# Selective inhibitors of the osteoclast vacuolar proton ATPase as novel bone antiresorptive agents

Carlo Farina and Stefania Gagliardi

The proton ATPase located on the apical membrane of the osteoclast is essential to the bone resorption process. This proton pump is, therefore, an attractive molecular target for the design of novel inhibitors of bone resorption, and potentially useful for the treatment of osteoporosis and related metabolic diseases of bone. Recently, several inhibitors with different degrees of selectivity for the osteoclast V-ATPase have been reported. In particular, systematic chemical modifications of the macrolide antibiotic bafilomycin $A_1$ have identified the minimal structural requirements for activity and allowed the design of simplified analogues that demonstrate high potency and selectivity for the osteoclast enzyme.

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture[1]. In postmenopausal osteoporosis, which is the most common form of disease, the decline in oestrogen levels causes an acute increase in bone turnover, with bone resorption exceeding formation of new bone. The current treatments, in addition to hormone replacement therapy, are therefore antiresorptive and entail mainly the use of bisphosphonates or calcitonins. None of these treatments, however, are fully satisfactory in terms of either tolerability or patient compliance, and the discovery of novel agents acting on molecular mechanisms specifically involved in bone resorption is eagerly awaited.

To effect bone resorption, osteoclasts ultimately need to lower the pH in the sealed microcompartment that underlies their site of attachment to the bone surface[2]. An acidic environment in this microcompartment is required to dissolve the bone mineral and to allow degradation of the bone matrix by acid cysteine proteinases, primarily cathepsin K (Refs 3,4). Acidification is carried out by a proton pump located on the ruffled border of the osteoclast, which belongs to the family of vacuolar $H^+$-ATPases (V-ATPases)[5].

It has been shown that expression of the proton channel part of V-ATPase is stimulated in resorbing osteoclasts[6] and that antisense RNA and DNA molecules targeted against two subunits of the V-ATPase inhibit bone resorption by rat osteoclasts[7]. Therefore, this enzyme is a major potential target for reducing osteoclast activity and, consequently, for designing novel agents useful for the treatment of osteoporosis.

Proton-translocating V-ATPases are ubiquitous components of eukaryotic organisms and are the major electrogenic pumps of vacuolar membranes. The V-ATPases pump protons from the cytoplasm to the lumen using the energy released by ATP hydrolysis. The electrical potential

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difference created across the membrane is used to drive the movement of ions and solutes into the vacuole. The acid pH of vacuoles is also used for other functions in the endocytic and secretory pathways[8]; for example, it leads to activation of hydrolytic enzymes in lysosomes. Low pH is also required for the dissociation of the receptor–ligand complexes[9].

Given the ubiquitous nature of vacuolar proton pumps, therapeutically useful V-ATPase inhibitors would need to be able to discriminate between the osteoclast enzyme and the other essential V-ATPases. This requirement constitutes a major challenge to this approach and the discovery of novel and selective inhibitors of the osteoclast V-ATPase has been hampered by the high complexity and the incomplete structural knowledge of the pump.

## Structure of the pump

Comprehensive reviews of the structure and function of V-ATPases have been published recently[10,11]. Briefly, vacuolar proton ATPases are heteromultimeric protein complexes composed of at least 10–12 different subunits (Fig. 1) arranged in a 'stalk and ball' structure. The cytoplasmic 'ball' ($V_1$) contains the ATP-binding catalytic site and is connected by the 'stalk' to the membrane sector ($V_0$), containing the proton channel. It has been proposed that, similar to mitochondrial F-ATPases[12,13], the net movement of protons across the bilayer catalyzed by this complex is achieved by a mechanism involving the rotation of one part of the assembly relative to other portions of the enzyme[14].

Molecular masses of between 560 kDa and 740 kDa have been reported for V-ATPases isolated from fungal, plant and animal sources[15–17]. Although sequence differences between the subunits of V-ATPases of different origins have been reported, the existence of biochemically distinct isoforms of the enzyme has not yet been demonstrated. With particular regard to the osteoclast, discordant evidence has appeared in the literature; alternately supporting or rejecting the existence of an isoform uniquely expressed in the plasma membrane of the osteoclasts. While osteoclasts do express the ubiquitous subunits B2 (Refs 18,19) and C-E (Ref. 20), 'osteoclast-specific' 67 kDa (Refs 21,22) and 116 kDa (Ref. 23) subunits were described that, however, were subsequently shown to be ubiquitously expressed in human tissues[24,25]. Also, the 'peculiar sensitivity' of chicken osteoclast V-ATPase to vanadate[26] and to nitrate[27] have not been confirmed by independent investigators in either chicken[5,28,29] or mammalian[30] osteoclasts. Taken together, all the above results suggest possible differences between the V-ATPase in osteoclasts compared with other cells, but data are conflicting and definitive evidence has still to be obtained.

