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Bone-Targeted Carbonic Anhydrase Inhibitors: Effect of a Proinhibitor on Bone Resorption *in Vitro* (42590A)

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Abstract. Many investigations have indicated a functional role for carbonic anhydrase in the mediation of hormone-stimulated bone resorption. These studies depend heavily on the use of heterocyclic sulfonamide inhibitors of carbonic anhydrase. These drugs have effects on many tissues other than bone, and some of these effects confound the interpretation of studies of the role of carbonic acid in bone metabolism. A novel, "bone-targeted" sulfonamide has been produced to obviate these extraosseous effects. This compound (designated WP-1) is the combination of tetracycline and acetazolamide, such that the acetazolamide is not an active inhibitor. Hydrolysis of WP-1 yields an active carbonic anhydrase inhibitor. WP-1 has a marked affinity for bone mineral, allowing deposition of the drug in bone. At a concentration of 10^{-5} *M*, WP-1 attenuates parathyroid hormone stimulated net release of calcium from neonatal rat calvaria in culture. WP-1 is the first member of a class of drugs which may prove useful as pharmacological probes in the study of bone metabolism.

The mechanism by which parathyroid hormone (PTH) and other agents stimulate bone resorption remains ill-defined. Most theories attempting to explain this action include as a final step the production of a local acidic environment by osteoclasts and/or osteocytes. This localized acidic environment would favor the solubilization of bone mineral and the destruction of the organic phase of bone by enzymes with a pH optimum in the acidic range.

Numerous investigators have suggested that PTH stimulates bone resorption by increasing the bone cell production of carbonic acid (1–5). The production of carbonic acid is catalyzed by the enzyme carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1). This enzyme plays a significant role in acid–base balance in many tissues (6). This enzyme is found in bone and is localized in bone resorbing cells (7–9).

Most of the information implicating carbonic anhydrase in bone resorption has been obtained utilizing heterocyclic sulfonamides that act as inhibitors of this enzyme. The agents used most often are acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), methazolamide (*N*-[5-(aminosulfonyl)-3-methyl-1,3,4-thiadiazol-2(3*H*)-ylidene]-acetamide), and ethoxzolamide (6-ethoxy-2-benzothiazolsulfonamide). The activity of these agents as attenuators of PTH-induced bone resorption was first demonstrated in chickens (1). However, initial reports indicated low concentrations of enzyme in mammalian bone and observed that classical enzyme inhibitors do not affect plasma calcium concentrations in mammals (6, 10). This led to the proposal that carbonic anhydrase is not important in skeletal events in mammals. However, data from our laboratory and other laboratories support a postulated role for carbonic anhydrase in mammalian bone metabolism (3–5, 11–16). We have observed that the effect of carbonic anhydrase inhibitors on mammalian bone resorption is masked by the effects of these agents on other carbonic anhydrase-containing tissues (11).

It appears clear that the utility of these agents to inhibit bone resorption could be greatly enhanced by the production of a compound that would specifically inhibit bone cell carbonic anhydrase while not influencing the enzyme activity of other tissues. This could be accomplished by the synthesis of a compound that would be rather specifically delivered to bone. This specificity, coupled with the use of appropriate dosing protocols, should minimize extraosseous effects. In an attempt to produce a specific inhibitor of bone cell carbonic anhydrase, we have synthesized a compound consisting of a known bone-seeking agent (tetracycline: 4-(dimethylamino)-1,4,4a,5,5a,6,-11,12a-octahydro-3,6,10,12,12a-pentahy-

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droxy-6-methyl-1,11-dioxo-2-naphthacene-carboxamide) coupled via an acid-labile bond to an inhibitor of carbonic anhydrase (acetazolamide) such that the sulfonamide nitrogen atom is no longer unhindered, and thus this compound should be inactive as an enzyme inhibitor. Most synthetic inhibitors of carbonic anhydrase are sulfonamides and the unsubstituted $-SO_2NH_2$ grouping is a requirement for inhibitor activity (6).

This compound should be a specifically "bone-targeted" proinhibitor of carbonic anhydrase. We propose that this and similar agents may serve as prodrugs and will specifically accumulate in bone extracellularly (bind to the inorganic phase of bone) and upon the initiation of the resorptive process, the acidic milieu will promote hydrolysis of the labile bond thus liberating an active inhibitor of carbonic anhydrase, which is also an active inhibitor of bone resorption. This use would constitute a pharmacological negative feedback system, with the liberated acetazolamide inhibiting the process which generates it. Using this strategy, extraosseous effects of acetazolamide should be minimized due to specific accumulation of the agent in bone fluid and the relatively low inhibitor concentrations available systemically due to dilution. Some of these data have been presented in abstract form (17).

