Inhibition of Bone Resorption by H^+/K^+-ATPase Inhibitors

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We have found that 3-(3-(ethoxycarbonyl)propionyl)-8-methoxy-4-((2-methylphenyl)amino)quinoline (1, CP-113,411), a reversible inhibitor of gastric H^+/K^+-ATPase (IC_{50} 10–20 mM), is also a potent inhibitor of bone resorption by osteoclasts in a bone slice assay at concentrations as low as 10^{-7} M, with an IC_{50} of 2 mM. By contrast, the structurally related H^+/K^+-ATPase inhibitor 2 (3-(ethoxycarbonyl)-8-methoxy-4-((2-methylphenyl)amino)quinoline) disclosed by Robins is slightly more potent as an inhibitor of the gastric enzyme (IC_{50} 3–10 μM in our hands) but less efficacious than 1 as an inhibitor of osteoclasts in the bone slice assay at the lower concentrations (no effect at $\leq 10^{-6}$ M, IC_{50} 4 mM). These findings suggest that osteoclasts contain an H^+/K^+-ATPase-like enzyme which differs from the gastric one.

Introduction

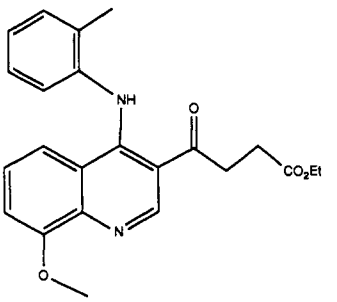
Osteoclasts are the principal cells responsible for bone resorption. The resorption process involves acid secretion into the space between the ruffled border of the osteoclast and the bone surface as the first step in the solubilization of the mineral content and the organic matrix of the bone surface.[1] It has been proposed that an ATP-dependent proton pump causes the acid secretion, although the exact nature of this pump is still not known.[2-6] Some evidence suggests that a gastric H^+/K^+-ATPase-like enzyme exists on the ruffled border of the osteoclast.[2,3] Others have suggested that the osteoclast proton pump resembles the vacuolar proton pump.[4-6] Recent data indicate that osteoclasts contain a unique proton pump which is different from either the gastric or the vacuolar pump.[7] In this study, we have prepared and tested two reversible inhibitors of the gastric H^+/K^+-ATPase and found that both compounds are potent inhibitors of bone resorption. Our findings suggest that osteoclasts contain a H^+/K^+-ATPase-like proton pump. However the difference in inhibitory patterns between these compounds indicate that the osteoclast proton pump is not identical to the gastric enzyme.

Chemistry

In the course of studies aimed at developing an affinity column for H^+/K^+-ATPase, we prepared compound 1 (3-(3-(ethoxycarbonyl)propionyl)-8-methoxy-4-((2-methylphenyl)amino)quinoline) as a chemical intermediate. Compound 1 was prepared by an adaption of the procedures used in the preparation of 2[8] and the related SK&F compound 3-butyryl-8-methoxy-4-((2-methylphenyl)amino)quinoline, SK&F 96067.[9] Thus *o*-anisidine was condensed with triethyl orthoformate and diethyl β-ketoadipate to a 4-hydroxyquinoline derivative, which was then treated with $POCl_3$ and with 2-methylaniline to give compound 1.

Results and Discussion

Compounds 1 and 2 were tested for their ability to inhibit gastric H^+/K^+-ATPase using isolated porcine gastric microsomes by the method of Saccomani et al.[10] As shown in Table I, compound 1 inhibited the enzyme reversibly with an IC_{50} of 10–20 μM (we observed close to 50% inhibition at 10 μM, higher concentrations of 1 could not be obtained due to limiting solubility of the compound in the assay medium containing 0.1% DMSO). By contrast, 2, which is reported to have an IC_{50} of 0.85 μM against gastric H^+/K^+-ATPase,[11] had in our hands an IC_{50} of 3–10 μM.



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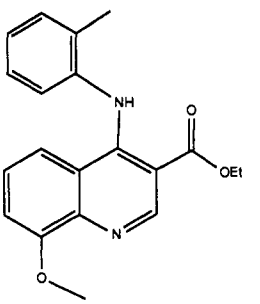

2

Both compounds 1 and 2 were reversible inhibitors of gastric H^+/K^+-ATPase because preincubation of either compound at a concentration near the IC_{50} at 37 °C for 10 min, followed by 200-fold dilution into the assay mixture led to a nearly total recovery of enzymatic activity (see Table I). SCH 28080, a reversible competitive inhibitor of gastric H^+/K^+-ATPase, served as a positive control in these experiments.[12]

Compounds 1 and 2 were assessed for their ability to inhibit bone resorption by osteoclasts in a bone slice assay, using the method of Chambers et al.[13] In this assay 1 inhibited lacunae formation by isolated osteoclasts in a dose-responsive manner, with statistically significant inhibition at concentrations of 10^{-7} M and above and with an IC_{50} of about 2 μM (Table II). By contrast, compound 2 showed significant inhibition of bone excavation only at 10^{-5} M, with an IC_{50} of about 4 μM (Table III). The osteoclast inhibition is unlikely to be explained by cytotoxicity since drug treatment did not lead to a decrease in the number of spread osteoclasts. Furthermore, as an indicator for lack of toxicity, neither 1 nor 2 inhibited basal Mg^{2+}-ATPase at any concentration up to 10 μM in isolated gastric microsomes.