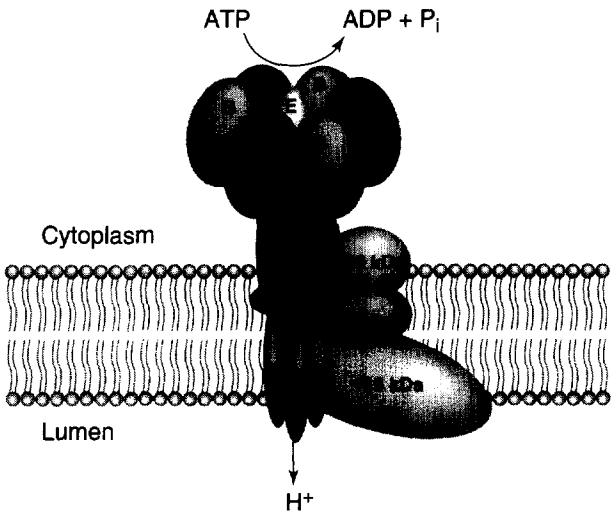


***Figure 1.*** *Simplified schematic model of V-ATPases, built from the data available for yeast, fungal, plant and mammalian V-ATPases, showing the multisubunit complex with a 'ball and stalk' structure. The peripheral catalytic domain ($V_1$) contains three copies each of subunits A (70 kDa) and B (60 kDa) where ATP is hydrolyzed. Additional subunits C (40 kDa), D (34 kDa), E (33 kDa), F (16 kDa) and the recently identified subunit G (14 kDa) are present. The integral membrane-embedded sector ($V_0$) contains six copies of the proteolipid subunit c (17 kDa) which forms the proton channel, together with one copy each of accessory 100–116 and 39 kDa subunits. An additional accessory 50-kDa subunit has been identified in yeast. Several observations support the hypothesis that the V-ATPases operate by a rotational mechanism similar to that described for F-ATPases[14–16]. In this biological motor the stalk would act as a rotating cam transmitting conformational changes to the proteins in the trimeric complex formed by A and B subunits and in the proteolipid (c) hexamer.*

## Inhibitors of V-ATPase

Since their discovery in the early 1980s, several molecules have been found to inhibit both ATP hydrolysis and proton translocation in V-ATPases (Fig. 2). Compounds, such as *N*-ethylmaleimide (NEM, **1**)[31,32] or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, **2**)[33], can react covalently with cysteines or tyrosyl-OH residues, respectively, in the ATP-binding site of catalytic subunit A and inhibit the function of the enzyme at low micromolar concentrations. Similarly, the thiol reagent omeprazole (**3**), which inhibits the $H^+/K^+$-ATPase of gastric parietal cells at low micromolar

concentrations, is also able to inhibit the V-ATPase in osteoclasts at concentrations above 100 μM (Ref. 34). This compound has also been reported to inhibit bone resorption in humans[35].

As in the case of mitochondrial F-ATPases, micromolar concentrations of dicyclohexylcarbodiimide (DCC, **4**) give complete and irreversible inhibition of proton pumping in V-ATPases by covalently binding a glutamic residue in the 16 kDa proteolipid[36]. Lipophilic alkyl tin derivatives, such as tributyl tin chloride (TBT, **5**) and organotin–flavone complexes[37], are potent inhibitors of the V-ATPase from bovine adrenal chromaffin granules, with $K_i$ values ~0.3 μM, but are not specific as they also inhibit mitochondrial F-ATPases and some P-type ATPases. Their mechanism of interaction with the vacuolar enzyme has been studied and evidence for binding either to membrane $V_0$ domain[38] or to the catalytic subunit A have been obtained[37].

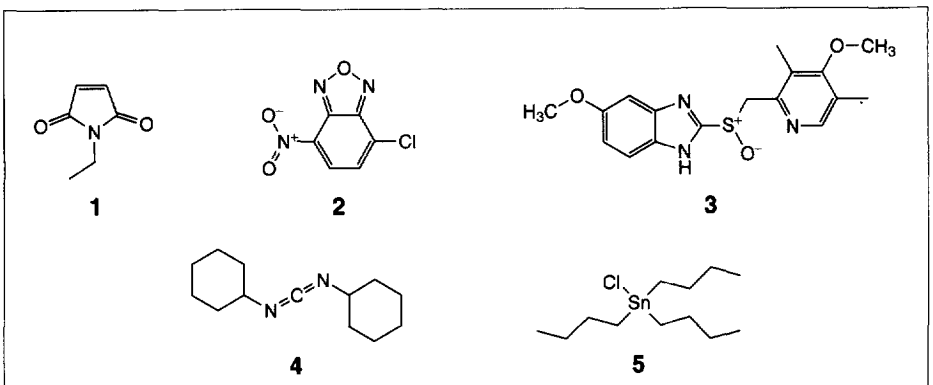


***Figure 2.*** *Non-specific inhibitors of V-ATPase.* ***1****, N-ethylmaleimide;* ***2****, NBD-Cl;* ***3****, omeprazole;* ***4****, dicyclohexylcarbodiimide;* ***5****, tributyl tin chloride.*

Bone-bound gallium is able to inhibit bone resorption by isolated avian osteoclasts at lower concentrations (10 pmol $\mu g^{-1}$ of bone) than those toxic to the osteoclasts (100 μM)[39]. It has only limited utility, however, because of associated adverse effects, such as anaemia, nausea and vomiting, and renal toxicity. It has been recently discovered that EDTA complexes of thallium and indium, which localize to bone with high affinity, inhibit V-ATPase-mediated proton transport in chicken osteoclasts with $ID_{50}$ values of 3 and 20 μM, respectively[40]. The same complexes were three orders of magnitude more potent in inhibiting bone resorption, possibly owing to hydroxyapatite partitioning, which increases their concentration locally in the resorption compartment. The two complexes were effective *in vivo* at the dose of 10 μmol $kg^{-1}$ in preventing both the parathyroid hormone (PTH)-induced hypercalcaemia in parathyroidectomized rats and the decrease of bone mineral density in ovariectomized rats.

