APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 9, 111–119 (1995)

Anti-osteoporotic Activity of Metal Complexes of Amine Carboxyboranes

K. G. Rajendran,* A. Sood,† B. F. Spielvogel,† I. H. Hall,* V. M. Norwood III‡ and K. W. Morse‡

* Division of Medicinal Chemistry and Natural Products, School of Pharmacy, CB#7360, University of North Carolina, Chapel Hill, NC 27599-7360, USA, † Boron Biologicals, Inc., 620 Hutton Drive, Raleigh, NC 27606, USA, and ‡ Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300, USA

Metal complexes of trimethylamine carboxyborane successfully suppressed calcium flux from both paired pup calvaria bones and rat UMR-106 osteosarcoma cultured cells over a 48 h period. These agents increased uptake of calcium into the cell cultures and accelerated [^{3}H]proline incorporation into collagen. Copper and iron complexes of the trimethylamine carboxyborane were more potent compared with the cobalt and chromium complexes. The agents effectively reduced lysosomal enzyme activity and also proteolytic enzyme activities of macrophages. Since macrophages invade the bone surface and assist in the demineralization and digestion of collagen, those agents may be potentially useful to retard diseases involving bone reconstruction. Influx of white blood cells and macrophages to sites of degradation most probably would be inhibited by the agents, based on sponge test observations in mice. Osteoporosis induced by ovariectomy was minimized by injections of tetrakis[μ-(trimethylamine-borane-carboxylato)-bis(trimethylamine-carboxyborane)-dicopper(II) into rats at 3.5 mg kg^{-1} day^{-1} for 14 days. Bone volume, density, weight and calcium content returned to normal baseline control values. In addition, the copper complex returned serum calcium, serum parathyroid hormone (PTH) and vitamin D_3 values to normal levels. One possible mode of action of these derivatives is the regulation of the production and release of chemical mediators initiating bone loss, e.g. tumor necrosis factor, TNFα and interleukins I1 or I1-2.

Keywords: osteoporosis; metal complexes; boron; bone

INTRODUCTION

In addition to the influence of nutrition components and exercise, trace mineral elements play a major role in increasing bone density during growth and are altered in certain bone diseases, e.g. osteoporosis, osteopenia and rheumatoid disease. Mineral elements, including trace elements copper and magnesium as well as iron, zinc, sodium, potassium, chloride and iodine, are needed for bone metabolism, and most of these agents are provided in milk and food components.[1] Cadmium and high doses of iron have been linked with increased bone resorption.[1] Boron, as boron salts contained mainly in fruit and vegetables, is important for magnesium and calcium metabolism preventing bone loss in postmenopausal women.[1] Mineral elements in bone change with age and in the development of osteoporosis; for example, copper and iron concentrations increase with age and are related to decreased levels of bone collagen, bone calcium and bone density.[2] Copper, when administered to children suffering from alimentary copper deficiency with general skeletal osteoporosis, retards metaphyseal and multiple fractures.[3] Oral copper supplements results in complete healing of fractures and copper treatment increases ossification of the bone.[4,5] Mineral changes in aged mice prone to developing osteoporosis demonstrated changes in calcium, iron and copper with a 9% decrease in boron levels.[6] Boron salt supplements at 3 mg day^{-1} in post-menopausal women prevent calcium loss and bone demineralization.[7] Boron deprivation in the diet depresses growth and serum 25-hydroxycholecalciferol levels, whereas serum calcitonin and osteocalcium levels and plasma alkaline phosphatase activity were elevated in humans. The changes in alkaline phosphatase levels induced by boron mimic the biochemical effects caused by estrogen on bone metabolism.[8,9]

Previous studies have demonstrated that trimethylamine carboxyboranes were very potent anti-osteoporosis agents in rodents at 8 mg kg^{-1} day^{-1}. By chemically complexing these

CCC 0268–2605/95/020111–09

Received 4 August 1994
Accepted 11 November 1994

compounds with copper, iron, cobalt or chromium, their effects on bone metabolism may be improved.[10] The present study is directed towards the investigation of the effects of these complex metal compounds rather than free metal cations on bone metabolism of rodents, to determine whether they could be useful therapeutically to improve bone metabolism in disease states.

