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The Cytotoxicity of Trifluoromethyl Boron Derivatives and Mode of Action in Human $Tmolt_3$ T Leukemic Cells

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Trifluoromethylboron derivatives proved to be cytotoxic in a number of murine and human leukemic and lymphoma screens. Solid tumor growth, e.g. glioma HS 683, breast Mck-7 and colon adenocarcinoma SW480, was reduced significantly by the compounds. Human $Tmolt_3$ T-cell leukemia DNA synthesis was inhibited preferentially by the derivatives, with marginal effects on RNA and protein syntheses, after 60 min at 100 μM. The agents appeared to act by multiple mechanisms in that they inhibited the activities of DNA polymerase α, dihydrofolate reductase and nucleosides, significantly within 60 min at 100 μM. Deoxyribonucleotide pools were reduced after 60 min incubation with the compounds. $Tmolt_3$ DNA fragmentation and reduced ct-DNA viscosity were evident after 24 h of incubation at 100 μM. ct-DNA thermal denaturation studies indicated that the agents caused some type of interaction with the bases of DNA.

Keywords: trifluoromethylboron; cytotoxicity; leukemic; lymphoma

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INTRODUCTION

Boron-containing agents have been investigated in the past for the purpose of boron neutron capture therapy for human brain glioma, e.g. *p*-carboxyphenylboric acid. Soloway and co-workers initially investigated the use of *p*-carboxybenzeneboric acid,[1] sodium decahydrodecaborates[2] and sulfhydryl-containing hydroborates[3] for the treatment of brain tumors. Subsequently, arylboronic acids,[4,5] *p*-boronophenylalanine, 3-amino-4-carboxybenzeneboronic acid and *m*-boronsuccinanilic acid[5] were observed to cross the blood–brain barrier and were taken up efficiently by the tumor cells. Since these original studies, boron has been included in a number of carrier molecules (i.e. heterocyclic borones,[6] amino acids,[7] tumor-specific antibodies,[8] lipoproteins,[9] porphyrins,[10] anti-estrogens,[11] polyhedral boranes,[12] pyrimidines and nucleosides,[13,14] and antibiotics[15–17]) which are taken up preferentially by brain tumor cells. Boron compounds were then examined as antitumor agents not involving boron neutron capture. Soloway and Butler synthesized 4-[bis(2-carboroethyl)amino]phenylcarborane[17] and boron-containing acridines[18] which were tested *in vivo* in mouse ependymoma tumors. Shealy and Struck[19] prepared bis(2-chloroethyl)amino analogues which were tested against rat Walker 256 carcinosarcoma and mouse L1210 lymphoid leukemia growth. These derivatives were also active *in vitro* in the KB nasopharynx and H. Ep-2 larynx carcinoma cytotoxic screens. Bollag[20] prepared a boron derivative of 1-(3,4-dichlorophenyl)-5-isopropylbiguanide, a folic acid antagonist, as an antitumor agent. Alam *et al.*[21] prepared 1,2-*o*-carboranes and *nido*-carboranes linked to phenyl rings to treat B16 melanoma in mice.

This led to the synthesis of novel isoelectronic and isostructural boron analogues of α-amino acids which proved to be potent antineoplastic and cytotoxic agents.[22–25] Trimethylamine–carboxyborane inhibits rat Walker 256 carcinosarcoma

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growth and mouse Lewis lung growth,[1] and has been demonstrated to have potent cytotoxicity.[23,24] Further structural modifications have included amidation and esterification of trimethylamine–carboxyboranes,[26] heterocyclic amine–carboxyboranes,[27] di- and tri-peptide carboxyboranes,[28] boronated nucleic acids,[29] metal complexes of trimethylamine–carboxyborane,[15,30] and substituted carboranes and polyhedral hydroborate salts.[31] All of these derivatives were very effective in inhibiting DNA synthesis in L1210 lymphocytic leukemia cells.[22–32] The major target of the boron agents appeared to be the purine synthetic pathway and nucleoside kinases along with considerable DNA fragmentation. The ethylamine–carboxyborane derivative and the heterocyclic amine adducts were shown to inhibit HeLa-S^3 DNA topoisomerase II activity at 200 μM and the trimethylamine–carboxyboranes at 1000 μM.[33,34] The metal complexes of trimethylamine–carboxyborane, e.g. copper(II), cobalt, iron and chromium (II), were very effective inhibitors of L1210 DNA topoisomerase II activity *in vitro* at 10–100 μM, but did not themselves cause DNA protein-linked breaks.[35] Other metal complexes of polypeptides and trimethylamine have been examined.[36,37] Ferratricarbadecarbaranyl complexes have recently been shown to have similar cytotoxicities and effects on nucleic acid metabolism, including inhibition of DNA topoisomerase activity.[22] The present study involves the evaluation of a different type of boron complex, trifluromethylboron derivatives, for their cytotoxicity and their mechanism of action in tumor cells to determine whether their effects are similar to those of the previous boron complexes.

