Development and Characterization of Biphenylsulfonamides as Novel Inhibitors of Bone Resorption

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Increased osteoclastic bone resorption plays a central role in the pathogenesis of many bone diseases, and osteoclast inhibitors are the most widely used treatments for these diseases. We have identified and characterized a series of novel biphenylsulfonamide derivatives that have potent inhibitory effects on osteoclastic bone resorption in vitro and that prevent ovariectomy-induced bone loss in vivo. A number of aromatic substituted derivatives were prepared and a QSAR model was generated, which allowed accurate prediction of compound potency. Using this model, we have prepared compounds able to inhibit osteoclast formation and bone resorption in vitro at concentrations in the nanomolar range. One such compound, **55** (ABD295) (Greig, I. R.; Mohamed, A. I.; Ralston, S. H.; van't Hof, R. J. Alkyl Aryl Sulfonamides as Therapeutic Agents for the Treatment of Bone Conditions. GB Patent WO2005118528, 2005), fully reversed ovariectomy-induced bone loss in mice at a dose of 5 (mg/kg)/day. In conclusion, biphenylsulfonamides like **55** form a new class of potent antiresorptive agents with possible therapeutic use in diseases characterized by increased bone resorption.

Introduction

Bone is a dynamic tissue that is constantly renewed in the process of bone turnover. Osteoclasts are multinucleated cells closely related to macrophages, which are responsible for the resorption of bone.[1] Increased osteoclastic bone resorption plays an important role in the pathogenesis of common bone diseases such as osteoporosis, Paget's disease of bone, cancer-associated bone disease, and periarticular bone loss associated with inflammatory conditions,[2−5] and the most successful forms of treatment of these disorders are based on osteoclast inhibition.[6]

Bisphosphonates[7] are the most widely used osteoclast inhibitors in clinical practice, but calcitonin has also been used successfully in the treatment of various conditions characterized by increased bone loss.[8] However, calcitonin appears to be less effective than the bisphosphonates in the treatment of osteoporosis and other bone diseases.[9] While the bisphosphonates are highly effective antiresorptive drugs, intestinal absorption is poor and inhibited by food. Therefore, bisphosphonates have to be given on an empty stomach,[10] which leads to problems with compliance, especially in the elderly. Treatment with aminobisphosphonates is also associated with adverse effects including gastrointestinal intolerance[11,12] and an acute phase response after initial administration.[13]

Hormone replacement therapy and selective estrogen receptor antagonists also act by inhibiting bone resorption, but they are only effective in the prevention and treatment of osteoporosis associated with oestrogen deficiency,[14] and hormone replacement therapy has been associated with an increased risk for cancer.[15−17] The range of available treatments for diseases characterized by increased osteoclast activity therefore falls into relatively few mechanistic classes, highlighting the need to identify new classes of antiresorptive agents.

We previously reported that the 4-hydroxybutyl ester of biphenylcarboxylic acid derivative **67** (ABD56)[18] inhibits osteoclastic bone resorption in vitro and in vivo.[18] The mechanism of action for **67** is not fully understood. However, we have previously reported that **67** inhibits activation of the transcription factor nuclear factor κB (NFκB) stimulated by both tumor necrosis factor (TNF) and the receptor activator of the NFκB ligand (RANKL), indicating that the molecular target may be a signaling molecule in the NFκB pathway.[18] Our studies showed that **67** causes osteoclast and macrophage apoptosis at concentrations in the range 5−20 μM but had no effect on apoptosis of unrelated cells such as osteoblasts (IC_{50} > 100 μM) and hepatocytes (IC_{50} > 200 μM). In spite of extensive optimization of the structure, we were unable to make significant improvements to the potency of **67** derived esters. Since the low potency and potentially labile ester linkage made **67** unsuitable as a drug candidate, we have carried out studies involving bioisosteric replacement of the ester moiety (Figure 1) in an attempt to identify more promising drug candidates. Here, we report our findings on a set of sulfonamide derivatives as alternatives to the ester-linked compounds described in our original report.[18] We have developed simple synthetic methods for preparing a large number of derivatives of this lead compound, which has allowed us to generate a QSAR model from which we were able to predict and make large improvements in the potencies of derivatives. The most promising of these drugs has been tested in osteoclast cultures in vitro and in an in vivo model for bone loss.