### *Natural inhibitors*

Among the natural products (Fig. 3), the red pigment prodigiosins, obtained from *Streptomyces hiroshimensis*, inhibits the acidification mediated by V-ATPases without inhibiting ATP hydrolysis or membrane potential formation[41]. In rat liver lysosomes, prodigiosin 25-C (**6**) inhibits the proton pump activity with an $IC_{50}$ of ~30 nM, but does not affect ATPase activity up to 1 μM. This uncoupling of proton translocation from ATPase hydrolysis is peculiar and not shared by other inhibitors. An explanation has been obtained very recently with the discovery that prodigiosins dissipate pH gradients through promotion of $H^+/Cl^-$ symport activity[42] and, therefore, are not true inhibitors of proton V-ATPases.

The most potent inhibitors of V-ATPases are the bafilomycins and concanamycins, which are macrolide antibiotics isolated from the fermentation of *Streptomyces* spp. Among these unusual macrolides, which are characterized by the presence of a vinylic methoxy at position 2 and by a long side chain bearing a hemiketalic ring, bafilomycin $A_1$ (**7**) and concanamycins (**8**) are very potent and highly specific inhibitors of the V-ATPases[43,44] Bafilomycin $A_1$ exerts its inhibitory effect by tight binding to one or more subunits in the transmembrane $V_0$ domain of the enzyme complex[45], and does not produce any covalent modification even though washing does not readily reverse its effect *in vitro*. Because of its high specificity for V-ATPases, bafilomycin $A_1$ has been widely used to study the biochemistry and the physiological role of these enzymes. Using this compound, it was possible to demonstrate that pharmacological inhibition of osteoclast V-ATPase effectively prevents bone resorption both *in vitro*[46] and *in vivo*[47,48]. However, bafilomycin $A_1$ does not distinguish among the various types of V-ATPases; therefore, its administration to animals causes inhibition of all the essential V-ATPases and leads to systemic alteration of cellular physiology and high toxicity.

Other natural products that inhibit V-ATPase less potently are the destruxins, a family of cyclic depsipeptide mycotoxins that was first isolated from the antomogenous fungi *Metarhisium anisopliae*[49]. Destruxin B (**9**) specifically inhibits yeast V-ATPase with an $IC_{50}$ of 5.4 μM and, unlike bafilomycin $A_1$ or concanamycin A, its action is almost completely reversed after washing[50].

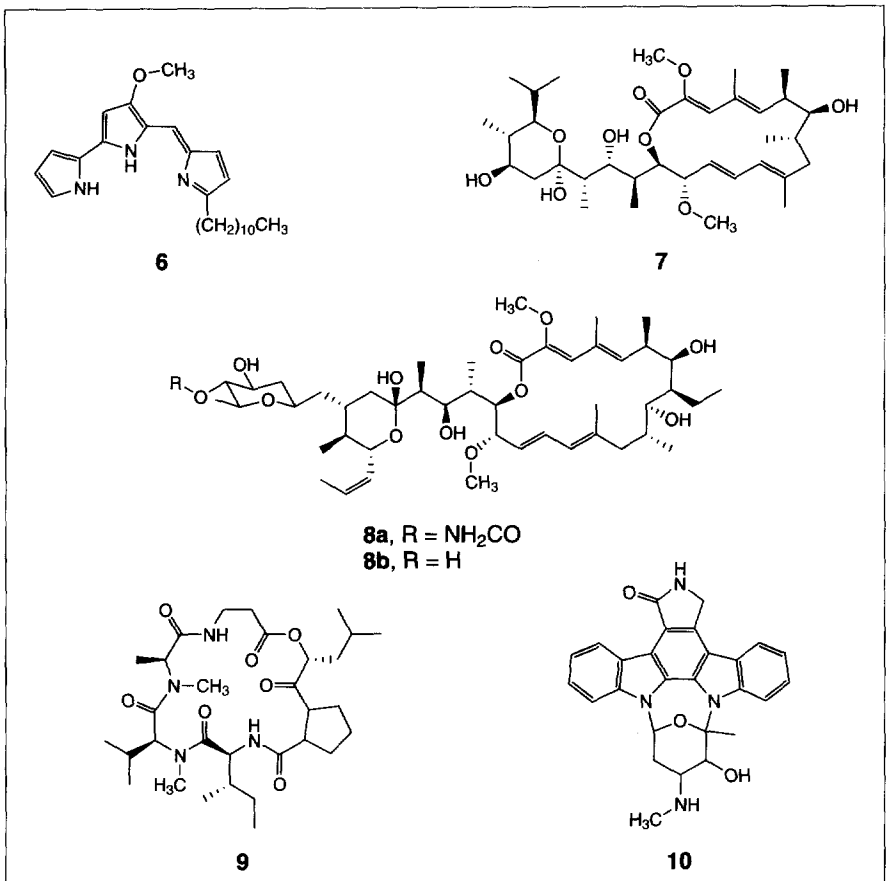


***Figure 3.*** *Natural products with V-ATPase inhibitory activity.* ***6****, prodigiosin 25-C;* ***7****, bafilomycin* $A_1$*;* ***8a****, concanamycin A;* ***8b****, concanamycin C;* ***9****, destruxin B;* ***10*** *indolocarbazole derivative.*

Activation of protein kinase C (PKC) promotes proton extrusion by V-ATPases[51]. Some inhibitors of PKC have been reported to also be strong inhibitors of the V-ATPases, such as the indolocarbazole derivative **10**, which is extremely potent against the vacuolar proton pump isolated from clathrin-coated vesicles in bovine brain, with an $IC_{50}$ of 0.6 nM (Ref. 52).

### *Synthetic inhibitors*

Bisphosphonates bind avidly to bone and inhibit bone resorption by a variety of mechanisms that are still being elucidated[53]. Tiludronate (**11**), a bisphosphonate used in therapy for the treatment of osteoporosis, has been reported to inhibit V-ATPase-dependent proton transport in chicken osteoclasts with an $IC_{50}$ of 466 nM, while being about 2000-fold less potent in the same assay using chicken kidney-derived vesicles[54]. A subsequent report, however, suggests that tiludronate is a weak inhibitor of V-ATPase activity in both chicken osteoclast- and chicken adrenal-derived membrane vesicles with an $IC_{50}$ of 1 mM (Ref. 55).