CHEMISTRY

Replacement of a hydrogen or a methyl group by a trifluoromethyl group changes the chemical and physical properties of the boron derivatives. Because of the inductive effect of the F_3C groups, the B–NB bond in amine–trifluoromethylboranes $(F_3C)_2R_1B{\cdot}NHR_2$, where R_1 is an arbitrary ligand, is stronger than in non-fluorinated analogues. In general, the attachment of the F_3C group stabilizes the tetracoordinated state of the boron to an extent that such compounds are better described as neutral 'ammonium' ions rather than as adducts. Little is known about the physiological properties of trifluoromethylated boron species, and nothing about their cytotoxicity. The selection of test compounds from the approximately 200–300 trifluoromethylboron compounds synthesized to date was arbitrary but these compounds were representative of various interesting species of this chemical class: compound **1**, cesium tris-(trifluoromethyl)nitroborate $\{Cs[(CF_3)_3BNO_2]\}$, is a salt with high water solubility; compound **2**, amino–tris(trifluoromethyl)borane tetrahydrate $\{(CF_3)_3B{\cdot}NH_3{\cdot}4H_2O\}$, is a water-soluble covalent species; compound **3**, potassium 2,2-bis(trifluoromethyl)-3-methyl-3-azonia-2-boratabutanesulfonate hydrate $\{K[O_3SCH_2B(CF_3)_2{\cdot}NHMe_2]{\cdot}H_2O\}$, is a salt-like ammonium ion; compound **4**, dimethylpropargylamine–bis(trifluoromethyl)borane $\{HB(CF_3)_2\ NMe_2CH_2C{\equiv}CH$ contains a B–H bond; and compound **5**, dimethylamine–bis(carboxy)-methylbis(trifluoromethyl)borane $\{[HOOC)_2CH]B(CF_3)_2\ NHMe_2\}$, has a carboxylic acid function.

SOURCE OF COMPOUNDS

The synthesis of **2**,[38] **3**[39] and **4**[40] has been reported previously. The X-ray crystal structure of **2** has been reported recently.[41] Compound **1**[42] was prepared from **2** by ozone oxidation in aqueous CsOH solution and has been published (Eqn [1]).[42]

$$(CF)_3)_3BNH_3 + CsOH \xrightarrow{O_3} Cs[(CF_3)_3BNO_2] + 2H_2O \quad [1]$$

Compound **5** was prepared from dimethylaminobis(trifluoromethyl)borane, $(CF_3)_2BNMe_2$ and malonic acid t-butyl ester $(C_4H_9OOC)_2CH_2$ to yield $[C_4H_9OOC)_2CH]B(CF_3)_2NHMe_2$. The t-butyl ester was subsequently cleaved by trifluoroacetic acid to form free carboxylic acid, $[(HOOC)_2CH]B(CF_3)_2NHMe_2$. Mass spectrum MS (70 eV): *m/z* (%) = 92 (100) $[F_3BC_2H_3O^+]$, 94 (52) $[F_2BNH(CH_3)_2{}^+]$, 44 (24) $[C_2H_6N^+]$, 74 (13) $[FBN(CH_3)_2{}^+$, 253 (2) $[M^+{-}COA]$, 228 (1) $[M^+{-}CF_3]$, 277 (1) $[M^+{-}HF]$. Elemental analysis: Calcd: C, 28.32; H, 3.39; N, 4.72; Found: C, 28.00; H, 3.33; N, 5.56%. All compounds were recrystallized and were more than 99.4% pure for the pharmacological studies.

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).

CYTOTOXICITY

Compounds **1** and **5** (Table 1) were tested for cytotoxic activity by homogenizing the drugs as a 1 *m*g ml^{-} solution in 0.05% Tween 80 H_2O. These solutions were sterilized by passing them through an Acrodisc (0.45 μm). Different histological types of murine and human tumors were selected for cytotoxic testing of the agents to evaluate their potency in a wide range of tumors. Included in this series of tumors were some human tumors which do not have effective drugs which inhibit their cell growth.

The following cell lines were maintained by literature techniques:[43] murine L_{1210} lymphoid and P388 lymphocytic leukemias, human $Tmolt_3$ and $Tmolt_4$ T-cell leukemias, Hl-60 leukemia, HuT-78 cutaneous lymphoma, THP-1 monocytic leukemia, SW480 colorectal adenocarcinoma, HCT-8 ileocecal adenocarcinoma, MB-9812 lung bronchogenic, A-549 lung carcinoma, Saos-2 osteosarcoma, KB epidermoid nasopharynx, A431 skin epidermoid, HeLa-S^3 suspended and HeLa solid cervical carcinomas, Mck-7 breast effusion, clear cell renal Caki, Sk-MEL-2 malignant melanoma and U-87-MG glioma. The NCI protocol was used to assess the cytotoxicity of the test compounds and standards in each cell line.[43] The number of cells was determined by the Trypan Blue exclusion technique[43] and the percentage inhibition of growth for each concentration of the compound was calculated and averaged ($N = 4$). The percentage inhibition was plotted against the log of the concentration of the compound and the ED_{50} value estimated. Solid tumor cytotoxicity was determined utilizing Crystal Violet/MeOH and read at 580 nm (Molecular Devices).[44] Values of cytotoxicity were expressed as ED_{50} (g ml^{-}), i.e. the concentration of the compound inhibiting 50% of cell growth [Tables 1 and 2]. A value of less than 4 μg ml^{-} was required for significant activity for inhibition of tumor cell growth.