Materials and Methods. *Synthesis.* Unless otherwise noted, all reagents used were obtained from Sigma Chemical Co. (St. Louis, MO). Tetracycline was recrystallized twice: once from methanol, once from dichloromethane. Two novel compounds were synthesized. The first, WP-1, is the product of the condensation of one equivalent each of tetracycline and acetazolamide with adipoyl dichloride (1,6-hexanedioyl-dichloride). This yields a hexanedioic acid bridge between the sulfonamide nitrogen of acetazolamide and tetracycline. The second compound, acetazolamide-adipate-ethyl ester (AAOEt), is a similar condensation product of acetazolamide, adipoyl dichloride and ethanol, with a hexanedioic bridge between the sulfonamide nitrogen and the ethyl ester.

Each of these compounds was synthesized in two steps; the first step was the condensation of adipoyl dichloride and acetazolamide. In a 500-ml round-bottom flask, 4.44 g (0.02 mole) acetazolamide was slurried in 260 ml pyridine. To this mixture 0.02 mole adipoyl dichloride (Aldrich, Milwaukee, WI) was added dropwise over a period of 2 hr. The mixture was stirred at room temperature for 24 hr. The resultant was divided into two portions, and one was taken for synthesis of acetazolamide-adipate-ethyl ester. The remainder was used for synthesis of WP-1.

AAOEt was synthesized by adding the above described acetazolamide-adipoyl hemichloride to boiling ethanol and maintaining the solution at reflux for 1 hr. The solvent was removed under vacuum, and the product was recrystallized from methanol.

WP-1 was synthesized by adding 4.44 g (0.01 mole) of tetracycline to the remaining 0.01 mole (theoretical) of acetazolamide–adipoylhemichloride, along with 100 ml of dimethyl sulfoxide (DMSO) and 20 ml of pyridine. The mixture was maintained at 50°C for 48 hr with stirring. Two hundred milliliters of water was added to the flask, with stirring, and 50 ml of a 15% (v/v) slurry of hydroxyapatite were added. The mixture was then allowed to stir gently overnight at room temperature. Hydroxyapatite was recovered by filtration, washed three times with 200 ml each of hot water, methanol, and ethyl acetate, and allowed to dry under vacuum at room temperature. The resultant was then suspended in 1.0 *M* sodium phosphate buffer (pH 7), filtered again, and resuspended in 2.0 *M* sodium phosphate (pH 7). This suspension was mixed with 100 ml of 1-butanol to extract the product, which was recovered by stripping the solvent under vacuum. Product yield was 1.2 g (15% of theoretical).

Physicochemical and biochemical characterization. Melting points were determined using a Fisher-Johns apparatus with a heating rate of 2°C per minute.

Infrared spectra were obtained using a Perkin–Elmer spectrometer (Perkin–Elmer, Norwalk, CT) and KBr discs, scanning from 680–4000 cm^{-1}.

Ultraviolet-visible spectroscopy was performed using 1-cm quartz cuvettes and a Cary 19 spectrophotometer (Varian Associates, Palo Alto, CA). Spectra were obtained on 10^{-6} *M* solutions in 0.1 *N* HCl and 0.1 *N* NaOH, scanning from 600–190 nm at a rate of 2 nm/sec.

Chromatographic analysis was performed using high-performance liquid chromatography (HPLC). The chromatograph was a Hewlett–Packard Model 1084 (Hewlett–Packard, Palo Alto, CA) fitted with a 25-cm LC-18-DB reverse-phase column (Supelco, Bellefonte, PA). The elution mode was isocratic using a mobile phase of 8% tetrahydrofuran/9% acetonitrile/1% methanol/82% 5 m*M* sodium phosphate, pH 2. Solvents were HPLC grade obtained from Fisher (Fisher Scientific, Pittsburgh, PA). Flow rate was maintained at 1.0 ml/min and detection was accomplished using a Hewlett–Packard Model 1040 photodiode array detector.

Carbonic anhydrase activity and inhibition were determined using the method of Maren (18). Inhibitory activity of test compounds was determined before and after incubation in 0.1 *N* HCl at 60°C for 1 hr.