EXPERIMENTAL

Source of compounds

$[Cu_2(Me_3NBH_2COO)_4\ (Me_3NBH_2COOH)_2]$ (**1**), $[Fe_3O\{(CH_3)_3NBH_2COO\}_6\ (CH_3OH_3)NO_3 \cdot CH_3CN]$ (**2**), $[Fe_3O\{(CH_3)_3NBH_2COO\}_6 \cdot (CH_3OH)_3Cl]$ (**3**), $[Cr_3O\{(CH_3)_3BH_2COO\}_6(H_2O)_3]NO_3 \cdot CH_3OH \cdot CH_3CN$ (**4**), *cis*-$[Co(en)_2\{(CH_3)_3NBH_2COO\}_2]Cl \cdot 2.5H_2O \cdot 5CH_3OH$ (**5**) and $Co\{(CH_3)_3NBH_2COO]NO_3 \cdot CH_3CN \cdot CH_3OH$ (**6**) have previously been synthesized. Physical and chemical properties have been reported.[11–13] Sodium tetraborate, $Na_2B_4O_7 \cdot H_2O$ (borax) (Aldrich Chemical Co., Milwaukee, WI, USA) and disodium (1-hydroxyethylidene)bisphosphate (sodium etidronate biphosphonate) (Sigma Chemical Co., St Louis, MO, USA) were used as standards. All tissue culture cell lines were purchased from American Type Culture Collection, Rochville, MD, USA.

Mouse calvaria cultures

Pregnant CF_1 female mice (32 g) {Jackson Laboratory, Bar Harbor, MA, USA} on day 17 of gestation were injected with 5 μCi of $^{45}CaCl_2$ (34.12 mCi mg^{-1}). When pups were four days old, they were decapitated after their heads had been swabbed with 70% EtOH.[14,15] Parietal bones were collected, washed three times with Dulbecco's modified Eagle's medium (DMEM), and divided into two along the sagittal suture. After their placement in adjoining wells, 1 ml of DMEM + 15% horse serum + 10 U ml^{-1} heparin + 100 U ml^{-1} penicillin was added. Drugs (10^{-4}–10^{-8} M) were added to one well and vehicle (0.05% Tween 80/wter) to the other. Aliquots from the media were taken at 24, 48, and 72 h and counted. The bone fragment was digested with HCl and counted. Total calcium and percentage of calcium released were calculated.

^{45}Ca uptake by cells

Rat UMR-106 osteosarcoma cells [DMEM + 10% fetal calf serum (FCS) + penicillin/streptomycin (P/S)] and IC-21 mouse macrophages (RPMI 1640 + 10% FCS + P/S) were grown to confluency. $^{45}CaCl_2$ (0.2 mCi) was added to these cells as well as to pup calvaria bones and incubated for 48 h. The medium was decanted and the cells were washed four times with phosphate-buffered saline (PBS), pH 7.2. The cells were taken up in NaOH and the radioactive calcium counted.[16,17]

[^{3}H]Proline incorporation into collagen

Rat UMR-106 cells (70% confluent) and pup calvaria cells were incubated with 1 μCi of [2-, 3-, 4- and 5-^{3}H]proline (102 Ci $mmol^{-1}$) and drugs. After 24 h, the medium was discarded and the cells were washed with PBS. The cells were harvested in NaOH. After treatment with 20% trichloroacetic acid (TCA), the cells were centrifuged at 3500*g* for 5 min. The supernatant was discarded and the pellet was added to 1 ml 50 mM Tris + 5 mM $CaCl_2$ + 20 ml collagenase (10 mg ml^{-1} buffer) and incubated for 2 h at 37 °C. Tannic acid/TCA (5%:20%) solution was added and incubated for 15 min at room temperature, followed by centrifugation.[18,19] [^{3}H]Proline incorporation into collagen and non-collagen protein was counted.

Ovariectomized rat model for the induction of osteoporosis

Female Sprague–Dawley rats (~400 g) were purchased from Charles River Laboratory Inc. (Raleigh, NC, USA) and received on day 16 of gestation. On day 2 *post partum*, lactating rats were ovariectomized via bilateral paraspinal incisions.[20–22] On day 6 *post partum*, pups were removed from the cages and pooled. At this time, litters of 12 pups were randomly assigned to the dams. The pups were weighed on day 6 and also at the end of the experiment.

Dosing

Six animals were randomly assigned to each group. Non-mated control animals were of the same age and body weight. Drugs were dissolved in 0.05% Tween 80/H_2O and administered daily at 3.5 mg kg^{-1} orally, in a volume of 0.2 ml, for 14 days starting on day 6 *post partum*. Etidronate

and sodium borate were administered orally to pups at 8 mg kg^{-1} day^{-1} and 3 mg kg^{-1} day^{-1}, respectively. Rats were killed 24 h after the last dose of each agent.