INCORPORATION STUDIES

The effects of drugs on the incorporation of radiolabeled precursors [^{3}H]thymine (72 *Ci* mmol^{-1}), [^{3}H]uracil (12 Cimmol^{-}) or [^{3}H]leucine (120 Cimmol^{-}) into DNA, RNA or protein, respectively, for 10^6 $Tmolt_3$ T-cell leukemia cells at 25, 50 and 100 μM was determined for 60 min.[45] The acid-insoluble labeled DNA, RNA or protein was collected on discs [GF/A] which were counted in a Packard beta-counter. Incorporation of [^{14}C]glycine (53.0 mCimmol^{-1}) into purines was obtained by the method of Cadman *et al.*[46] Incorporation of [^{14}C]formate (53.0 mCimmol^{-1}) into pyrimidines was determined by the method of Christopherson *et al.*[47] The final purines or pyrimidines were separated by TLC from the starting components using the appropriate R_f for standard nucleoside bases, and counted.

ENZYME ASSAYS

Since the trifluoromethylboron derivatives **1–5** inhibited RNA and DNA syntheses in $Tmolt_3$ cells effectively, their effects on a number of enzymes involved in nucleic acid metabolism were determined at 25, 50 and 100 μM after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler *et al.*'s method.[48,49] The DNA polymerase α assay was described by Sawada *et al.*[50] with 2-deoxy[^{3}H]ribothymidine-5′-triphosphate [TTP] (53 Ci mmol^{-1}). Messenger-, ribosomal- and transfer-RNA polymerase enzymes were isolated from the nuclei with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using [^{3}H]uridine-5′-triphosphate [UTP] (35 Ci mmol^{-1}).[51,52] The following enzyme activities were determined using $Tmolt_3$ homogenates. Ribonucleotide reductase activity was measured using [^{14}C]cytidine-5′-diphosphate (CDP) (19.4 Ci mmol^{-1}) with dithioerythritol.[53] 2′-Deoxy[^{14}C]ribocytine- 5′-diphosphate was separated from the [^{14}C]CDP by TLC on polyethyleneimine cellulose (PEI) plates. Thymidine, thymidine-5′-monophosphate (TMP) and thymidine-5′-diphosphate (TDP) kinase activities were determined using [^{3}H]thymidine (58.3 mCi mmol^{-1}) in the medium of Maley and Ochoa[54] and separated by TLC. Carbamyl phosphate synthetase activity was determined by the method of Kalman *et al.*[55] and the product citrulline was determined colorimetrically.[56] Aspartate transcarbamylase activity was measured using the incubation medium of Kalman *et al.*,[55] the product carbamyl aspartate was determined

Table 1 The cytotoxicity (ED_{50}, $\mu g\ ml^{-1}$) of the trifluoromethylboron derivatives against the growth of suspended tumor cells[a]

Compound	Murine		Human					
	L_{1210} leukemia	P388 leukemia	THP-1 acute monocytic leukemia	$Tmolt_3$ T-cell leukemia	$Tmolt_4$ T-cell leukemia	HuT 78 lympho	H1-60 leukemia	HeLa$-S^3$ uterine carcinoma
1 $Cs[(CF_3)_3BNO_2]$	3.62	2.96	3.16	3.58	3.95	1.13	4.86	4.27
2 $(CF_3)B{\cdot}NH_3{\cdot}4H_2O{\cdot}$	3.85	3.42	2.05	2.86	2.64	2.25	4.81	3.73
3 $K[O_3SCH_2B(CF_3)_2{\cdot}NHMe_2]{\cdot}H_2O$	3.17	3.32	2.64	3.44	3.04	2.00	4.27	2.34
4 $HB(CF_3)_3{\cdot}NMe_2CH_2{=}CH$	3.72	2.07	4.81	3.01	2.80	3.00	3.08	4.45
5 $[(HOOC)_2HC]B(CF_3)_2{\cdot}NHMe_2$	3.14	2.10	4.56	2.44	3.27	2.25	3.67	3.12
Standard agents for comparison								
6-MP	2.43	2.04	3.03	0.43	2.67	1.63	1.63	2.12
Ara-C	2.43	0.79	2.54	1.29	2.36	2.50	2.50	2.13
Hydroxyurea	2.67	2.87		4.47	6.68	3.87	5.22	1.96
5-FU	1.41	0.99	0.49	2.14	2.75	1.50	1.50	2.47
VP-16	1.83	0.99	3.27	1.00	1.92	1.13	1.13	7.87

[a] $N = 4$; $ED_{50} \leq 4\ \mu g\ ml^{-1}$ for significant activity.
6-MP = 6-Mercaptopurine; Ara-C = Cytarabine or Cytosine Arabinosine; 5-FU = 5-Fluorouracil; VP-16 = Etoposide.