To assess binding of drugs to hydroxyapatite, 10^{-5} *M* solutions of test drugs in 10 m*M* sodium phosphate buffer (pH 7) were constructed, including 5% by volume hydroxyapatite. These mixtures were gently agitated for 30 min. Hydroxyapatite was separated by centrifugation and treated with 1.0 and then 2.0 *M* sodium phosphate buffers (pH 7). Percentage of drug bound and eluted under these conditions was determined by uv-visible spectroscopy.

In vitro bone organ culture studies. A neonatal rat calvarial organ culture system was employed to assess effects of these agents on PTH-stimulated and basal bone resorption rates. The use of such a system has been described previously (13). Essentially, calvaria are harvested from 1 to 5 day neonatal rat pups and incubated in BGJb medium supplemented with 2.5% calf serum, 15% horse serum, 0.025 *M* Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, streptomycin sulfate (10 mg/liter), and penicillin (10^5 U/liter). Medium and sera were obtained from GIBCO (Grand Island, NY). To some cultures, parathyroid hormone (bovine 1-34 fragment, Beckman, Palo Alto, CA) was added at a concentration of 10^{-7} *M* (9 U/ml). Some cultures contained drugs in a concentration of 10^{-5} *M*. These were made by addition of DMSO solutions such that the final DMSO concentration was 0.01%. The same concentration of DMSO was included in control cultures. Each group contained 8–12 calvaria and individual calvaria were incubated in wells of a 24-well multiplate. Medium was collected after 24 hr of culture and taken for determination of calcium concentration using the method of Kessler and Wolfman (19).

Results. Physiochemical properties of the two novel compounds were studied in parallel with those of the parent compounds, acetazolamide and tetracycline (Table I). Melting ranges were determined, and AAOEt was shown to have a melting range approximately the same as that of acetazolamide. WP-1 decomposes at 220°C, as does tetracycline. Values derived from the available literature for tetracycline and acetazolamide are listed in brackets throughout Table I.

Spectroscopic data were obtained as well. Infrared and uv-visible major absorbances, consistent with the structures of WP-1 and AAOEt, are shown in Table I. In addition, pH-dependent shifts in absorbance of uv-visible light are consistent with the structures for WP-1 and AAOEt; each manifests the properties of its parent compounds.

Chromatographic data were obtained using the HPLC system described. As one would predict, AAOEt is somewhat more lipophilic than is its parent, acetazolamide. WP-1 was shown to be more lipophilic than either of its parent compounds.

Binding to hydroxyapatite. Binding of test compounds to hydroxyapatite was studied, and results are shown in Table II. Acetazolamide and AAOEt have no particular affinity for hydroxyapatite; only 19% of acetazolamide and 3% of the AAOEt were bound to hydroxyapatite under these conditions. In contrast, 92% of the WP-1 and 90% of the tetracycline bound to hydroxyapatite. Most of the tetracycline was eluted with 1.0 *M* sodium phosphate. WP-1 was equally or more tightly bound, requiring 2.0 *M* sodium phosphate to achieve elution of most of the WP-1.

Inhibition of carbonic anhydrase. Acetazolamide, tetracycline, AAOEt, and WP-1 were all tested for carbonic anhydrase inhibitory activity (Table III). All were tested before and after being subjected to hydrolytic conditions with the final assay concentration being 5×10^{-6} *M*. For these experiments, the dependent variable is the amount of time required for acidification of a CO_2/bicarbonate buffer

TABLE I. PHYSIOCHEMICAL CHARACTERIZATION

Acetazolamide	Tetracycline	AAOEt	WP-1
	Melting range (°C)		
258–260 [258][a]	dec. 220 [dec. 214][b]	256–258	dec. 220
	ir spectral bands (cm^{-1})		
A 1550 [1548]	A 1580 [1580]	A 1540	A 1610
B 1345 [1363]	B 1400	B 1360	B 3300
C 1170 [1167]	C 3300	C 1270	C 2060
	uv visible absorbance maxima (nm)		
292 (0.1 *N* NaOH)[c]	385 (0.1 *N* NaOH)	298 (0.1 *N* NaOH)	383,290 (0.1 *N* NaOH)
266 (0.1 *N* HCl)	359,268 (0.1 *N* HCl)	264 (0.1 *N* HCl)	359,265 (0.1 *N* NaOH)
	HPLC capacity factor (k')[d]		
1.14	3.55	1.73	3.95

[a] Values in brackets are literature data (26).
[b] dec., decomposes.
[c] Denotes solvent for spectroscopic analysis.
[d] k' is defined as $(V_r - V_e)/V_e$ where V_r is the retention volume and V_e is the void volume of the system.

system to a defined endpoint. With no additions, the time required is 54 ± 2 sec. Addition of purified carbonic anhydrase decreased reaction time to 23 ± 1 sec. Finally, test drugs were added to enzyme-containing reaction mixtures, and any increase in reaction time is indicative of enzyme inhibition. Drug vehicles were tested independently to assure that they had no influence on reaction time.