Chemistry

All compounds were synthesized using starting materials purchased from Sigma-Aldrich or Lancaster. 4-Bromo-2-ethylphenylsulfonyl chloride was purchased from Maybridge. Products were characterized using ^{1}H and ^{13}C NMR obtained at 250 or 62.9 MHz, respectively, on a Brucker AC250 spectrometer. Purity was assessed using TLC and LC (column, HIRPB, C18; mobile phase, 60% 25 mM ammonium acetate, pH 4, 40% acetonitrile; λ_{max} = 235−275 nm). Elemental analysis was performed on all compounds included in the QSAR

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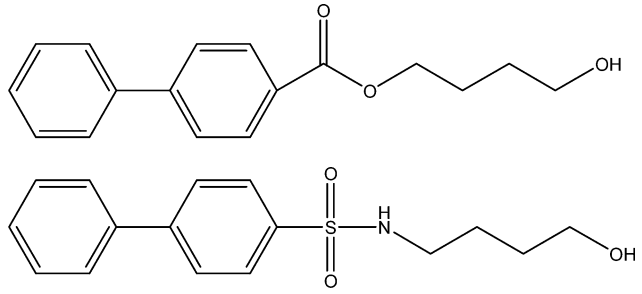
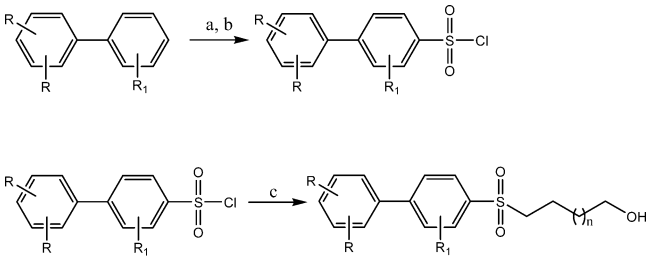


Figure 1. Parent compound **67** and its sulfonamide bioisostere.

Scheme 1. Synthesis of Biphenylsulfonamides from Biphenyls[a]



15. R = H	R_1 = H	n = 0
16. R = H	R_1 = H	n = 1
17. R = H	R_1 = H	n = 2
18. R = H	R_1 = H	n = 3
19. R = 4-F	R_1 = H	n = 1
20. R = 4-Br	R_1 = H	n = 1
21. R = 4-Br	R_1 = H	n = 2
22. R = 4-Br, 2-F	R_1 = H	n = 1
23. R = 2-NO_2	R_1 = H	n = 1
24. R = 3-NO_2	R_1 = H	n = 1
25. R = 2-I	R_1 = H	n = 1
26. R = 2-F, 4-F	R_1 = 3-OMe	n = 2

[a] Reaction conditions: (a) $CHCl_3$, SO_2Cl_2, room temp, 4 h; (b) $SOCl_2$, reflux, 3 h; (c) CH_2Cl_2, pyridine, amino alcohol.

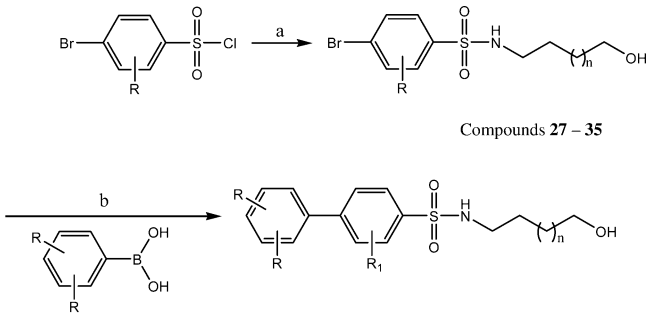
model. All samples showed only one component unless otherwise stated.

Sulfonamide derivatives were prepared either by direct sulfonylation of the required biphenyl,[19] which was then chlorinated[19] and coupled with the required amino alcohol in the presence of pyridine (Scheme 1) to give sulfonamides **15**−**26**, or when the biphenyl was unavailable or where sulfonylation was favored at a different site, from the required bromophenyl sulfonamides **27**−**35** (Scheme 2), which was then used in parallel syntheses and coupled to phenylboronic acids by standard Suzuki coupling methods, to give sulfonamides **36**−**61** (Scheme 2)[19] and to prepare a sufficiently large and diverse selection of derivatives to permit determination of the structure−activity relationship.