Trihydroxybenzamide derivatives have been claimed to possess V-ATPase inhibitory properties[56]. Compound **12** (Fig. 4) produced maximal inhibition of the V-ATPase in pig kidney cortex at a concentration of 4 μM. When tested in an *in vitro* assay of bone resorption, it was able to reduce the PTH-induced $^{45}Ca^{2+}$ release from foetal rat long bones in a dose dependent manner, with statistically significant inhibition (63%) at 100 μM. When assayed in the chicken osteoclast V-ATPase assay by independent investigators, compound **12** displayed an $IC_{50}$ of 17 μM, thus, appearing a less potent inhibitor of the osteoclast enzyme when compared with pig kidney V-ATPase.

A series of aminoquinoline derivatives, exemplified by the structure **13**, are claimed to cause full inhibition of the V-ATPase-dependent proton transport on membrane vesicles from mouse peritoneal macrophages at 1 μM (Ref. 57). At the same concentration, the compounds fully inhibited (98–100%) PTH-induced bone resorption in rat calvaria, thus, suggesting that they also inhibit the V-ATPase in osteoclasts. The structurally related 4-aminoquinoline **14** is a weak inhibitor of the gastric $H^+/K^+$-ATPase ($IC_{50}$ = 10–20 μM) and was able to inhibit lacunae formation and the resultant bone resorption by isolated osteoclasts at a lower concentration (0.1 μM). Compound **14** and analogues are stated to inhibit V-ATPase in osteoclasts, even though no direct biological data are reported in the patent application[58].

Among all the inhibitors mentioned above, many are useful only as pharmacological tools or are described in patent applications that do not provide sufficient biological evidence to allow us to assess their actual potential as possible pharmacological agents. The few reports delineating some structure–activity relationships that have appeared in the literature concern omeprazole analogues and the derivatives of bafilomycin $A_1$.

### *Omeprazole-like derivatives*

The prototype of this series is WY47766 (**15**), which was reported to inhibit proton transport in chicken osteoclasts dose dependently, with an $IC_{50}$ of 10 nmol $\mu g^{-1}$ protein[59]

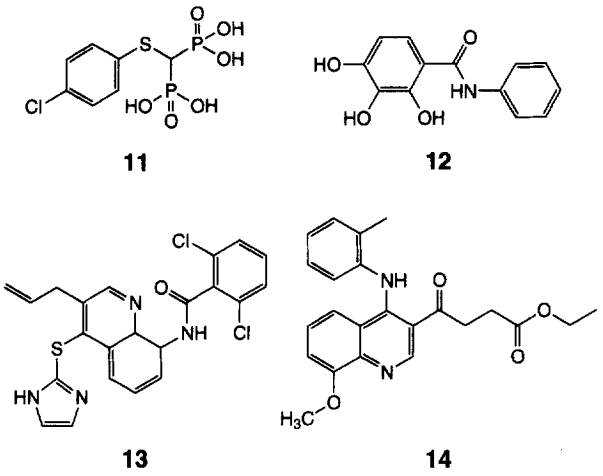


***Figure 4.*** *Small-molecule inhibitors of bone resorption with V-ATPase inhibitory activity.* ***11****, tiludronate.*

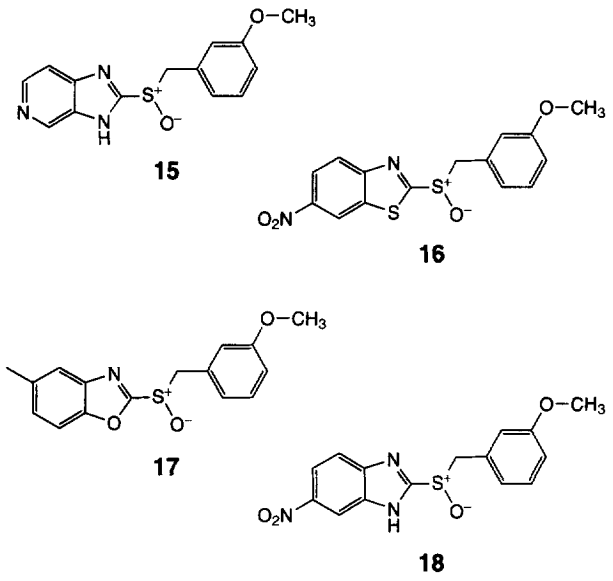


***Figure 5.*** *Omeprazole-like V-ATPase inhibitors.* ***15****, WY47766;* ***16****, XS238;* ***17****, XT533;* ***18****, XT531.*

and to inhibit bone resorption by rat osteoclasts with an $IC_{50}$ of 45 μM (Ref. 30). V-ATPase inhibition was not reversed by washing but was reversed upon addition of membrane permeable (dithiothreitole, DTT) or impermeable (cysteine) disulphide-reducing agents. These data suggest that, like omeprazole (**3**), **15** is a sulphydryl reagent that inhibits the osteoclast V-ATPase by binding to a cysteine residue in the catalytic subunit(s). Proton transport in membrane vesicles prepared from chicken kidney was inhibited by **15** with a potency identical to that observed in osteoclast vesicles. Although **15** is a structural analogue of omeprazole, it does not share with omeprazole the ability to inhibit potently the gastric $E_1E_2$-type $H^+/K^+$ ATPase. Proton transport in vesicle preparations from the gastric mucosa was resistant to inhibition by **15** at all concentrations tested.