Blood collection

Blood was collected from the abdominal vein above the kidneys of ether-anesthetized animals at the end of the experiment. Serum was separated by centrifuging at 3500 rpm for 10 min (IEC B 28 centrifuge) and stored below −70 °C until assayed.

Serum assays

Serum total calcium was assayed by the colorimetric method of Kessler and Wolfman[23] using a kit (no. 587-A from Sigma Chemical Co. (St Louis, MO, USA). Serum phosphorus was assayed by a colorimetric method.[24] Serum total estrogens were measured by radioimmunoassay using a [^{125}I] Total Estrogen kit from ICN Biomedicals Inc., serum calcitonin by a radioimmunoassay kit from INSPRA (Stillwater, MN, USA), serum parathyroid hormone (PTH) by an Intact *N*-Terminal Specific RIA kit from Nichols Institute (San Juan Capistrano, CA, USA) and serum 1,25-dihydroxy-vitamin D_3 by a radioimmunoassay kit from Amersham (Chicago, IL, USA). Serum tumor necrosis factor (TNFα) was assayed for cytotoxicity using L-929 cells. Cellular survival was quantified using a microplate reader after staining with 0.2% Crystal Violet in 20% MeOH.[25] The assay was validated using standard TNFα enzyme-linked immunosorbent assay (ELISA) kits (Genzyme, Cambridge, MA, USA).

Bone collection and ashing

One femur and one humerus were collected from each animal at the end of the experiment. Adhering connective tissue was removed manually. Femurs were dried overnight at 100 °C, ashed in a muffle furnace at 600 °C for 24 h, and the ash weighed. After ash had been dissolved in 1 M HCl, the calcium content (Sigma kit 587-A) and phosphorus content were determined by a colorimetric method.[24]

Bone density

The cleaned bones were immersed in water and kept *in vacuo* for 90 min to drive out any trapped air. The bones were blotted with filter paper and weighed, then completely immersed in water and reweighed. Bone density in g/cm^3 bone volume was calculated using Archimedes' principle.

Bone lipids

The humerus from each animal was dried overnight at 100 °C and ground to a fine powder in a mortar. After bone lipids had been extracted with chloroform/methanol (2:1)[26] for 48 h, total lipids, cholesterol, triglycerides, neutral lipids and phospholipids were assayed.

Macrophage tissue culture and determination of macrophage lysosomal enzymes

Mouse macrophages, J774 A, were maintained in DMEM, 10% FCS, and P/S.[21] Cells [10^8] were harvested and incubated for 60 min with drugs from 10^{-4} to 10^{-8} M at 37 °C. Free and total acid phosphatase activities were determined using 0.1 M β-glycerophosphate in 0.1 M acetate buffer, pH 5.0.[27] Total enzyme activity was released with Triton X-100. The reaction was terminated with 10% TCA and centrifuged at 3500*g* for 10 min. The inorganic phosphate in the supernatant was determined spectrophotometrically at 800 nm by the method of Chen *et al.*[24] Aryl sulphatase activity was measured using 0.72 mmol of *p*-nitrocatechol sulfate as a substrate in 0.2 M acetate buffer, pH 5.0. The reaction was terminated with 4 M NaOH. The formation of *p*-nitrocatechol in the supernatant was measured at 510 nm.[28] Cathepsin activity was determined with 2% azocasein as substrate in acetate buffer, pH 5 or pH 7.0 for 60 min at 37 °C. The acid-soluble fragment in the supernatant was determined at 366 nm.[29,30] All assay values had the appropriate tissue blanks subtracted from them before inhibition was calculated.

Macrophage proteolytic enzyme assays

Trypsin proteolytic activity was determined by the method of Schleuning and Fritz[31] using 2.0 ml of 0.1 M Tris buffer, pH 8.0, and 6 mM *N*-benzoyl-L-arginine ethyl ester (BAEE) substrate. BAEE hydrolysis over 60 min was determined at 253 nm and was subtracted. Elastase activity was determined by the method of Kleinerman *et al.*,[32] using 2.9 ml of 0.2 M Tris–HCl buffer, pH 8.0, and 20 ml *N*-succinyl-L-alanyl-L-alanine-*p*-nitroanilide

(Sigma; 100 mg in 5 ml of methyl-2-pyrrolidone). The cleaved product (*p*-nitroanilide) was determined at 410 nm. Collagenase I and II activities were determined by using 1 ml of 50 mM Tris + 5 mM $CaCl_2$ buffer, pH 7.6, 10 mg [^{3}H]collagen-*N*-[2,3-^{3}H]propionate (0.53 mCi mg^{-1}) and 10 mg *Clostridium histolyticum* type I or II (Sigma)[33–35] incubated for 24 h at 37 °C. The reaction was stopped with 1 ml of 50 mM EDTA . The tubes were centrifuged at 10 000*g* for 15 min and the supernatant was counted and corrected for quenching.

