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Novel bone antiresorptive agents that selectively inhibit the osteoclast V-H$^+$-ATPase

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Abstract

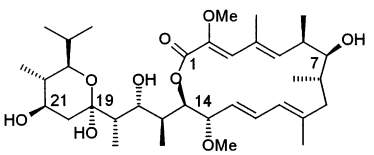
The vacuolar proton pump (V-ATPase) located on the plasma membrane of the osteoclast is a potential molecular target for the discovery of novel bone antiresorptive agents useful for the treatment of osteoporosis. In order to design novel compounds able to selectively inhibit the osteoclast V-ATPase we firstly identified the minimal structural requirements of bafilomycin A1, a macrolide antibiotic which potently inhibits all V-ATPases. This information allowed the design of 2-(indole)pentadienamide derivatives whose optimization led to a novel class of potent inhibitors that demonstrated a high degree of selectivity for the osteoclast V-ATPase. The most interesting derivative, SB-242784, was able to inhibit bone resorption by human osteoclasts in vitro and to completely prevent ovariectomy-induced bone loss in rats when administered orally at 10 mg kg^{-1} day^{-1}. Structure activity relationships of this class of compounds were investigated further by replacing the 2,4-pentadienoyl chain with suitable spacers able to maintain the correct orientation and distance between the indole ring and the amide moiety.

Keywords: Antiresorptive therapy; Osteoporosis; Selective inhibition; Osteoclast V-ATPase inhibitors

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and consequent increase in fracture risk [1,2]. It is due to an imbalance of the bone remodeling process, with bone resorption by osteoclasts exceeding new bone formation by osteoblasts. The bone resorption process requires secretion of protons by osteoclasts into the resorption lacuna in order to produce the acidic environment essential for dissolution of bone mineral and for the degradation of the bone matrix by proteolytic enzymes. This proton extrusion is energized by a proton pump belonging to the class of the vacuolar-type H$^+$-ATPases (V-H$^+$-ATPases) [3]. This class of proton translocating pumps is present in all eukaryotic cells, where they transport protons across the membrane of various intracellular compartments and play an important role in membrane trafficking, protein sorting and protein degradation.

The V-H$^+$-ATPase complex, as determined by electron microscopy [4–6] is a multi-subunit enzyme composed of at least 13 different types of protein, which couple ATP hydrolysis to proton translocation through a rotary mechanism, similar to that reported for the mitochondrial ATP synthases, where a proton gradient is utilized to synthesize ATP [7,8].

A gene (*Atp6i*) encoding a putative osteoclast-specific pump subunit, 116 kDa, was recently identified [9,10] whose disruption in mice generated severe osteopetrosis with osteoclasts unable to resorb bone. Interestingly, the acidification mediated by V-H$^+$-ATPase in other tissues was not affected by this gene deletion [11]. This finding provided the molecular basis for the design of novel antiresorptive agents working through selective inhibition of the osteoclast enzyme.



Bafilomycin A_1

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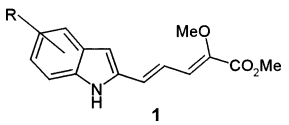
Bafilomycin A_1, a natural macrolide that is a potent and specific inhibitor of V-H^+-ATPases, inhibits bone resorption both in vitro and in vivo. To exploit the therapeutic potential of bafilomycin for the treatment of osteoporosis, it is necessary to modify the structure and confer high selectivity for the osteoclast enzyme compared with other essential V-H^+-ATPases. The structure of bafilomycin A_1 was used as a starting point to identify the minimal structural requirements for V-ATPase inhibitory activity.

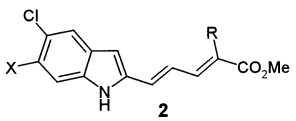
Table 1
Substitution of the indole ring



1

	R	cOc ATPase assay IC_{50} (μM)
a	5,6-Cl	1.9
b	5-Cl	6.9
c	4,5-Cl	15
d	5,6,7-Cl	24
e	H	30
f	5-sBu	>30
g	5,6-OMe	>30
h	1-Me	inactive

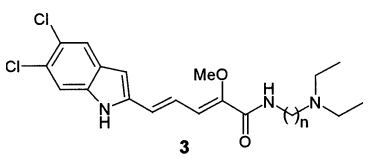
Table 2
Replacement of the enol-ether function