Acetazolamide was shown to be the only compound which inhibits carbonic anhydrase activity. Following incubation under hydrolytic conditions, acetazolamide, solutions derived from AAOEt, and WP-1 each inhibited carbonic anhydrase activity. Tetracycline was without effect.

Hydrolysis of WP-1 at 37°C revealed that 2.5 to 4.1% of a 10^{-3} *M* solution was hydrolyzed at pH 1.5 when incubated for 24 to 96 hr. At pH 5.5, hydrolysis liberated 0.1 to 0.3% of the acetazolamide contained in WP-1. At pH 7.5, no hydrolysis was detected.

In vitro inhibition of bone resorption. Rat calvaria were cultured as described. Some cultures included the addition of PTH in the presence and absence of test compounds (10^{-5} *M*). Results are shown in Table IV. Treatment with PTH leads to a significant stimulation of the net release of calcium from cultured bone. None of the test compounds have a demonstrable effect on basal rates of calcium release. PTH-stimulated calcium release from calvaria was inhibited by acetazolamide and WP-1. Tetracycline was without effect at this concentration.

Discussion. There is ample evidence that carbonic anhydrase plays a role in the mediation of the bone resorptive response to PTH,

TABLE II. BINDING TO HYDROXYAPATITE

	Percentage not bound	Percentage bound	Percentage of total eluted by 1.0 *M* phosphate	Percentage of total eluted by 2.0 *M* phosphate
Acetazolamide	81	19	18	2
Tetracycline	10	90	81	12
AAOEt	97	3	3	1
WP-1	8	92	30	63

Note. Hydroxyapatite suspensions were incubated with 10^{-5} *M* test compound. Percentages of total drug bound and eluted under the stated conditions were monitored using uv-visible spectroscopy.

TABLE III. INHIBITION OF CARBONIC ANHYDRASE ACTIVITY

	Reaction time	
	Preincubation	Postincubation
Acetazolamide	53 ± 2	52 ± 3
Tetracycline	21 ± 1	22 ± 1
AAOEt	24 ± 1	38 ± 2
WP-1	25 ± 1	41 ± 1

Note. Times are given for carbonic anhydrase assays. Values shown are means ± SD for three replicate assays. Reaction time for no added enzyme or complete enzyme inhibition = 54 ± 2 sec. Reaction time for added enzyme and no inhibitor = 23 ± 1 sec. Compounds were incubated in 0.1 *N* HCl at 60°C for 1 hr.

1,25-dihydroxycholecalciferol, prostaglandin E_2, and forskolin (3–5, 7–9, 11–16, 20–23). Most of the studies supporting this contention are of three types: (i) enzyme localization studies, using antibodies, staining techniques, and fluorescent probes; (ii) *in vitro* organ culture studies; or (iii) *in vivo* studies using large doses of inhibitor or rather contrived models which attempt to minimize the effects of carbonic anhydrase inhibition in extraosseous tissues. These studies demonstrate that carbonic anhydrase inhibitors suitable for further study of *in vivo* bone metabolism do not exist.

To meet this need we have synthesized the first bone-targeted carbonic anhydrase inhibitor, a simple combination of tetracycline and acetazolamide. Specific tissue-directing of drugs is a common modern strategy in medicinal chemistry. Most attempts to date involve the use of monoclonal antibodies to achieve specificity of interaction. We have used the rather unique physiochemical environment of bone cells (i.e., the bone mineral phase) to similar advantage.

The condensation of an acyl chloride with the sulfonamide nitrogen of acetazolamide in the first synthetic step is the committed step in the synthesis of a prodrug, since a free sulfonamide group is required for enzyme inhibitory activity. In the reaction of the remaining acyl chloride with tetracycline, the tricarbonylmethane region of tetracycline must be preserved for calcium binding capacity to remain intact (24). The synthesis of WP-1 does not involve protection of this region, which diminishes the yield; however, purification using hydroxyapatite selects for "bone-seeking" products. The demonstrated affinity of WP-1 for hydroxyapatite indicates that the calcium binding domain is intact.