Macrophage prostaglandin synthetase activity

The incubation procedures of Tomlinson *et al.*[36] and Glatt *et al.*[37] were used to determine prostaglandin formation from [^{3}H(n)arachidonic acid (100 Ci mmol^{-1}) and cells (10^6). After 1 h, the reaction was terminated with 2 M HCl and the mixture was extracted with ether and evaporated. The residue was dissolved in ethyl acetate and applied to silica gel TLC plates eluted with chloroform/methanol/water/acetic acid (90:8:1:0.8). The plates were developed with iodine vapor and the area corresponding to the prostaglandin standards was scraped and counted. The disintegrations per minute (dpm) in each area were calculated as a percentage of the total dpm applied to the plate.

Macrophage 5′-lipoxygenase assay

Cells were incubated for 30 min with phosphate buffer (pH 7.2) containing 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mg calcium ionophore A23 187, and 1 μCi [^{14}C]arachidonic acid (100 Ci mmol^{-1}). The reaction was terminated with 2 volumes of EtOAc/CH_2Cl_2 containing 12 mg cold arachidonic acid. The organic phase was evaporated to a residue which was applied to silica gel plates. Plates were eluted with chloroform/methanol/water/acetic acid (90:8:1:0.8). The 5-HETE (hydroxyeicosate tetraenoic acid) area corresponding to the standard was scraped and counted.[38]

In vivo TNFα,Il-1 and Il-2 measurements

CF_1 male mice (~30 g) were administered agents at 8 mg kg^{-1}, i.p. Three hours later, lipopolysaccharide (LPS) (*Salmonella abortus equi*. Lot no. 69F4003, Sigma) at 5 mg kg^{-1} i.p. was administered. Blood was collected in non-heparinized tubes 90–180 min after LPS injection. Serum was obtained by centrifuging at 3500 rpm × 10 min (IEC). Serum TNFα levels were determined in a bioassay using cultured L_{929} mouse cells[19] grown in DMEM + 10% FCS + P/S. Cytotoxicity was determined with 0.2% Crystal Violet in 20% MeOH; the number of living cells was determined at 580 nm using SOFTmax (Molecular Devices). The bioassay was quantitated with TNFα standards and confirmed with a mouse ELISA kit (Genzyme Corp., Cambridge, MA, USA). Blood levels of Il-1 and Il-2 were determined in an analogous manner using mouse ELISA kits (Cytokine Research Products).

In vitro TNFα and Il-1 measurements

IC-21 mouse macrophages were maintained in RPMI-1640 + 10%FCS + P/S. After the cells had grown to confluency, *E. coli* LPS at 10 μg ml^{-1} was added to the medium. Agents were incubated at 1, 10 and 100 μM final concentration for 18 h.[39] The medium (100 μl) was collected for TNFα determinations. Il-1 release was determined using P_{388D} cells. The L_{929} bioassay was used to quantitate Il-1 levels released by P_{388D} cells.

Assays of chemical mediators from inflammatory exudates

In order to determine the effects of agents on the release of chemical mediators, i.e. cytokines or lymphokines, a number of biological fluids were collected from *in vivo* experiments. A modified sponge test[40,41] was performed in CF_1 male mice (~30 g). A 1.0 cm^2 × 5 mm thick [~30 mg] sponge saturated with 2.0 mg carrageenan was implanted subcutaneously in the abdominal wall. The mice were treated with agents i.p. at 8 mg kg^{-1}, 2 h prior to surgery, and 2 h post-surgery. Polymorphoneutrophils (PMN) migration to the site of inflammation was estimated after collection of sponges 6 h post-implantation by preparing a 0.5% homogenate of the sponge in hexadecyltrimethylammonium bromide (HTAB).[42] Aliquots of the homogenate were used to determine PMN myeloperoxidase (MPO) activity using *o*-dianisidine dihydrochloride at 460 nm. Monocyte and/or macrophage migration to the site was determined by leaving sponges in place for six to nine days. Homogenates of the sponge were prepared in HTAB; *N*-acetyl-glucosaminidase (NAG) lysosomal activity was

Table 1 ^{45}Ca displacement (%) to medium by paired pup calvaria after 48 h[a]