2

	X	R	cOc ATPase assay IC_{50} (μM)
a	Cl	OMe	1.9
b	Cl	Me	>10
c	Cl	OPh	>30
d	H	OMe	6.9
e	H	OEt	30
f	H	H	>30
g	H	F	>30
h	H	Cl	>30

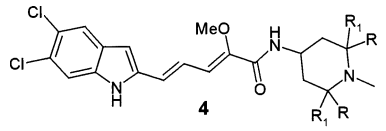
Table 3
Optimization of the length of the amino-amidic function



3

	n	cOc ATPase assay IC_{50} (μM)
a	2	0.60
b	3	0.18
c	4	>1

Table 4
Piperidine substitution in the amino-amidic moiety



4

	R	R_1		cOc ATPase assay IC_{50} (nM)
a	H	H		120
b	Me	H	*cis*	70
c	Me	H	*trans*	90
d	Me	Me		29

The C1–C7 fragment bearing the enol-ether at position 2, the dienic system and the free hydroxy group at position 7 were identified as the key features important for biological activity [12]. This fragment was attached to different aromatic, alicyclic and heterocyclic rings and, when this residue was linked to an indole ring at position 2, a moderate inhibitor (**1e**) was obtained with an IC_{50} of 30 μM. Insertion of lipophilic and electron-withdrawing substituents at positions 5 and 6 of indole afforded a 15-fold increase of potency (**1a**), whereas substitution at other positions or electron-donating substituents were detrimental for activity (Table 1).

The requirement for the vinylic methoxy α to the carbonyl group was confirmed when methoxy was replaced by hydrogen, methyl or halogens (Table 2). Replacement by ethoxy, as in **2e**, caused a fivefold decrease of potency, indicating that even very small modifications of this function are hardly tolerated.

A further significant step toward highly potent compounds entailed conversion of the ester in **2a** into amides **3**, bearing a strongly basic nitrogen group separated by a three-carbon-atom spacer from the amidic nitrogen (Table 3).

To identify the correct spatial orientation of the basic nitrogen with respect to the amidic nitrogen, some conformationally constrained analogues were prepared. 4-Amino-substituted piperidine **4a** was slightly more potent than the linear amide; this potency was improved by increasing the substitution alpha to the basic nitrogen (Table 4), leading to (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-*N*-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide (**4d**, SB 242784), which was a potent and selective inhibitor of the osteoclast V-H^+-ATPase [13].

The in vitro V-H^+-ATPase inhibitory profile of SB 242784 has been reported previously [14]. It was a low-nanomolar inhibitor of the bafilomycin-sensitive (vacuolar) Mg^{2+}-ATPase in membrane preparations of osteoclasts obtained either from egg-laying hens or

human osteoclastoma ($IC_{50} = 29$ nM and 22 nM, respectively).

In addition, **4d** was a very potent inhibitor of bone resorption ($IC_{50} = 3.4$ nM) in an assay measuring the collagen fragments released from bone slices after a 48 h incubation with human osteoclasts. The biochemical and pharmacological actions of **4d** were further characterized [15], demonstrating that it is highly selective for the V-H^+-ATPase of human osteoclasts ($IC_{50} < 5$ nM) compared with V-H^+-ATPases from kidney, liver, brain, spleen, stomach and heart ($IC_{50} \geq 5$ μM) measured in tissue slice in situ using a cytochemical colorimetric assay.

In vivo, SB 242784 was able to prevent fully the retinoid-induced hypercalcemia in thyroparathyroidectomized rats at 10 mg/(kg day) p.o. In an animal model of osteoporosis, **4d** (5 or 10 mg/kg p.o. daily for 6 months) prevented dose dependently the decrease of bone mineral density caused by ovariectomy in rats with efficacy similar to 17β-estradiol. Histomorphometric analysis confirmed that **4d**, similar to estradiol, normalized trabecular number and prevented the bone resorption induced by ovariectomy (Fig. 1). In the same experiment, **4d** had no significant effects in urinary acidification, confirming the selectivity observed in vitro [15].

