103	79
BUN (mg/dL)	100	108	86	69
LDH (Sigma Unit/L)	100	61	81	99
SGPT (mol/min/L)	100	100	90	87
Acid phosphatase	100	—	25	18
Direct bilirubin (g/100 mL)	100	—	60	68
Uric acid (g/100 mL)	100	—	60	97

* No rouleaux formation

in the percent of lymphocytes and PMNs after treatment with compound **9**. There was some modulation of the lesser white cell population after treatment. There was no evidence of rouleaux by red blood cells. The organ weight of the treated animals showed some changes, e.g. brain and thymus showed an increase. Heart, reproductive organs and spleen showed increases in weight with higher doses of the drug. The stomach and small intestine showed decreases in weight after treatment. There were no histological or morphological changes in the liver, kidney and spleen of the mice treated with **9** at 8, 16 and 40 mg/kg/day. No clinical chemistry values changed significantly. Serum cholesterol levels were elevated at 16 and 40 mg/kg/day. Serum glucose, BUN, acid phosphatase and

direct bilirubin levels were decreased at 40 mg/kg/day treatment. The LD_{50} in CF1 mice was >500 mg/kg IP for compounds **9** and **10**.

Discussion

The boron containing peptides were shown to possess potent cytotoxicity against murine and human tissue cultured cancer cells. Significant activity was observed against the growth of single cell suspensions, e.g. L_{1210}, $Tmolt_3$ and HeLa-S^3, by most of the agents. In the cells derived from human solid tumors, selectivity in inhibiting cell growth was demonstrated.

The mode of action of compounds **9** and **10** in L_{1210} cells demonstrated significant inhibition of DNA, RNA and protein synthesis at higher doses, e.g., 50 and 100 μM. The observed increase in L_{1210} DNA and protein synthesis after treatment with **10** may be due to feedback control mechanisms involving regulatory enzymes to override drug inhibition at specific sites in the biochemical pathway. Elevation of DNA and protein synthesis followed by their reduction has been observed after treatment with cisplatinum agents (Harder and Rosenberg, 1970; Howle and Gale, 1970; Oswald et al., 1990). For the other types of boron derivatives, protein synthesis inhibition lagged behind inhibition of DNA synthesis in the cancer cells.

Whereas both de novo purine and pyrimidine synthesis were inhibited by both agents, the primary site of drug action in the pyrimidine pathway appeared to be at OMP decarboxylase. Other regulatory enzymes in this pathway were marginally inhibited to account for the overall suppression of pyrimidine synthesis. IMP dehydrogenase and not PRPP amido transferase was the site of inhibition in the purine pathway by the peptide boron derivatives. The magnitude of inhibition of IMP dehydrogenase activity was sufficient to account for the observed de novo purine synthesis inhibition in L_{1210}. Most of the amine-carboxyborane derivatives reported previously were more potent inhibitors of the purine pathway rather than the pyrimidine pathway; usually both regulatory enzymes, i.e. IMP dehydrogenase and PRPP amidotransferase, were inhibited (Hall et al., 1990; Hall et al., 1991; Sood et al., 1991). With the boron-containing peptide derivatives, there was no inhibition of dihydrofolate reductase and thymidylate synthetase. However, these two key enzyme sites are inhibited by metal complexes of boron and salts of hydroborates (Hall et al., 1991). But the heterocyclic adducts of amine carboxyboranes (Sood et al., 1991) and trimethylamine carboxyboranes (Hall et al., 1990) did not cause inhibition of thymidylate synthetase activity. These differences in ability to inhibit the activity of a specific enzyme is probably due to the longer peptide structure containing an aromatic group which did not bind to the pharmacophore or enzyme site in the same manner as the simple trimethylamine carboxylboranes. This finding would suggest that the PRPP amide transferase receptor site is too small to accommodate the binding of the boron derivative. The ability to cause different effects on specific enzyme activities may be based on the individual chemical moieties containing the boron atom rather than the presence of the boron alone or a boron salt. The electron distribution within the molecule around the boron atom caused differences in its abilites to bind to the active sites of the enzymes.

The peptide derivatives were unique in that they significantly inhibited m-RNA and r-RNA polymerase activity. The inhibition of t-RNA polymerase activity has been demonstrated by the other boron derivatives and the inability to affect DNA polymerase α has been reported previously (Hall et al., 1990, 1991; Sood et al., 1991). The peptide derivatives were very potent inhibitors of ribonucleoside reductase activity which is not true of the other methyl complexes, e.g. amino-carboxylboranes (Hall et al., 1990), hydroborates, and their heterocylic adducts (Sood et al., 1991) in addition to hydroborates (Hall et al., 1991). Suppression of this enzyme markedly reduces d(NTP) pool levels. The inhibition of the pyrimidine pathway by boronated peptide derivatives would account for the reduction of dCTP and dTTP. The levels of the deoxyribonucleoside pools after 60 min of drug incubation may not reflect blockage of de novo purine and pyrimidine syntheses since scavenger pathway are high in cancer cells partially where cells die rapidly.

DNA strand scission induced by **9** and **10** would also account in part for the reduction in DNA synthesis. All DNA inhibition occurred from the drugs' inhibition of de novo synthetic pathways. Previous studies have shown that compound **9** is neither incorporated into protein of $Tmolt_3$ leukemic cells in lieu of the phenyalanine residue nor incorporated into DNA or RNA. Unpublished data also indicate that compound **9** is not hydrolyzed in tissue culture cells over 24 hr. Apparently, the boron atom protects the amide bond from hydrolysis by most tissues, including GI mucosa secretions and cells possessing digestive proteolytic enzymes. The acute toxicity of the phenylalanine derivative **9** did not suggest that the agent had deleterious effects in CF_1 mice, particularly at the therapeutic dose of 8 mg/kg/day for antineoplastic activity (Sood et al., 1990). Thus, these peptide boron derivatives appear promising as antineoplastic agents.

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