Compound	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
1	+1.26±0.32	−1.23±0.16	−2.07±0.22	−6.66±0.97	−3.55±0.27
2	+1.08±0.18	−0.89±0.07	−1.12±0.16	−1.74±0.19	−6.58±0.33
3	—	−7.23±0.09	+1.92±0.14	+1.86±0.05	−0.28±0.05
4	—	—	−2.29±0.27	−1.14±0.09	−0.90±0.08
5	—	—	−4.28±0.35	−0.45±0.03	−1.69±0.12
6	—	—	+1.04±0.09	−1.26±0.06	−1.84±0.15
Calcitonin	−1.45±0.08	−1.88±0.07	−2.40±0.11	−3.97±0.17	—
Etidronate	—	—	−4.12±0.17	−6.79±0.23	—
PTH	+2.91±0.11	+4.50±0.21	+8.00±0.51	—	+5.38±0.25

[a] Positive (+) values indicate reabsorbed calcium displaced to medium; negative (−) values, calcium maintained in cell; all values are ±standard deviations (SD). Control demonstrated +20±2.5% displacement to medium.

determined by using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. The hydrolysis product, *p*-nitrophenol, was determined at 400 nm. Statistical analysis for the data in Tables 1–7 is presented as the means of the control and treated groups ± standard deviation. A Student's test was used to obtain *P* values between control and treated groups; ANOVA was applied among treatment groups to obtain *P* values; $n = 6$ for all groups of test animals.

RESULTS

Compounds **1–6** when incubated with paired mouse calvaria bone cultures reduced the percentage of ^{45}Ca flux into the medium (Table 1). Compound **1** resulted in the highest reduction of calcium resorption (−6.66% at 10^{-5} M) and compound **3** was the most active with −7.23% at 10^{-7} M. Individual compounds, percentage reductions in ^{45}Ca flux, and doses at which maximal inhibition of ^{45}Ca flux was obtained, were: Compound **1**, −6.66% at 10^{-5} M; compound **2**, −6.58% at 10^{-4} M; compound **3**, −7.23% at 10^{-7} M; compound **4**, −2.29% at 10^{-6} M; compound **5**, −4.28% at 10^{-6} M; and compound **6**, −1.84% at 10^{-4} M (Table 1). Generally the compounds did not afford a concentration-dependent effect on reduction of calcium resorption. ^{45}Ca uptake in UMR-106 osteosarcoma cells grown in culture was increased by compounds **1–3** in a concentration-dependent manner. Incubation with several compounds at 10^{-4} M revealed that compound **1** increased ^{45}Ca uptake threefold, compound **2** resulted in a ninefold increase, and compound **3** resulted in a twofold increase. Etidronate resulted in a 1.2-fold increase. Macrophages also increased calcium uptake in the presence of compounds **1–3** at 10^{-4} M (Table 2).

Compounds **1–3** increased collagen synthesis by promoting [^{3}H]proline uptake into collagen within UMR-106 cells. Compounds **4–6** were not

Table 2 ^{45}Ca uptake (% of control ± SD[a]) by rat UMR-106 cells and macrophages

	Rat UMR-106 cells				IC-21 macrophages
Compound	5×10^{-7} M	5×10^{-6} M	5×10^{-5} M	10^{-4} M	
1	121±7	144±6	150±7	340±8	595±7
2	108±4	137±5	286±6	953±9	738±6
3	114±5	209±6	223±8	231±7	918±8
Etidronate	—	134±4	194±6	123±4	—
PTH	—	—	—	66	—
Control	100[b]±6	100±6	100±6	100±6	100[c]±6

[a] $n = 6$. [b] 88527 dpm (mg of protein)$^{-1}$. [c] 2553 dpm (mg of protein)$^{-1}$.

Table 3 [^{3}H]Proline uptake (% of control ± SD; $n = 6$) into collagen of rat UMR-106 cells

Compound	10^{-6} M	10^{-5} M	10^{-4} M
1	325±8*	183±5*	223±5*
2	118±5	177±6*	110±4
3	26±5*	237±7*	634±6*
4	60±3*	106±6	102±5
5	61±4*	62±5*	63±4*
6	56±4*	57±7*	32±2*
Etidronate	223±6*	33±5*	332±7*
Control	100[a]±5	100±5	100±5

[a] 5107 dpm (mg of collagen isolated)$^{-1}$.
* $P \leq 0.001$.