These findings are remarkable since they show for the first time that a reversible gastric proton pump inhibitor

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Table I. Reversibility of Gastric H^+/K^+-ATPase Inhibition by 1 and 2[b]

compd	concentration	% activity of control
1	10 μM	60
	0.05 μM[a]	100
2	2 μM	66
	0.01 μM[a]	90
SCH 28080	2 μM	47
	0.01 μM[a]	100

[a] 200-fold dilution after preincubation. [b] The standard deviation in these experiments was less than 9%; 0.1% DMSO was used as vehicle control. Gastric microsomal H^+/K^+-ATPase was incubated with the compounds at a concentration near their IC_{50} at 37 °C for 10 min and then diluted 20-fold with 50 mM Tris-HCl (pH 7.5) and 0.14 μM nigericin and then with a 10-fold dilution into the assay mixture. For comparison the activity of compounds was assayed near their IC_{50} concentrations.

can interfere with osteoclast function at a low concentration. Earlier studies by Hall and Chambers[14] had shown that omeprazole, a potent irreversible inhibitor of gastric H^+/K^+-ATPase (IC_{50} 1 μM), inhibited osteoclast function only at 100 μM but not at 10 μM in the same lacunae assay used in these experiments. The mechanism of action of omeprazole is well understood.[15-18] As a weak base, omeprazole accumulates in the acidic canaliculi of the parietal cells of the stomach. The irreversible inactivation of gastric H^+/K^+-ATPase occurs through a covalent modification of a mercapto group near the active site. The relative inactivity of omeprazole as an inhibitor of bone resorption[14] versus the potent inhibition by the reversible gastric H^+/K^+-ATPase inhibitors 1 and 2 may be related to the fact that omeprazole needs to be activated by acidic pH[16] which may not be present in the bone slice assay.

It is generally accepted that bone resorption by osteoclasts is associated with acidification of the extracellular space underneath the ruffled border of the osteoclast and that this acidification involves proton pumps, although the type of proton pump involved is still unknown. Findings by Sundquist et al.[19] that bafilomycin A_1 inhibits bone resorption in osteoclast cultures with an IC_{50} of 1 nM suggested that a vacuolar-type H^+-ATPase might be involved. Others have postulated that a gastric-like H+/K+-ATPase is present in osteoclasts, and our findings support this suggestion. However, since 1 appears to be more potent that 2 as an inhibitor of bone resorption despite their inverse potency against gastric H^+/K^+-ATPase, it is possible that either the specificity of the bone H^+/K^+-ATPase is different from that of the gastric enzyme or that compound 1 has other properties that make it an effective inhibitor of osteoclast function, particularly at the lower concentrations.

In order to resolve the uncertainty as to whether or not the osteoclast enzyme is identical to the gastric enzyme, further experiments are needed to isolate and characterize the osteoclast enzyme or to establish homology by molecular cloning techniques.

Experimental Section

Chemistry. 3-(3-(Ethoxycarbonyl)propionyl)-8-methoxy-4-((2-methylphenyl)amino)quinoline (1, CP-113,411). A mixture of 5.0 g (41 mmol) of o-anisidine (Aldrich), 8.86 g (41 mmol) of β-ketoadipic acid diethyl ester (Sigma), and 6.07 g (41 mmol) of triethyl orthoformate (Aldrich) was heated to 140 °C in an open flask until no more ethanol evolved (3 h). After the mixture cooled overnight, the solid residue was triturated with petroleum ether and filtered to give 11.53 g (82%) of o-(1-(ethoxycarbonyl)-1-((3-(ethoxycarbonyl)propionyl)methylidene)amino)anisole: MS *m/e* 349; NMR ($CDCl_3$) consistent with product. This material was added in portions to 100 mL of boiling diphenyl ether. After refluxing for 4 h, the mixture was allowed to cool to room temperature, petroleum ether was added, and the precipitated product was filtered to give 4.52 g (45%) of 3-((3-ethoxycarbonyl)propionyl)-4-hydroxy-8-methoxyquinoline: MS *m/e* 302; NMR ($CDCl_3$) consistent with product. A 4.44-g (14.7 mmol) batch of this material was combined with 10 mL of $POCl_3$ and warmed to reflux for 1.5 h. After cooling, the mixture was evaporated in vacuo, and the residue was dissolved in methylene chloride, washed with water, dried over Na_2SO_4, and evaporated to an oil. Chromatography