For this compound to be effective as a pharmacological probe in studies of bone metabolism, it must have at least two germane properties. First, it must bind avidly to bone so that, *in vivo,* this target organ is specifically influenced. Acetazolamide does not have this property, and since it inhibits carbonic anhydrase in many tissues, it is of limited further utility in the study of bone metabolism. As shown in Table II, of the agents which are inhibitors or proinhibitors of carbonic anhydrase, only WP-1 binds avidly to hydroxyapatite. It appears that WP-1 should have an affinity for bone at least as great as that of tetracycline.

The second requisite property for a useful bone-targeted inhibitor is that it either inhibit carbonic anhydrase or give rise to such an inhibitor under bone metabolic conditions. The data shown in Table III indicate that acetazolamide is the only test compound which is per se inhibitory. Under these *in vitro* hydrolytic conditions, AAOEt and WP-1 liberate an inhibitor of carbonic anhydrase. This inhibitor co-chromatographs with acetazolamide using a reverse-phase thin-layer chromatographic system (data not shown). Thus, it appears that WP-1 has potential as a bone-targeted carbonic

TABLE IV. EFFECTS ON CALCIUM RELEASE FROM RAT CALVARIA IN CULTURE

Hormone treatment	Drug treatment	24-hr calcium release (μg/calvarium)
None	None	7.2 ± 2.0
PTH	None	16.1 ± 1.0[a]
None	Acetazolamide	8.7 ± 1.3
PTH	Acetazolamide	11.0 ± 1.3[b]
None	Tetracycline	9.4 ± 1.5
PTH	Tetracycline	16.7 ± 0.9[a]
None	WP-1	10.2 ± 1.0
PTH	WP-1	11.8 ± 1.6[b]

Note. Values are means ± SE for each group. PTH (1–34) concentration was 10^{-7} *M* (9 U/ml); all other drug concentrations were 10^{-5} *M*. Statistical analysis by analysis of variance and Duncan's new multiple range test.

[a] Different from non-hormone-treated control ($P < 0.05$).

[b] Different from treatment with PTH only ($P < 0.05$).

anhydrase proinhibitor. Under conditions outlined in Table III, acetazolamide is liberated at the rate of approximately 0.5% per hour. However, under less severe hydrolytic conditions, WP-1 is not hydrolyzed to the same extent. At pH 5.5, only 0.3% of available acetazolamide is liberated during 96 hr of incubation (see Results). The implications are twofold: (i) the link between the sulfonamide nitrogen of acetazolamide and the remainder of WP-1 is sufficiently acid-stable to allow oral administration of the drug, with subsequent delivery of intact WP-1 to bone, and (ii) this bond is sufficiently labile to deliver small amounts of acetazolamide to bone fluid. This rather high degree of stability should decrease the potency of the compound, but not its efficacy.

As has been shown repeatedly by various laboratories, addition of PTH to calvarial organ cultures stimulates the release of calcium from bone. While none of the drugs tested in these studies (Table IV) have an effect on the basal rate of calcium efflux, acetazolamide and WP-1 attenuate PTH-stimulated net calcium release. In this system, at a concentration of 10^{-5} *M*, tetracycline was without effect on either basal or PTH-stimulated calcium release. A previous report (25) demonstrated an inhibition of PTH-stimulated calcium release from bone by higher concentrations of tetracycline (45 and 450 μM). In our studies no such effect was observed. Gomes *et al.* (25) have postulated that tetracycline inhibits collagenolysis. If tetracycline or a derivative from WP-1 has the same effect, one would predict an additive or synergistic effect with a carbonic anhydrase inhibitor. Such possibilities will be addressed in future mechanistic studies. While the mechanism is not defined in these studies, WP-1, which binds avidly to hydroxyapatite and is a latent carbonic anhydrase inhibitor, inhibits PTH-stimulated net release of calcium from neonatal rat calvaria *in vitro*.

While WP-1 is not an extremely potent inhibitor of bone resorption, its efficacy has been demonstrated, and the data support the fundamental hypothesis that sulfonamide (or other) inhibitors or proinhibitors of carbonic anhydrase can be delivered to bone and subsequently inhibit hormone-stimulated bone resorption. Future studies will examine other members of this family of bone-targeted carbonic anhydrase inhibitors. Some of these will be produced such that they are prodrugs (as is WP-1), others will retain the active sulfonamide moiety and serve as carbonic anhydrase inhibitors without metabolic activation. Such drugs may prove to be useful pharmacological probes in the study of bone metabolism.

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