Results and Discussion

Since osteoclast inhibition assays are time-consuming and are unsuitable for high-throughput screening, we conducted initial screening of candidate compounds in cultures of the mouse macrophage cell line J774. This cell line has been used before as a model system to screen for inhibitor effects of bisphosphonates on osteoclast activity,[20] and our previous studies showed that inhibitory effects of biphenyl carboxylate esters on J774 survival correlated closely with inhibitory effects on authentic osteoclasts.[18] The unsubstituted derivative, compound **16**, was tested in this assay and found to inhibit J774 survival with an IC_{50} value of approximately 20 μM (Table 1), which is comparable to that of the original ester-linked compound **67**. We found that increasing the length of the alkyl chain to five or six carbons had little effect on potency, but reducing it to three caused a dramatic reduction (Table 1). The five-carbon starting material 5-aminopentanol has been used for many derivatives. However, for the most potent compounds we have found that the four-carbon chain gave compounds with a better solubility profile for testing in aqueous medium, and 4-aminobutanol has mostly been used for these.

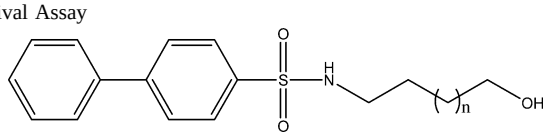
Scheme 2. Synthesis of Biphenyl Sulfonamides from Bromophenylsulfonyl Chlorides[a]



36. R = 4-Me	R_1 = H	n = 2
37. R = 4-Ac	R_1 = H	n = 2
38. R = 4-SMe	R_1 = H	n = 1
39. R = 4-CN	R_1 = H	n = 2
40. R = 2-OMe	R_1 = H	n = 2
41. R = 4-CO_2H	R_1 = H	n = 2
42. R = 4- NMe_2	R_1 = H	n = 2
43. R = 4-OH	R_1 = H	n = 2
44. R = 2-Cl, 4-Cl	R_1 = H	n = 2
45. R = 2-CF_3, 4-CF_3	R_1 = H	n = 1
46. R = 4-OCF_3	R_1 = H	n = 1
47. R = 4-Ph	R_1 = H	n = 2
48. R = 3-Me	R_1 = H	n = 1
49. R = 2-CF_3	R_1 = H	n = 2
50. R = 4-CF_3	R_1 = H	n = 1
51. R = 3-F, 4-F	R_1 = H	n = 2
52. R = 2-F, 4-F	R_1 = H	n = 2
53. R = 2-F, 4-F	R_1 = 3-Cl	n = 1
54. R = 2-F, 4-F	R_1 = 3-OCF_3	n = 1
55. R = 2-F, 4-F	R_1 = 2-Me	n = 1
56. R = 4-F	R_1 = 2-Me	n = 1
57. R = 2-F, 4-F	R_1 = 3-Et	n = 1
58. R = H	R_1 = 3-CF_3	n = 1
59. R = 2-F, 4-F	R_1 = 3-CF_3	n = 1
60. R = 4-CF_3	R_1 = 3-F	n = 1
61. R = 4-CF_3	R_1 = 2-F	n = 1

[a] Reaction conditions: (a) CH_2Cl_2, pyridine, amino alcohol; (b) $(Ph_3P)_4Pd$, Na_2CO_3, H_2O, toluene, ethanol, reflux, 3 h.

Table 1. Effects of Alkyl Chain Length on Drug Potency in the J774 Survival Assay

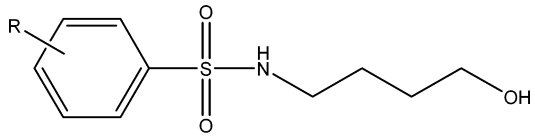


compd	*n*	IC_{50} (μM)
15	0	>50
16	1	18
17	2	18
18	3	13

We next studied the importance of the ring orientation as shown in Table 2. As expected from our studies on biphenyl carboxylates, only linear structures are highly active, and the biphenyl-2-sulfonamides and 3-sulfonamides were of much lower potency. Also studied were the effects of N-alkylation. We found that such variations had little effect on potency; IC_{50} values of 30 and 20 μM were obtained for the *N*-methyl (**65**) and *N*-pentyl (**66**) derivatives of compound **16**, respectively.