Table 2 The cytotoxicity (ED_{50}, $\mu g\ ml^{-1}$) of the trifluoromethylboron derivatives against the growth of human tumor cells derived from solid tumors

Compound	Breast Mck-7	Solid HeLa	Renal Caki-1	KB nasopharynx	Bone Saos-2	SW 480 colon	HCT-8 ileum	Skin 431	Melanoma SK2	Lung MB 9812
1	3.01	9.98	13.89	5.84	7.36	2.33	6.08	5.09	8.24	6.45
2	2.47	10.91	6.21	6.98	9.15	2.44	7.83	2.55	10.60	6.40
3	3.95	8.81	10.40	6.43	8.53	1.44	8.20	10.70	7.15	6.06
4	2.80	7.01	15.55	6.23	9.77	5.47	5.96	7.80	10.29	6.45
5	2.47	6.26	8.00	4.51	7.62	6.74	5.10	7.89	7.87	5.91
Standard agents for comparison										
6-MP		5.61	9.35	11.04	7.16	3.61	1.15	9.13	6.86	4.29
Ara-C		4.74	1.38	2.84	8.90	3.42	2.54	0.86	10.53	6.16
Hydroxyurea		8.12		5.27		7.33	1.77	2.87		7.18
5-FU		4.11	5.29	1.25	8.75	3.09	1.12	1.80	5.93	5.64
VP-16		3.05	7.01	3.32	7.19	3.34	3.78	1.97	3.53	3.50

[a] $N = 4$; $ED_{50} \leq 4\ \mu g\ ml^{-1}$ for significant activity.

Table 3 Effects (% control) of cesium tris (trifluoromethyl)nitroborate (**1**) on $Tmolt_3$ T-cell leukemia nucleic acid metabolism after 60 min incubation

Assay ($N = 4$)	Control	25μM	50μM	100μM
DNA synthesis	100 ± 5^a	86 ± 5	72 ± 4*	14 ± 2*
RNA synthesis	100 ± 6^b	94 ± 6	69 ± 4*	66 ± 4*
Protein synthesis	100 ± 5^c	123 ± 5	108 ± 5	95 ± 5
DNA polymerase α	100 ± 6^d	70 ± 5	55 ± 4*	53 ± 5*
m-RNA polymerase	100 ± 7^e	104 ± 4	109 ± 4	119 ± 5
r-RNA polymerase	100 ± 4^f	98 ± 4	101 ± 3	102 ± 5
t-RNA polymerase	100 ± 7^g	83 ± 4	81 ± 4*	48 ± 3*
Ribonucleotide reductase	100 ± 5^h	88 ± 5	88 ± 4	87 ± 5
Dihydrofolate reductase	100 ± 5^i	79 ± 5	42 ± 3*	23 ± 2*
Purine *de novo* synthesis	100 ± 5^j	81 ± 4*	73 ± 4*	68 ± 3*
PRPP amidotransferase	100 ± 6^k	88 ± 5	80 ± 4*	77 ± 4*
IMP dehydrogenase	100 ± 5^l	140 ± 6*	136 ± 5*	95 ± 5
Pyrimidine *de novo* synthesis	100 ± 6^m	129 ± 6	113 ± 7	94 ± 5
Carbamyl phosphate synthetase	100 ± 7^n	124 ± 5	111 ± 6	110 ± 4
Aspartate transcarbamylase	100 ± 6^o	98 ± 5	93 ± 5	87 ± 4
Thymidylate synthetase	100 ± 5^p	104 ± 5	80 ± 4*	71 ± 3*
Thymidine kinase	100 ± 6^q	68 ± 4*	63 ± 3*	53 ± 3*
Thymidine monophosphate kinase	100 ± 7^r	61 ± 4*	43 ± 4*	42 ± 3*
Thymidine diphosphate kinase	100 ± 6^s	98 ± 5	24 ± 2*	14 ± 2*
d[ATP]	100 ± 5^t			98 ± 4
d[GTP]	100 ± 6^u			97 ± 5
d[CTP]	100 ± 5^v			92 ± 3
d[TTP]	100 ± 4^w			86 ± 4

Control values for 10^6 cells/h:

[a] 7 719 dpm
[b] 1 014 dpm
[c] 17 492 dpm
[d] 9 019 dpm
[e] 1 343 dpm
[f] 325 dpm
[g] 400 dpm
[h] 48 780 dpm
[i] 0.144 OD unit
[j] 28 624 dpm
[k] 0.0878 OD unit
[l] 19575 dpm
[m] 19758 dpm
[n] 0.807 mol *N*-carbamyl a sparate
[o] 0.273 mmol citrulline
[p] 77616 dpm
[q] 1371 dpm
[r] 179 dpm
[s] 1891 dpm
[t] 17.07 pmol
[u] 13.58 pmol
[v] 33.60 pmol
[w] 31.04 pmol

* $P \leq 0.001$ (Student's *t*-test).