A series of benzimidazole, benzothiazole and benzoxazole derivatives, substituted at position 2 by a sulphoxide group (Fig. 5), were identified as novel inhibitors of the osteoclast V-ATPase[60]. Their effect was compared with that of **15** using the V-ATPase isolated from bovine kidney and purified by immunoprecipitation with the antibody to the 31 kDa subunit. Inhibition of bone resorption *in vitro* was assessed by measuring the PTH-induced $^{45}Ca^{2+}$ release from mouse calvaria. Compound **15** inhibited this V-ATPase activity with an $IC_{50}$ of 200 μM (Table 1). The $IC_{50}$ values of the nitrobenzothiazole XS238 (**16**) in the V-ATPase and mouse calvaria assays were ~250-fold more potent in the enzyme assay ($IC_{50}$ = 0.7 μM) and tenfold more potent in the mouse calvaria assay ($IC_{50}$ = 5 μM) compared with that of **15** in the same assays. Conversely, the 2-substituted nitrobenzimidazole **18** was a less potent V-ATPase inhibitor ($IC_{50}$ = 300 μM). The 5-methylbenzoxazole **17** was equipotent to **15** in the mouse calvaria assay but more active in the enzyme assay ($IC_{50}$ = 4.6 μM). The benzothiazole **16** was also able to inhibit bone resorption in different assays, such as the $^3H$-proline release from rat long bones by chicken and mouse osteoclasts ($IC_{50}$ = 1 μM) and the pit formation by mouse osteoclasts ($IC_{50}$ = 100 nM).

The lack of correlation between the potencies of sulphoxides **16–18** in V-ATPase and resorption assays suggests that different mechanisms are involved in the two activities. The ability of DTT to significantly reverse the inhibitory effect of **16** strongly supports the suggestion that the mechanism of ATPase inhibition by **16** involves

**Table 1. Inhibition of bovine kidney V-ATPase and of bone resorption *in vitro* by omeprazole analogues[a]**

| | $IC_{50}$ (μM) | |
|---|---|---|
| **Compound** | **$H^+$-ATPase assay** | **Mouse calvaria assay** |
| **15** | 200 | 50 |
| **16** | 0.7 | 5 |
| **17** | 4.6 | 60 |
| **18** | 300 | n.t. |

[a]From Ref. 62; n.t., not tested.

the formation of one or more disulphide bonds in subunit A of the osteoclast V-ATPase. The mechanism responsible for the inhibition of the osteoclast-mediated bone resorption has not been elucidated. One hypothesis is that it could involve the formation of a cysteine-reactive intermediate in the resorption environment, determined by a nucleophilic attack at C-2 of **16** with expulsion of a highly reactive sulphenic acid, or by a rearrangement of **16** to a similarly reactive sulphenic acid ester.

### *Derivatives of bafilomycin $A_1$*

Extensive chemical modifications of bafilomycin $A_1$ have been performed by two independent research groups with the aim of increasing the selectivity of this type of molecule for the osteoclast V-ATPase (Fig. 6). Synthetic modifications performed at Astra Hässle[61,62] entailed replacement of the 21-hydroxy group by an amino function (**19**), enlargement of the macrolactone ring (**20**), degradation of the side chain to the carboxylic acid **21** and preparation of the sulphonamide derivatives **22** and **23**.

The assessment of tissue selectivity *in vitro* of the novel derivatives was made by comparing their relative potencies in inhibiting the V-ATPase activity in chicken osteoclast and chicken brain membrane vesicles. Selectivity *in vivo* was evaluated by comparing their bone antiresorptive activity, measured as the prevention of the retinoid-induced hypercalcaemia in thyroparathyrodectomized rats, with the toxicity threshold quantified as the highest dose that could be