as effective in increasing collagen synthesis; in fact, they all decreased proline uptake at 10^{-6} M (Table 3). Again, proline incorporation into collagen induced by the compounds did not follow concentration-dependent patterns. When compound **1** was tested in ovariectomized rats at 3.5 mg kg^{-1} day^{-1} orally, the body weight gain of the rats over 14 days decreased approximately 20% compared with the ovariectomized control group. Compound **1** in rats increased femur and humerus bone weight and density, and femur total ash weight significantly (Table 4) ($P \leq 0.005$). These changes were comparable with etidronate at 8 mg kg^{-1} day^{-1} and generally better than the borax salt at 3 mg kg^{-1} day^{-1}. Calcium and phosphorus contents were increased in the femur ash after drug treatment for 14 days (Table 4) ($P \leq 0.005$). Humerus total lipids after treatment with compound **1** at 3 mg kg^{-1} day^{-1} were elevated significantly above the ovariectomized control values ($P \leq 0.005$). These increases appeared to reflect osteal increases in triglycerides and phospholipids. In rats treated with compound **1**, serum calcium levels were increased whereas serum phosphorus levels were decreased. Femoral ash calcium levels were elevated significantly above the ovariectomized control after treatment with compound **1** and etidronate. PTH levels in rats treated with **1** and the standard etidronate were returned to normal levels after ovariectomy. Serum 1,25-dihydrovitamin D_3 levels were also significantly elevated by treatment with compound **1**, but not by etidronate or the borax salt. There were no changes in the blood levels of calcitonin, estrogen or its metabolites and TNFα (data not shown).

Compounds were able to suppress PMN migration into the inflammation sites after 6 h, measured by the MPO activity (Table 5). Compounds **3**, **4** and **6** reduced PMN migration by more than 60%. Macrophage migration, measured as NAG activity on day 9, was moderately suppressed by **1** and **3**, and significantly inhibited by **2** (64%).

Incubation of cells with compounds **1–6** in macrophages (Table 6) demonstrated that IC_{50} values for inhibiting cathepsin, aryl sulfatase, acid

Table 4 Effects of compound **1**, an amine carboxyborane, on body weight, femur, humerus and lipid measures in ovariectomized lactating rats (mean ± SD)

Assay	Compound 1	Etidronate	Borax	OVX control	Sham control
Body weight gain over 14 days (g)	30.50 ± 6.190	48.17 ± 8.420	45.67 ± 5.430	49.17 ± 5.100	50.50 ± 3.850
Femur					
Weight (g)	0.93 ± 0.028	0.96 ± 0.054	0.88 ± 0.015	0.87 ± 0.062	0.99 ± 0.018**
Volume (ml)	0.67 ± 0.029	0.69 ± 0.048	0.62 ± 0.006	0.63 ± 0.018	0.67 ± 0.012
Density (g ml^{-1})	1.41 ± 0.046	1.41 ± 0.033	1.43 ± 0.014	1.37 ± 0.023	1.49 ± 0.010**
Ash weight (g)	0.33 ± 0.008**	0.33 ± 0.010**	0.30 ± 0.013	0.26 ± 0.006	0.38 ± 0.320**
Ash calcium (mg g^{-1})	6.973 ± 0.1225**	6.9645 ± 06.08**	7.1238 ± 04.22**	6.1292 ± 0.2266	7.083 ± 0.0871**
Ash phosphorus (mg g^{-1})	4.477 ± 0.0343	4.870 ± 0.1260**	4.598 ± 0.2462	4.408 ± 0.0302	4.213 ± 0.1510
Humerus					
Weight (g)	0.53 ± 0.016*	0.5068 ± 0.009**	0.4252 ± 0.0108	0.451 ± 0.0079	0.4537 ± 0.01
Volume (ml)	0.35 ± 0.012	0.3475 ± 0.006**	0.2944 ± 0.0093*	0.322 ± 0.0056	0.3045 ± 0.0072
Density (g ml^{-1})	1.51 ± 0.009**	1.4557 ± 0.0145*	1.4474 ± 0.0219	1.401 ± 0.0112	1.4885 ± 0.014**
Total lipids (mg/0.2 g wet wt)	3.725 ± 0.1916*	3.91 ± 0.36	3.00 ± 0.27	2.94 ± 0.32	3.29 ± 0.06
Cholesterol (mg g^{-1})	58.875 ± 3.235	58.06 ± 2.53	57.25 ± 0.69	60.25 ± 2.03	47.25 ± 0.8**
Triglycerides (mg g^{-1})	199.06 ± 10.16**	206.56 ± 9.41**	137 ± 7.1	134.44 ± 7.17	188.31 ± 5.87**
Neutral lipids (mg g^{-1})	681.63 ± 13.93	667.62 ± 24.02	16.13 ± 7.28	707.69 ± 15.94	683.31 ± 8.88**
Phospholipids (mg g^{-1})	80.44 ± 6.43*	68.81 ± 10.71	23.75 ± 1.7**	52.0 ± 8.56	19.88 ± 1.55**
Serum levels					
1,25-Dihydroxy-vitamin D_3 (pg ml^{-1})	91.0 ± 37.150*	22.6 ± 20.15*	52.0 ± 33.97	36.0 ± 5.15*	76.4 ± 19.87
PTH (pg ml^{-1})	32.58 ± 16.3	37.08 ± 8.22**	25.13 ± 2.81	25.37 ± 5.1	32.21 ± 3.87
Calcium (mg dl^{-1})	9.375 ± 0.1569	8.5783 ± 0.0954	9.065 ± 0.5739	8.3992 ± 0.4073	8.3983 ± 0.0958
Phosphorus (μg dl^{-1})	3.828 ± 0.1019**	3.407 ± 0.1139**	4.0917 ± 0.3667	4.4933 ± 0.159	4.1967 ± 0.4426