colorimetrically by the method of Koritz *et al.*[57] Thymidylate synthetase activity was analyzed by the method of Kampf *et al.*[58] The ^3H_2O separated by charcoal was proportional to the amount of TMP formed from 2′-deoxy[^{3}H]ribouridine-5′-monophosphate (UMP) (20 Ci mmol^{-1}). Dihydrofolate reductase activity was determined by the NADH disappearance spectrophotometric method of Ho *et al.*[59] at 340 nm. Phosphoribosyl pyrophosphate (PRPP) amidotransferase activity was determined by Spassova's method as the generation of NADH[60] and inosine −5′-monophosphate (IMP) dehydrogenase activity was analyzed with 8-[^{14}C]IMP (54 mCi mmol^{-1}) (Amersham, Arlington Heights, IL, USA) after separating [^{14}C] xanthosine-5′-monophosphate (XMP) on PEI plates (Fisher Scientific) by thin-layer chromatography (TLC)[61] which was then counted. Protein content was determined for the enzymatic assays by the Lowry technique.[62] Results are given in Tables 3–6.

DNA STUDIES

After deoxyribonucleoside triphosphates (d[NTP]) were extracted[63] from $Tmolt_3$ cells, d[NTP] levels were determined by the method of Hunting and Henderson[64] with calf thymus DNA, *E. coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of [^{3}*H−methyl*]-d[TTP] (53.1 Ci mmol^{-1}) or [5-^{3}H]-d[ATP]

Table 4 The effects of amine–tris(trifluoromethyl)borane tetrahydrate (**2**) on $Tmolt_3$ T-Cell leukemia nucleic acid metabolism after 60 min incubation

Assay ($N = 4$)	Control	25μM	50μM	100μM
DNA synthesis	100 ± 5	94 ± 6	88 ± 5	24 ± 5*
RNA synthesis	100 ± 6	$84 + 5$	79 ± 4*	68 ± 4*
Protein synthesis	100 ± 5	03 ± 6	98 ± 4	91 ± 5
DNA polymerase α	100 ± 6	93 ± 6	84 ± 5	69 ± 4*
m-RNA polymerase	100 ± 7	106 ± 4	125 ± 5*	130 ± 4*
r-RNA polymerase	100 ± 4	106 ± 5	97 ± 4	77 ± 3*
t-RNA polymerase	100 ± 7	95 ± 4	86 ± 4	80 ± 3*
Ribonucleotide reductase	100 ± 5	136 ± 5*	114 ± 4	78 ± 4*
Dihydrofolate reductase	100 ± 5	101 ± 5	101 ± 4	34 ± 3*
Purine *de novo* synthesis	100 ± 5	75 ± 5	69 ± 4	56 ± 4
PRPP amidotransferase	100 ± 6	84 ± 6	71 ± 4	67 ± 3
IMP dehydrogenase	100 ± 5	136 ± 5*	116 ± 4	79 ± 4*
Pyrimidine *de novo* synthesis	100 ± 6	123 ± 4	123 ± 5	116 ± 5
Carbamyl phosphate synthetase	100 ± 7	116 ± 7	113 ± 5	89 ± 5
Aspartate transcarbamylase	100 ± 6	94 ± 6	77 ± 5*	71 ± 4*
Thymidylate synthetase	100 ± 5	139 ± 6	107 ± 5	91 ± 5
Thymidine kinase	100 ± 6	97 ± 5	54 ± 4*	50 ± 4*
Thymidine monophosphate kinase	100 ± 7	53 ± 4	36 ± 5	29 ± 2*
Thymidine diphosphate kinase	100 ± 6	61 ± 4*	21 ± 3*	14 ± 2*
d[ATP]	100 ± 5			98 ± 4
d[GTP]	100 ± 6			71 ± 3*
d[CTP]	100 ± 5			90 ± 4
d[TTP]	100 ± 4			86 ± 4

* $P \leq 0.001$ (Student's *t*-test).
See control values in Table 3.

(30 Ci mmol^{-1}). Thus, 2′-deoxyriboadenosine-5′-triphosphate (d[ATP]) 2′-deoxyriboguanosine-5′-triphosphate (d[GTP]) 2′-deoxyribocytidine-5′-triphosphate (d[CTP]) and thymidine-5′-triphosphate (d[TTP]) levels were determined after incubation with the drugs for 60 min at 100 μM. The effects of compounds **1–5** on DNA strand scission were determined by the methods of Suzuki *et al.*[65], Pera *et al.*[66] and Woynarowski *et al.*[67] $Tmolt_3$ lymphoid leukemia cells were incubated with 10 μCi [*methyl*-^3H]thymidine (84.0 Ci mmol^{-1}) for 24 h at 37 °C. $Tmolt_3$ cells (10^7) were harvested and then centrifuged at 600 g × 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–KC1 and 0.01 M EDTA); this was followed by 0.2 ml of the cell preparation. After the gradient was incubated for 2.5 h at room temperature, it was centrifuged at 12 000 g × 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.