Table II. Inhibition of Bone Resorption by 1

	number of pits/slice ± SEM		mean pit size ($\mu m^2 \times 10^{-3}$) ± SEM		mean total resorption ($\mu m^2 \times 10^{-3}$) ± SEM	
control	5.28 ± 0.90		1309 ± 137		6689 ± 1200	
	n = 18	sd 3.80	n = 93	sd 1317		sd 5092
10^{-5} M	2.22 ± 0.65[a]		948 ± 164[b]		2037 ± 631[a]	
	n = 18	sd 2.74	n = 38	sd 1012		sd 2675
10^{-6} M	3.39 ± 0.91[c]		1189 ± 125		4039 ± 1048[c]	
	n = 18	sd 3.87	n = 59	sd 963		sd 4446
10^{-7} M	4.67 ± 0.83		1052 ± 115[c]		4793 ± 962[c]	
	n = 18	sd 3.53	n = 83	sd 1052		sd 4081
10^{-8} M	5.89 ± 1.15		1446 ± 145		8397 ± 1699	
	n = 18	sd 4.87	n = 107	sd 1498		sd 7210

[a] <0.005. [b] <0.01. [c] <0.05.

Table III. Inhibition of Bone Resorption by 2

	number of pits/slice ± SEM		mean pit size ($\mu m^2 \times 10^{-3}$) ± SEM		mean total resorption ($\mu m^2 \times 10^{-3}$) ± SEM	
control	7.67 ± 1.13		956 ± 147		7526 ± 1609	
	n = 12	sd 3.91	n = 92	sd 1414		sd 5573
10^{-5} M	2.67 ± 0.54[a]		477 ± 99[b]		1272 ± 321[a]	
	n = 12	sd 1.88	n = 32	sd 562		sd 1111
10^{-6} M	9.17 ± 1.00		798 ± 77		7296 ± 967	
	n = 12	sd 3.46	n = 110	sd 814		sd 3349
10^{-7} M	9.5 ± 1.14		919 ± 97		9063 ± 1180	
	n = 12	sd 3.94	n = 114	sd 1032		sd 4088
10^{-8} M	9.83 ± 1.59		926 ± 101		8745 ± 1466	
	n = 12	sd 5.51	n = 117	sd 1092		sd 5079

[a] <0.005. [b] <0.01.

over silica gel with 1% MeOH in chloroform and isolation of the major LP (more lipophilic) material gave an orange oil which crystallized on standing to give 1.72 g (36%) of 4-chloro-3-(3-ethoxycarbonyl)propionyl)-8-methoxyquinoline: MS *m/e* 321; NMR ($CDCl_3$) consistent with product. This material (5.35 mmol) was placed into a flame-dried, three-necked, round-bottomed flask equipped with condenser, stirring bar, and addition funnel, dissolved in 15 mL of THF (tetrahydrofuran), and treated dropwise with a solution of 0.573 g (5.35 mmol) of o-toluidine (Aldrich) dissolved in 15 mL of THF. The mixture was warmed to 60 °C and kept at that temperature overnight. After cooling the yellow precipitate was filtered and recrystallized from methylene chloride/ethyl acetate/petroleum ether to give 1.75 g (76%) of the title compound as the hydrochloride: mp 210–211 °C; MS *m/e* 392; NMR (DMSO-d_6) consistent with product. Anal. ($C_{23}H_{24}N_2O_4$·HCl) C, H, N.

Biology

Microsomes from hog gastric mucosa were prepared by a modification[20] of the procedure described by Saccomani et al.[10] The H^+/K^+-ATPase was assayed according to Chang[21] in a medium containing 10 mg protein, 3 mM ATP, 50 mM Tris-HCl (pH 7.4), and 2 mM $MgCl_2$ with or without 10 mM KCl in a final volume of 1.1 mL. Incubation was carried out at 37 °C for 15 min. The reaction was terminated by the addition of 100 μL of 50% TCA. Phosphate release was measured spectrophotometrically as described by Fiske and Subbarow.[22]

The reversibility of the inhibition was assayed by preincubating the H^+/K^+-ATPase with a concentration of the drug near the IC_{50} at 37 °C for 10 min and then diluting 200-fold into the assay mixture. Controls were carried out by preincubating with vehicle (0.1% DMSO) under identical conditions. SCH 28080 was used as the positive control.[12]

Bone resorption was assessed as previously described.[13] Osteoclasts were mechanically disaggregated from neonatal rat long bones into Hepes-buffered medium 199 (Flow Laboratories, Irvine, UK). The suspension was agitated with a pipet, and the larger fragments were allowed to settle for 30 s. The cells were then added to two wells of a multiwell dish containing bone slices (each measuring 2.5 mm^2). After 15 min at 37 °C the bone slices were removed, washed in medium 199, and placed in individual wells of a 96 well plate (Flow, UK). These were incubated overnight in a total volume of 200 μL of culture medium consisting of 10% newborn calf serum (Flow) in Hanks-buffered MEM (Flow) in the presence or absence of drug. Bone resorption was quantified by scanning electron microscopy. Bone slices were immersed in 10% NaOCl for 10 min to remove cells, washed in distilled water, dried, and sputter-coated with gold. The entire surface of each bone slice was then examined in a Cambridge S90 (Cambridge Instruments, Cambridge, UK) scanning electron microscope. The number and size of the osteoclastic excavations and the plan area of bone resorbed was recorded. Differences between groups were analyzed by means of Student's *t*-test.

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