[a] OVX, ovarectomized female rats.

* $P \leq 0.001$. ** $P \leq 0.050$.

Table 5 The effects of metal complexes on PMN and macrophage influx into sponges inplanted subcutaneously in CF_1 male mice[a]

Compound	PMNs: MPO	Macrophages: NAG
1	84 ± 3	74 ± 3
2	97 ± 4	36 ± 2
3	35 ± 3	78 ± 5
4	37 ± 2	—
5	63 ± 5	—
6	34 ± 4	—
1% CMC	100 ± 4[a]	100 ± 4

[a] Results are expressed as percentage of control ± SD ($n = 6$).
[b] 249 mmol h^{-1}.

phosphatase, trypsin and prostaglandin cyclo-oxygenase were between 1×10^{-6} and 3.5×10^{-6} M. Elastase activity was inhibited by the agents resulting in IC_{50} values between 3.9×10^{-6} M (Table 6). The drugs inhibited *Clostridium histolyticum* collagenase I activity (IC_{50} values were between 6×10^{-4} and 8×10^{-4} M), whereas they inhibited collagenase II activity somewhat better [IC_{50} values approximately 10^{-5} M (data not shown)]. The compounds inhibited bovine seminal vesicle prostaglandin synthetase activity (IC_{50} values were between 2.9×10^{-6} and 4.2×10^{-6} M). Macrophage 5′-lipoxygenase activity was inhibited both by **1** ($IC_{50} = 2.5 \times 10^{-5}$ M) and **3** ($IC_{50} = 1.1 \times 10^{-5}$ M). In LPS-induced IC21 macrophages, Il-1 release was reduced by **1** and **2** (43% and 44%, respectively), but not by **3** (Table 7). TNFα release was reduced by **2** and **3** (57% and 50%, respectively), but not by **1**. After *in vivo* treatment in mice for 90 min, TNFα levels were increased 168% for **1**, 145% for **2**, and 197% for **3**. However, after 3 h, the TNFα levels fell 86% for **1**, 77% for **2** and 76% for **3**. In mice treated similarly for 90 min, lower Il-1 levels (22% by **1**, 32% by **2** and 40% by **3**) and Il-2 levels (44% by **1**, 75% by **2** and 58% by **3**) were observed.

DISCUSSION

The metal complexes of amine carboxyboranes proved to be effective anti-osteoporotic agents, increasing both calcium and collagen contents of paired pup calvaria bones and rat UMR-106 cells. The drugs appeared to function on several levels: first, they inhibited calcium flux out of the bone and macrophage cells, which should slow the osteoporotic process; second, they increased calcium uptake by the cells, which should be beneficial and facilitate mineralization of bone; and third, they increased collagen synthesis in bone cells, which should theoretically improve the tensile strength of the bone as well as the bone's density and volume—this was reflected in the bone studies of rats after *in vivo* administration of drugs. In addition, since osteoporosis is a process initiated by white blood cells invading the osteal surface, it is relevant that amine carboxyboranes reduced the migration of PMNs and macrophages. The macrophages may be more important than PMNs in initiating bone resorption, but both types of cells will add to the subsequent tissue degradation. Whereas cellular and macrophage migrations were measured only with the sponge assay, the same principle may apply to migration of these cells to the bone surface.

Boron salts, when used to treat postmenopausal women, were thought to function in osteoporosis by increasing 17β-estradiol and testosterone levels.[8,9] There was no evidence of increased levels due to increased production of steroids by the adrenal cortex in these ovariectomized ani-

Table 6 Inhibition of mouse macrophage J774 enzyme activity

Compound	IC_{50} ($\times 10^{-6}$ M)[a]					
	Cathepsin D, pH 5.0	Acid phosphatase	Aryl sulfatase	Trypsin	Elastase	Prostaglandin cyclo-oxygenase
1	2.51	2.13	1.98	1.92	4.08	1.89
2	2.85	2.09	1.95	2.37	3.92	2.34
3	3.39	2.44	2.75	1.81	4.11	2.08
4	2.49	2.48	1.20	2.10	5.09	1.62
5	3.83	2.92	1.95	1.38	9.77	2.09

[a] Mean values ($n = 6$).