Calf thymus DNA thermal denaturation studies were conducted from 37 to 100 °C in order to determine the T_m values for the control and drugs at 100 μM for 24 h. Changes in DNA UV absorption from 220 to 340 nm and ct-DNA viscosity were measured after incubation of compounds **1–5** at 100 μM at 37 °C for 24 h.[68,69]

STATISTICS

The mean and standard deviations are designated by $x \pm$ SD The probable level of significance (P) between test and control samples was determined by Student's *t*-test using the raw data.

RESULTS

The trifluoromethylboron derivatives proved to be potent cytotoxic agents, reducing the growth of suspended (Table 1) as well as solid tumor cells (Table 2). An ED_{50} value of less than 4 μg ml^{-1} is

Table 5 Effects (% control) of potassium 2,2-bis(trifluoromethyl)-3-methyl-3-azonia-2-boratabutanesulfonate hydrate (**3**) on $Tmolt_3$ T-cell leukemia nucleic acid metabolism after 60 min incubation

Assay ($N = 4$)	Control	25μM	50μM	100μM
DNA synthesis	100 ± 5	97 ± 4	48 ± 3*	43 ± 3*
RNA synthesis	100 ± 6	95 ± 4	81 ± 3*	76 ± 3*
Protein synthesis	100 ± 5	86 ± 5	84 ± 5	74 ± 4*
DNA polymerase α	100 ± 6	79 ± 5*	78 ± 4*	68 ± 4*
m-RNA polymerase	100 ± 7	107 ± 4	97 ± 5	94 ± 4
r-RNA polymerase	100 ± 4	$99 + 5$	$110 + 5$	$118 + 5$
t-RNA polymerase	100 ± 7	107 ± 6	110 ± 5	120 ± 6
Ribonucleotide reductase	100 ± 5	83 ± 4	111 ± 6	64 ± 4*
Dihydrofolate reductase	100 ± 5	114 ± 5	111 ± 4	64 ± 3*
Purine *de novo* synthesis	100 ± 5	91 ± 5	85 ± 5	81 ± 4*
PRPP amidotransferase	100 ± 6	81 ± 4	72 ± 4*	65 ± 3*
IMP dehydrogenase	100 ± 5	142 ± 6	95 ± 5	84 ± 4
Pyrimidine *de novo* synthesis	100 ± 6	132 ± 5*	132 ± 6*	129 ± 5*
Carbamyl phosphate synthetase	100 ± 7	139 ± 7	111 ± 5	109 ± 6
Aspartate transcarbamylase	100 ± 6	88 ± 5	88 ± 6	64 ± 4*
Thymidylate synthetase	100 ± 5	122 ± 5	87 ± 4	87 ± 3
Thymidine kinase	100 ± 6	106 ± 5	105 ± 5	54 ± 3*
Thymidine monophosphate kinase	100 ± 7	65 ± 3*	50 ± 4*	46 ± 4*
Thymidine diphosphate kinase	100 ± 6	76 ± 5*	56 ± 4*	53 ± 5*
d[ATP]	100 ± 5			77 ± 4*
d[GTP]	100 ± 6			82 ± 4
d[CTP]	100 ± 5			95 ± 5
d[TTP]	100 ± 4			81 ± 5

* $P \leq 0.001$ (Student's *t*-test).
See control values in Table 3.

required in these screens for significant activity. The trifluoromethylboron derivatives were all active against the growth of murine L_{1210} lymphoid and P-388 lymphocytic leukemias, human $Tmolt_3$ and $Tmolt_4$ leukemias, and HuT-78 lymphoma. Compounds **1, 2** and **3** inhibited THP-1 acute monocytic leukemia growth. Only compounds **4** and **5** were effective against the growth of human Hl-60 leukemia. Compounds **2, 3** and **5** were active against the growth of suspended HeLa-S^3 uterine carcinoma growth. Compounds **1–3** were effective in reducing growth of colon adenocarcinoma SW480 but compound **2** was significantly active in the skin A431 screen. All five compounds were active against the growth of glioma Hs-683 and breast effusion Mck-7.' None of the compounds were active in the solid HeLa uterine carcinoma, KB nasopharynx', bone SOS', HCT-8 ileum adenocarcinoma, lung MB9812′, lung A549′, Sk-Mel-2 melanoma, or clear cell renal Caki-1 adenocarcinoma screens.