Compounds **4** undergo photo-induced isomerization of the double bonds. Exposure to daylight of solutions of **4d** caused the formation of 2*Z*,4*Z* (about 20%) and 2*E*,4*E* (about 10%) isomers. The two isomers were isolated and both of them were less potent than the reference compound ($IC_{50} = 0.66$ μM and 2.4 μM, respectively).

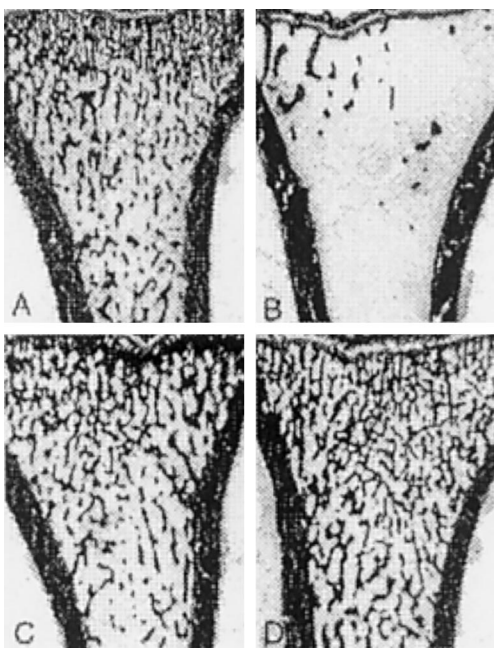
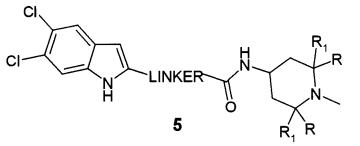


Fig. 1. Photomicrographs of proximal tibia metaphysis after 6 months treatment: (A) sham operated; (B) Ovx vehicle; (C) estrogen; (D) **4d**, 10 mg/kg (reprinted with permission from Ref. [15]).

Table 5
Replacement of the dienic chain



5

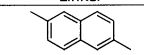
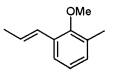
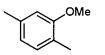
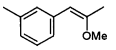
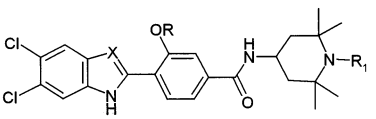
	Linker	IC_{50}, μM		Linker	IC_{50}, μM
a		10	**e**		0.5
b		2.5	**f**		0.6
c		1.6	**g**		0.9
d		2.3	**h**		0.03

Table 6
Indole replacement by benzoimidazole ring



	X	R	R_1	IC_{50} (nM)	
				hOc ATPase	hOc resorption
5h	CH	Me	Me	33	13
6a	N	Me	Me	51	29
6b	N	Et	H	8	6

This issue prompted us to replace the dienic chain of **4a** with different spacers (aromatic rings, such as phenyl, naphthyl and substituted phenyl, and heterocyclic rings, such as furanyl) that could mimic the electronic density of the double bonds and maintain the correct orientation of the amidic function relative to the indole ring (Table 5).

The replacement of the dienic chain by a phenyl ring (**5f**, Table 5) gave a moderately potent compound with $IC_{50} = 0.6$ μM. Insertion of a methoxy group, close to the indole ring (**5h**), restored potency ($IC_{50} = 30$ nM in hOc ATPase assay). Surprisingly, the insertion of the same substituent adjacent to the amidic function (**5g**) reduced the potency.

Finally, the replacement of 5,6-dichloroindole in **5h** by the 5,6-dichlorobenzoimidazole ring led to **6a**, which retained a similar inhibitory potency along with improved hydrophilicity and water solubility. The conversion of methoxy into ethoxy gave **6b**, which was the most potent inhibitor, with $IC_{50} = 8$ nM in the human osteoclast ATPase assay (Table 6).

In conclusion, it was possible to identify several different chemical classes of potent V-ATPase inhibitors starting from SAR of bafilomycin A_1 but completely unrelated to the macrolide structure. The selectivity of these compounds, and in particular of SB 242784, for the osteoclast plasma membrane proton ATPase was greatly increased in comparison with bafilomycin A_1. With this compound it was possible to demonstrate the utility of selective inhibition of the osteoclast V-ATPase in in vivo animal models of osteoporosis and to prove that this biological target can represent a novel approach for antiresorptive therapy for osteoporotic disorders.

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