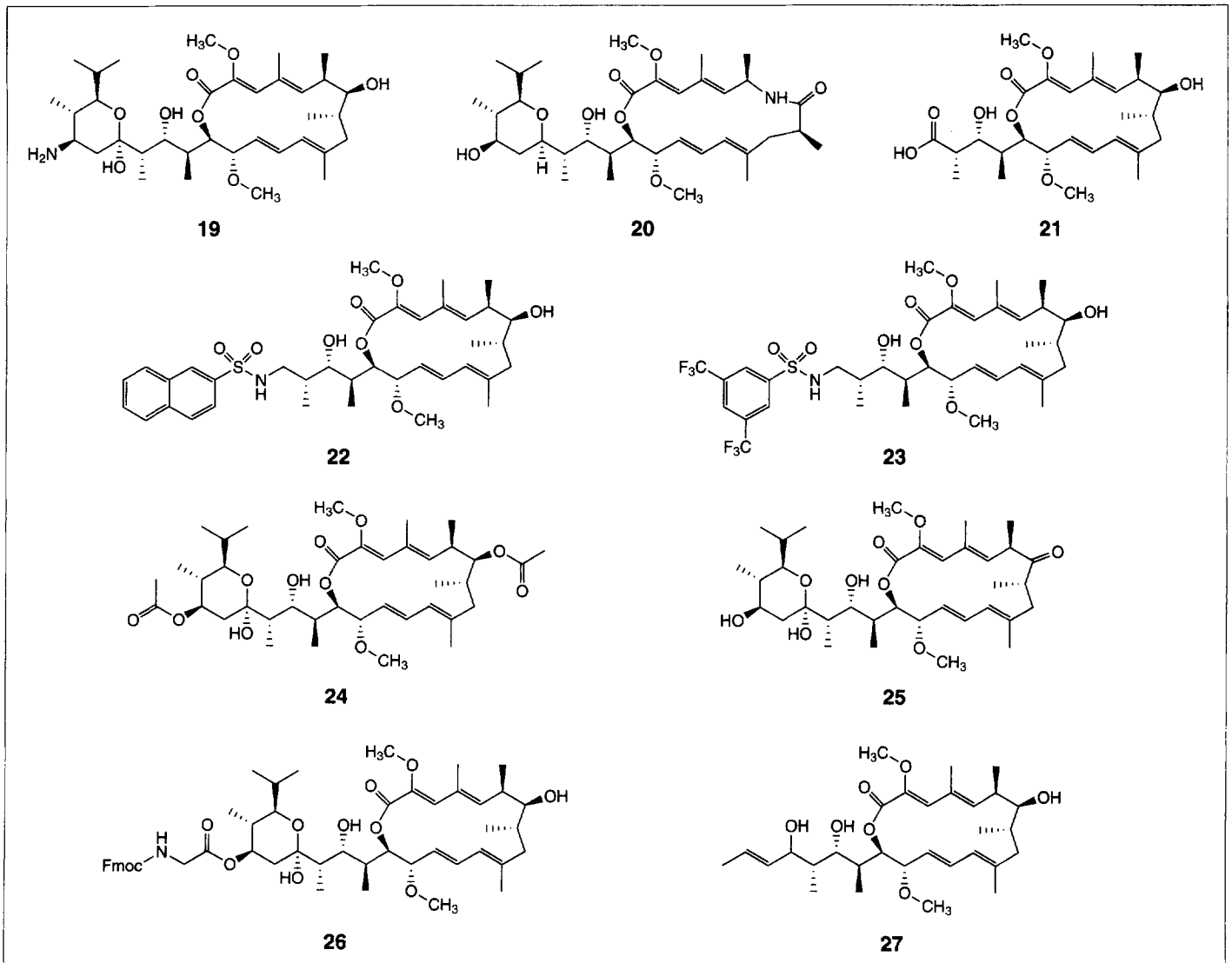


***Figure 6.*** *Derivatives of bafilomycin $A_1$.* ***19****, H31841;* ***20****, H41005;* ***21****, H31198;* ***22****, H35699;* ***23****, H36248.*

**Table 2. Inhibition of the vacuolar ATPase in chicken osteoclasts and brain and *in vivo* activity of bafilomycin derivatives[a]**

| Compound | V-ATPase assays, $IC_{50}$ (nM) | | | *In vivo* assays (mol $kg^{-1}$) | | |
|---|---|---|---|---|---|---|
| | Bone | Brain | Ratio | Potency | Toxicity threshold | Ratio |
| **7** | 0.36 | 0.84 | 2.4 | 0.5[b] | 0.4 | 0.8 |
| **8b** | 0.35 | 0.58 | 1.7 | >0.1[b] | 0.025 | <0.25 |
| **19** | 3.10 | 10.2 | 3.3 | 5.5 | 10 | 1.8 |
| **22** | 0.92 | 3.6 | 3.9 | 6.2 | 20 | 3.2 |
| **23** | 0.58 | 3.3 | 5.7 | 2.7 | 25 | 9.3 |

[a]From Refs 65 and 66.
[b]Extrapolated value.

administered intravenously without inducing any visible signs of toxicity[63,64]. A limited decrease in potency (4–10-fold) on osteoclast V-ATPase was observed *in vitro*; however, the bone/brain selectivity of the new derivatives varied consistently, with compound **23** being approximately sixfold less potent against brain compared with bone (Table 2). A minor increase in separation of *in vivo* effects was observed[65] in the amino derivative **19** retaining the intact bafilomycin skeleton and in compound **20**, where the lactone ring was enlarged via Beckman rearrangement of the corresponding 7-oxo derivative. Fragmentation of the pyran ring in bafilomycin $A_1$ resulted in the formation of the carboxylic acid **21** that was devoid of V-ATPase inhibitory properties. Compound **21** allowed the synthesis of analogues with greater structural diversity by several steps, entailing reduction to the alcohol, preparation of the corresponding azide and reduction to amine. The corresponding sulphonamide derivatives **22** and **23** maintained a high potency *in vitro* ($IC_{50}$ = 0.92 nM and 0.58 nM, respectively) and **23** displayed a tenfold separation between potency *in vivo* and toxicity threshold.

**Table 3. Inhibition of the vacuolar ATPase in chicken osteoclasts and bovine chromaffin granules of bafilomycin derivatives[a]**

| Compound | Potency ratio ($IC_{50}$ compound/$IC_{50}$ baf $A_1$) | | Selectivity ratio |
|---|---|---|---|
| | cOc | bCG | |
| **7** | 1 | 1 | 1 |
| **24** | 49 | 280 | 5.7 |
| **25** | 34 | 32 | 0.9 |
| **26** | 60 | 1.2 | 0.02 |
| **27** | 3.2 | 2.6 | 0.8 |

[a]From Ref. 68.

The relative toxicity of the five compounds in Table 2 correlate well with *in vitro* selectivity, suggesting that toxicity does indeed result from V-ATPase inhibition in other tissues. These results provided encouraging evidence that acid pumps isolated from different tissues could have distinct pharmacological properties and opened the way to novel and effective inhibitors of bone resorption lacking unwanted side effects. Researchers at SmithKline Beecham, who studied the minimal structural requirements for the inhibitory activity of bafilomycin[66], obtained a similar conclusion. Chemical modifications of the natural macrolide[67–69] involved alkylation, acylation or oxidation of the hydroxy groups, partial or total saturation of the double bonds and partial or total degradation of the side chain bearing the hemiketalic ring.