In the mode of action study performed in human $Tmolt_3$ T-cell leukemia cells, DNA synthesis was preferentially inhibited by the trifluoromethylboron derivatives in a concentration-dependent manner after 60 min, achieving more than 50% inhibition at 100 μM [Tables 3–6]. RNA synthesis was only marginally reduced by 20–33% after 60 min and protein synthesis was inhibited by 26% only by compound **3** at 100 μM after 60 min. DNA polymerase α activity was reduced by 21–47% at 100 μM. m-RNA polymerase activity was reduced by 23% by compound **5**. t-RNA polymerase activity was lowered by 52% by compound **1** and 20% by compound **2** after 60 min incubation at 100 μM. Ribonucleoside reductase activity was reduced by 22–36% by compounds **2** and **3** at 100 μM. Dihydrofolate reductase activity was significantly inhibited by 36–77% by the compounds at 100 μM after 60 min. Purine *de novo* synthesis after 60 min was reduced by 32% and 44% by compounds **1** and **2** after 60 min at 100 μM. PRPP amidotransferase activity was inhibited by 23–35% by the compounds while IMP dehydrogenase activity was reduced by 21% only by compound **2** at 100 μM after 60 min. Pyrimidine *de novo* synthesis and carbamyl phosphate synthetase activity were not affected by the compounds at these concentrations. Aspartate transcarbamylase activity was reduced by 27%, 29% and 36% by compounds **5, 2** and **3**,

Table 6 Effects (% control) of dimethylamine–bis(carboxy)methylbis(trifluoromethyl)borane (**5**) on $Tmolt_3$ T-cell leukemia nucleic acid metabolism after 60 min incubation

Assay ($N = 4$)	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	92 ± 6	80 ± 5	38 ± 4*
RNA synthesis	100 ± 6	88 ± 5	87 ± 4	80 ± 3*
Protein synthesis	100 ± 5	110 ± 6	106 ± 5	104 ± 5
DNA polymerase α	100 ± 6	93 ± 5	89 ± 5	79 ± 4*
m-RNA polymerase	100 ± 7	110 ± 4	107 ± 5	77 ± 4*
r-RNA polymerase	100 ± 4	91 ± 4	88 ± 4	86 ± 5
t-RNA polymerase	100 ± 7	109 ± 6	112 ± 5	124 ± 7
Ribonucleotide reductase	100 ± 5	139 ± 5*	118 ± 4	94 ± 5
Dihydrofolate reductase	100 ± 5	105 ± 6	61 ± 5*	55 ± 4*
Purine *de novo* synthesis	100 ± 5	96 ± 6	92 ± 5	88 ± 4
PRPP amidotransferase	100 ± 6	86 ± 5	74 ± 4	71 ± 4*
IMP dehydrogenase	100 ± 5	131 ± 5	111 ± 5	86 ± 4
Pyrimidine *de novo* synthesis	100 ± 6	113 ± 4	93 ± 5	90 ± 5
Carbamyl phosphate synthetase	100 ± 7	127 ± 6	121 ± 5	110 ± 5
Aspartate transcarbamylase	100 ± 6	77 ± 5*	76 ± 4*	73 ± 4*
Thymidylate synthetase	100 ± 5	269 ± 9	166 ± 7	101 ± 5
Thymidine kinase	100 ± 6	137 ± 6	68 ± 4*	51 ± 3*
Thymidine monophosphate kinase	100 ± 7	87 ± 5	62 ± 3*	38 ± 2*
Thymidine diphosphate kinase	100 ± 6	71 ± 4*	47 ± 4*	16 ± 2*
d[ATP]	100 ± 5			79 ± 3*
d[GTP]	100 ± 6			99 ± 5
d[CTP]	100 ± 5			97 ± 4
d[TTP]	100 ± 4			100 ± 5

* $P \leq 0.001$ (Student's *t*-test).
See control values in Table 3.

respectively at 100 μM after 60 min. Thymidylate synthetase activity was lowered by 29% by compound **1**. Thymidine kinase activity was reduced by 46–50% by the compounds at 100 μM after 60 min. Thymidine monophosphate kinase activity was inhibited by 54–71% by the derivative and thymidine diphosphate kinase activity was lowered by 47–86% by the compounds at 100 μM after 60 min. The d[GTP] pool level was lowered by 29% by compound **2** and the d[ATP] pool levels were reduced by 21% and 23% by compounds **5** and **3**, respectively, after 60 min at 100 μM.

ct-DNA studies after incubating the agents at 100 μM for 24 h demonstrated that there was no hyperchromic shift in the UV absorption from 220 to 340 nm, indicating that the agents did not cause any S_N1 or S_N2 alkylation of the deoxyribonucleotide bases. The T_m values for the denaturation of ct-DNA were: control, 92.5 °C, compound **1**, 77.5 °C; compound **2**, 85 °C and compounds **3** and **5**, 83.5 °C. This indicated that there was a possibility of the agents' intercalation between the base-pairs of DNA. ct-DNA viscosities times to move through the reservoirs, were: control 506 s; compound **1**, 485.6 s; compound **2**, 509 s; compound **3**, 488 s; and compound **5**, 484 s. This suggested that lower-molecular-weight DNA was present. Indeed, $Tmolt_3$ DNA strand scission studies after 24 h at 100 μM showed that DNA fragmentation did occur, with the radioactivity being displaced from fraction 17 to lower fractions (Fig. 1).