Table 7

(a) Effects of metal complexes of amine carboxyboranes on *in vitro* release of TNFα and Il-1[a]

Compound	IC-21: TNFα release (pg ml^{-1})	P_{388D_1} cells: Il-1 release (pg ml^{-1})
Control	150 ± 2	6.6 ± 1.0
LPS (10 μg ml^{-1})	375 ± 5	26.0 ± 3.9
1	197 ± 4*	15.0 ± 2.3*
2	160 ± 3*	14.5 ± 2.1*
3	187 ± 5*	27.0 ± 4.0

[a] Mean values ($n = 6$).
* $P \leq 0.001$ compared with LPS values.

(b) Effects of metal complexes of amine carboxyboranes on *in vivo* CF_1 mouse serum levels of TNFα, Il-1 and Il-2 after 90 min and 3 h[a]

Compound	TNFα (ELISA, pg ml^{-1}) 90 min	TNFα (ELISA, pg ml^{-1}) 180 min	Il-1 (pg ml^{-1}) 90 min	Il-2 (U ml^{-1}) 90 min
Control	19 ± 0.8	15 ± 0.9	5 ± 0.7	1.12 ± 0.10
LPS (5 mg kg^{-1})	316 ± 1.5	789 ± 3.5	69 ± 0.9	1.86 ± 0.14
1	847 ± 7.4*	107 ± 2.1*	54 ± 0.4*	1.05 ± 0.26*
2	460 ± 3.6*	185 ± 1.6*	47 ± 0.5*	0.47 ± 0.16*
3	623 ± 4.2*	187 ± 1.3*	42 ± 0.7*	0.78 ± 0.09*

[a] Mean values ($n = 6$).
* $P \leq 0.001$ compared with LPS values.

mals treated with metal complexes of trimethylamine carboxyboranes. Moreover, these drugs caused no changes in calcitonin levels compared with the ovariectomized control. However, 1,25-dihydroxy-vitamin D_3 levels were elevated with drug treatment above control values. Treatment with compound **1** at 3.5 mg kg^{-1} was more efficient than etidronate at 8 mg kg^{-1} or borax at 3 mg kg^{-1}. PTH levels in treated animals followed the same pattern of increases with compound **1** and the standard etidronate; however, the standard borax was ineffective in affecting PTH levels. Increases in serum levels of 1,25-dihydroxy-vitamin D_3 and PTH, two endogenous regulators of bone metabolism, should cause elevations in blood calcium, which was found after compound **1** treatment. These increments suggest that the agents caused bone remodeling. However, some other mechanism is needed to explain increases in bone density and calcium flux into the bone after drug treatment. Studies with other trimethylamine carboxyborane derivatives at 8 mg kg^{-1} day^{-1} in mice have shown that these agents increased blood calcium levels, decreased urinary calcium and reduced hydroxyproline levels in the blood and urine.[38] Further, these same agents lowered Il-1, Il-2 and TNFα levels in mice 1.5–3 h after administration. Suppression of Il-1 and TNFα, and their effects on bone cells, would explain the agents' abilities to block osteoporotic processes.

Il-1 from invading macrophages has been implicated in the initiation of bone osteoporosis, particularly in the demineralization process and loss of cellular calcium. Inhibition of the chemical mediator should retard the reabsorption process or the inorganic phase of osteoporosis.[43,44] That amine carboxyborane derivatives and other metal complexes also inhibit the organic phase of osteoporosis is indicated by the inhibition of enzymes such as neutral cathepsin, trypsin and elastase, in addition to the moderate inhibition of collagenase I and II.[45,46] Cells invading the bone surface during bone resorption, such as macrophages, monocytes, PMNs, and osteoclasts, release prostaglandins and leukotrienes locally.[46] The amine carboxyboranes and their metal complexes were potent inhibitors of cyclo-oxygenase and 5′-

lipoxygenase activities in a number of cell lines. These two enzymes are regulatory sites for *de novo* synthesis of prostaglandins and leukotrienes. Both of these are chemical mediators in inflammatory and osteoporotic processes participating in the degradation of dense bone which may lead to a higher incidence of bone fractures.[44]

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