The results obtained during this study confirm that the hydroxy group at position 21 can be acylated, alkylated or oxidized without jeopardizing the V-ATPase inhibitory potency. On the contrary, oxidation of the 7-hydroxy group to the ketone **25** caused a significant reduction of potency (Table 3), indicating that this group is strictly required for activity. Partial and total hydrogenation strongly reduced the activity, suggesting a role for the dienic systems, possibly in constraining the macrolactone configuration. Extensive chemical modifications could be performed on the tetrahydropyranic ring in the side chain, from which four carbon atoms could be removed (**27**) without affecting biological activity. Because the $IC_{50}$ of bafilomycin $A_1$ and its derivatives varies with the amount of membrane protein in the assay, all the results were normalized as potency ratios versus bafilomycin $A_1$ used as an internal standard in each experiment. A differential effect of two analogues in inhibiting the V-ATPase-dependent proton transport in chicken osteoclast and bovine chromaffin granule membrane vesicles confirmed that V-ATPases in different tissues have differential sensitivity to inhibitors. Indeed, the 21-Fmoc-aminoacetyl derivative **26** was 50-fold more potent in inhibiting proton transport in bovine chromaffin granules than in osteoclasts, while the 7,21-diacetyl derivative **24** was sixfold more potent against osteoclast V-ATPase.

### *Simplified bafilomycin derivatives*

As chemical modification of bafilomycin is limited by its high complexity and low chemical stability, a novel series

of simplified derivatives was designed with the aim of obtaining novel analogues endowed with a higher degree of selectivity for the osteoclast enzyme (Fig. 7). These 5-(2-indolyl)-2,4-pentadienoyl derivatives[70] contained the essential features for bafilomycin V-ATPase-inhibitory activity, that is, a vinylic methoxy group alpha to a conjugated carbonyl group with the indole-NH group possibly mimicking the necessary hydroxy group at position 7 of bafilomycin $A_1$. The inhibition of the bafilomycin-sensitive ATPase activity of these new compounds was measured in membrane vesicle preparations from chicken osteoclasts (cOc) and compared with their potency against the enzymes from chicken adrenal glands (cAG) and from human kidney cortex (hK). Compound **28** was a moderate inhibitor of ATP hydrolysis by the chicken osteoclast V-ATPase ($IC_{50}$ = 30 μM). Optimization of the aromatic substitution, by insertion of lipophilic and electron withdrawing substituents at positions 5 and 6 of the indole ring, led to the 5,6-dichloroderivative **29**, in which inhibitory potency was increased by 15-fold ($IC_{50}$ = 1.9 μM). Potency was further enhanced when the ester group was converted into the corresponding carboxamides bearing a strongly basic nitrogen group separated by a three carbon atom chain from the amidic nitrogen (Table 4). Compound **30** displayed a submicromolar potency ($IC_{50}$ = 180 nM) and a 15-fold selectivity against the vacuolar enzyme in chicken adrenal glands. Conformationally constrained piperidine derivatives **31** and **33** were even more potent (80 and 100 nM, respectively) with a similar degree of selectivity. The 2-pyrimidylpiperazine **34** was very potent in the V-ATPase assay with $IC_{50}$ = 60 nM.

When assayed *in vitro* using isolated human osteoclasts obtained from giant cell tumours of bone[71], compounds **30–34** were potent inhibitors of bone resorption, with potencies comparable with their ability to inhibit chicken osteoclast V-ATPase. The most potent compounds of Table 4 were **34** and **33**, with $IC_{50}$s of 10 and 30 nM, respectively, and a selectivity of ~40-fold if compared with the data from the human kidney V-ATPase assay. Compound **32** was shown to be a potent inhibitor of both chicken osteoclast V-ATPase ($IC_{50}$ = 26.3 nM) and bone resorption by human osteoclasts ($IC_{50}$ = 3.4 nM)[72]. When ATPase activity was measured in human tissue sections using a cytochemical assay, **32** was 1000-fold more potent as an inhibitor of the osteoclast enzyme when compared with kidney, liver, gastric parietal cells and heart[73]. Interestingly, compound **32** at the oral dose of 10 mg $kg^{-1}$ $day^{-1}$ was able to prevent completely the bone loss induced by ovariectomy in the rat[74]. The results obtained clearly suggest that the osteoclast V-ATPase is a unique enzyme, distinct from the V-ATPases in kidney, liver and other tissues, whose selective pharmacological inhibition can provide novel inhibitors of bone resorption, potentially useful as anti-osteoporotic agents.