DISCUSSION

The trifluoromethylboron derivatives exhibited cytotoxic action against the growth of a number of murine and human leukemias and lymphomas and suspended HeLa-S^3 cells. The ED_{50} values for the new compounds were in the range of the ED_{50} values of the standard clinical drugs. Very similar ED_{50} values have been demonstrated for boron derivatives of a variety of chemical types. Activity against solid tumor growth was more selective, but the compounds were active against the growth of human glioma Hs 683; however, the ferratricarbadecarbaranyl complexes and amine–carboxyboranes were not active in the glioma screen. The compounds also inhibited the growth of human

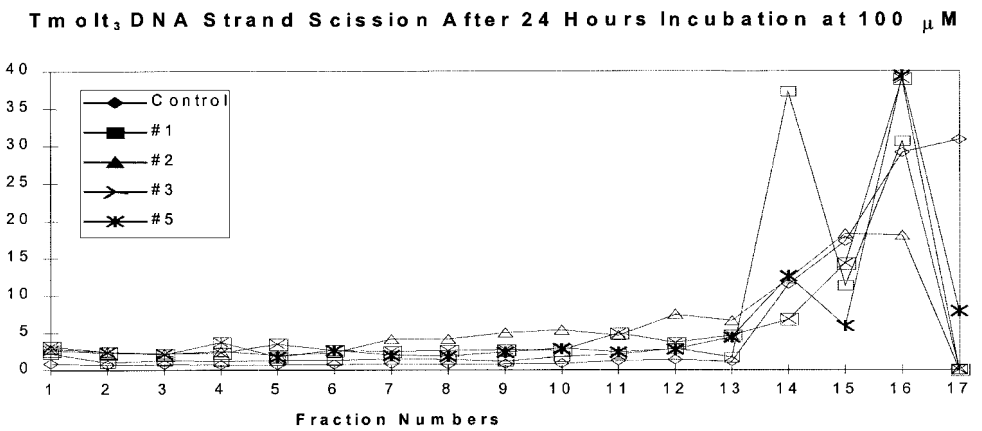

Figure 1 $Tmolt_3$ leukemia DNA strand scission after 24 h incubation with agents: $N = 4$; standard deviation 0.05%.

breast effusion MCK-7. This is an unusual property for boron complexes, since none has yet been shown to be active against the growth of human breast cells; furthermore, they were more potent in this screen than the standard clinical drugs. A number of the compounds were active against the growth of SW480 adenocarcinoma, with ED_{50} values similar to the ED_{50} values for amine–carboxylboranes but they were better than the values obtained for the ferratricarbadecarbaranyl complexes.

Mode-of-action studies in murine $Tmolt_3$ cells demonstrated that DNA synthesis was significantly reduced after 60 min and appears to be the major target of the agents. As observed with other boron–metal complexes, these compounds reduced *de novo* purine synthesis having the most effect on dihydrofolate reductase activity. The reduction of the activity of this enzyme would reduce one carbon transfer for purine synthesis, affecting DNA synthesis and to a lesser extent RNA synthesis, which is what was observed after 60 min incubations with the agents. The reason for this differential effect is that the ratio of ribonucleotide pools to deoxyribonucleotide pools is approximately 9:1 in mammalian cancer cells; thus, the effects of the drugs would appears first in DNA synthesis, followed later in RNA synthesis. In addition, the agents inhibited the activity of the first regulatory enzyme of the purine pathway, i.e. PRPP amidotransferase, moderately but did not affect the second regulatory enzyme, IMP dehydrogenase. Most of the amine–carboxyboranes and ferratricarbadecarbaranyl complexes actually inhibited both regulatory enzyme activities. The activity of ribonucleotide reductase was moderately reduced by the agents; this would suppress the conversion of ribonucleotides to deoxyribonucleotides for DNA synthesis. The fact that thymidine kinases and DNA polymerase α activities were reduced by the compounds would also be additive effects in inhibiting DNA synthesis rather than RNA synthesis, initially. The d[NTP] pools were reduced in certain cases, which reflects the inhibition of purine synthesis by the agents. If the major target of the agents was the inhibition of DNA polymerase α activity, this action would result in an increase in d[NTP] pools because the deoxyribonucleotides were not incorporated into the new strand of DNA. Thus, marginal reduction in the d[NTP] pools after 60 min indicates that the compounds' mode of action is probably as antimetabolities, and the inhibition of purine synthesis and related enzymes is more important than the effects observed on DNA polymerase α although the overall effects of the agents is additive in bringing about suppression of DNA synthesis. The different moieties within the individual compounds would account for the slight difference observed in the effects caused by each compound on the individual enzyme activity.

The agents did not appear to alkylate individual nucleotide bases of DNA through some type of S_N1 or S_N2 reaction, but that they did interact with the DNA molecule was suggested by the alteration of T_m values and DNA fragmentation over 24 h. Other boron derivatives have exhibited similar behavior in causing DNA strand scission and reduced DNA viscosity as a part of their mode of action. The metal complexes of amine–carboxyboranes and the ferratricarbadecarbonyl complexes were potent inhibitors of DNA topoisomerase II activity, which would explain the DNA fragmentation. The

trifluoromethyl boron derivatives have not yet been examined for this biochemical property; however, this pilot investigation of this chemical class of boron derivatives indicates that these derivatives should be evaluated for clinical use as antineoplastic agents by a more extensive investigation.

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