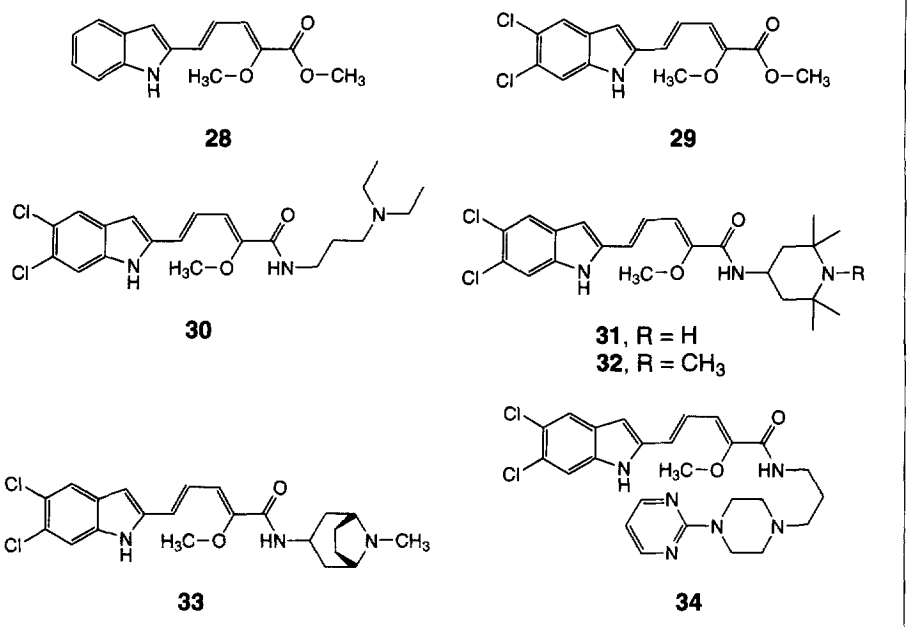


***Figure 7.*** *(2Z,4E)-5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienamide derivatives.*

## Conclusions and perspectives

Even though the structure of the V-ATPases has not yet been completely elucidated at the molecular level, the medicinal chemistry work performed on the bafilomycin framework has provided strong evidence that selective pharmacological modulation of V-ATPases can be achieved. The osteoclast enzyme, in particular, appears to be very sensitive to the new inhibitors at concentrations that do not affect other essential V-ATPases. This evidence opens a new avenue for the medicinal chemist to produce novel and selective inhibitors that are therapeutically useful for the treatment of osteoporosis. The research in this field is still in its infancy and, although some of the novel inhibitors, such as the indole derivatives **30–34**, look very promising, full pharmacological

**Table 4. Biological activity of 5-(2-indolyl)-2,4-pentadienoyl derivatives[a]**

| Compound | V-ATPase assays – $IC_{50}$ (nM) | | | Human osteoclast resorption assay – $IC_{50}$ (nM) | |
|---|---|---|---|---|---|
| | cOc | cAG | hK | Readout: pits/bs[b] | Readout: ELISA[c] |
| **7** | 0.1 | 0.2 | 0.1 | 0.1 | 0.01 |
| **30** | 180 | 2800 | 3000 | 180 | 140 |
| **31** | 80 | 820 | 440 | 40 | 30 |
| **33** | 100 | 2500 | 1320 | 30 | 30 |
| **34** | 60 | 930 | 370 | 30 | 10 |

[a]From Ref. 72.

[b]Number of resorption excavations (pits) per bone slice (bs) was counted.

[c]ELISA assay measures the amount of the C-terminal fragment of type I collagen (CTX) released during bone resorption.

and toxicological reports are awaited in order to evaluate exactly the validity and usefulness of these new agents. Safety and tolerability are a prerequisite for good compliance in elderly patients and for competing successfully with the available and upcoming treatments. From the scientific point of view, a new era might start. With the increasing knowledge of the structure and function of the various isoforms of this fascinating enzyme, it might be possible to design novel agents that selectively affect V-ATPases in specific target cells and organs, offering new therapeutic tools to treat a variety of diseases in which V-ATPase activity is involved.

*Other possible therapeutic uses of V-ATPase inhibitors*

Given the ubiquitous distribution of V-ATPases in eukaryotic cells, their modulation can affect a large variety of biological processes. For example, proton-extruding V-ATPases are functionally expressed on the plasma membranes of some human tumour cells[75] where they might contribute to maintain a high intracellular pH and might be directly responsible for the faster rates of proliferation of tumour cells. Bafilomycin $A_1$ reduced, in a dose-dependent manner, the intracellular pH and proliferation rate of transformed cells, but was without effect on normal cells[76]. Bafilomycin $A_1$ is also able to target selectively murine leukaemic cells[77] and its use as a possible means to treat cancer[78] and leukaemia[79] has been claimed. It is also possible that antitumour activity of V-ATPase inhibitors could be achieved via the induction of apoptosis[80]; it is known that granulocyte colony-stimulating factor delays programmed cell death in neutrophils by upregulating the V-ATPase[81], and this effect is inhibited by bafilomycin $A_1$. Bafilomycin $A_1$ also induces apoptosis in a human pancreatic cancer cell-line and inhibits tumour growth in nude mice[82].

A V-ATPase seems to be involved in the proteolytic processing of the β-amyloid precursor protein (APP) and treatment with bafilomycin $A_1$ of human 293 cells transfected with the 'Swedish' mutation of APP leads to a potent inhibition of the release of β-amyloid, possibly via inhibition of β-secretase[83,84]. These findings could have important consequences for the generation of pharmacological inhibitors of β-secretases as potential drugs to slow amyloid-plaque formation in Alzheimer's disease.

Finally, a vacuolar proton ATPase in the ocular ciliary epithelium is a key component of the mechanism controlling the production of aqueous humor and the regulation of intraocular pressure. This V-ATPase is a possible pharmacological target of several clinically used glaucoma medications, and could lead to novel treatments of glaucoma[85].

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## In short...

A recent acquisition has been made by **LeukoSite** (Cambridge, MA, USA) in a deal that provided the company with ~$14.5 million in cash and two compounds under clinical trial. The company acquired **CytoMed** (Cambridge, MA, USA), a biopharmaceutical company developing novel products for the treatment of inflammatory diseases. In the deal LeukoSite exchanged 935,625 shares of preferred stock based on a price of $11.88 per share for all the preferred and common shares of CytoMed. The conversion of LeukoSite preferred stock into common stock was made on a one for one basis. Additionally, in October 1999, upon receipt of a $6 million payment due to CytoMed from UCB Pharma (Atlanta, GA, USA) for certain research programmes and inflammation-related technology, LeukoSite will issue a further 631,313 shares to CytoMed stockholders.