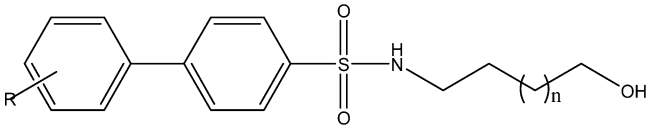
To test whether the substitution pattern for the aromatic rings was important for drug activity, we prepared derivatives with a wide range of substituents. Substitution on the outer ring showed that the addition of electron-withdrawing substituents leads to

Table 2. Effects of Ring Orientation on Potency in J774 Viability Assays



compd	R	IC_{50} (μM)
19	4-(4-fluorophenyl)	8
50	4-(4-trifluoromethylphenyl)	5
63	2-(4-fluorophenyl)	>50
64	3-(4-trifluoromethylphenyl)	>50

Table 3. IC_{50} of 2′- and 4′-Derivatives in the J774 Survival Assay

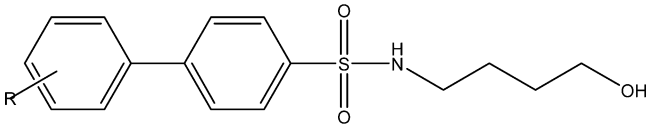


compd	R	*n*	IC_{50} (μM)
16	H	1	18 ± 3
17	H	2	20 ± 3
19	4-fluoro	1	8 ± 2.5
20	4-bromo	1	8 ± 2
21	4-bromo	2	9 ± 2
22	4-bromo-2-fluoro	1	3 ± 1
23	2-nitro	1	2 ± 0.5
25	2-iodo	1	5.5 ± 1.5
36	4-methyl	2	13 ± 2
37	4-acetyl	2	11 ± 2
38	4-methylthio	1	35 ± 6
39	4-cyano	2	12 ± 2
40	2-methoxy	2	31 ± 4
41	4-carboxy	2	33 ± 3
42	4-dimethylamino	2	32 ± 3
43	4-hydroxy	2	21 ± 3
44	2.4-dichloro	2	2.5 ± 0.5
45	2,4-bis(trifluoromethyl)	2	2 ± 0.5
46	4-trifluoromethoxy	1	8 ± 1
47	4-phenyl	2	33 ± 5
49	2-trifluoromethyl	1	6 ± 1.5
50	4-trifluoromethyl	1	5 ± 1
52	2,4-difluoro	2	4.5 ± 1
62	2-amino	1	24 ± 3

increased potency (Table 3), with halogen, haloalkyl, haloalkoxy, or nitro groups giving the most potent compounds. Comparison of 4′- and 2′,4′-derivatives showed that further addition of electron-withdrawing substitutions resulted in more potent compounds. In addition, hydrophobic derivatives were more potent than hydrophilic derivatives. This was shown by the relatively lower potency of the hydrophilic cyano, acetyl, and carboxy derivatives (Table 3), in spite of these substituents being electron-withdrawing. The most striking anomaly in these findings is the high potency of the nitro derivative, which has previously been reported as being either weakly hydrophobic[21,22] or hydrophilic.[23] Nitro groups are not normally considered to act as binding groups and therefore are unlikely to form an extra interaction.[24] It is possible therefore that a resonance structure confers a change in the aromatic nature of the ring itself that is particularly favorable in promoting interactions within the putative binding site.

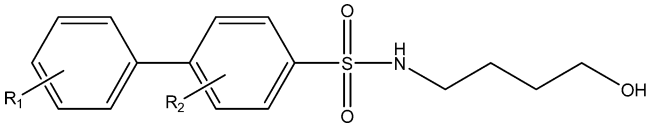
These findings are in accordance with our earlier studies on ester compounds[18] where we noted that addition of electron-withdrawing substituents such as fluorine resulted in a slight increase in potency. Addition of an electron-donating substituent such as methyl to biphenyl carboxylate esters resulted in a dramatic reduction in potency, while we were unable to find any derivatives that gave a significant increase in potency. In

Table 4. Comparison of 2′-, 3′-, and 4′-Substituted Derivatives



compd	R	IC_{50} (μM)
23	2-nitro	2
24	3-nitro	33
36	4-methyl	13
45	2,4-bis(trifluoromethyl)	2
48	3-methyl	28
49	2-trifluoromethyl	6
50	4-trifluoromethyl	5
51	3,4-difluoro	16
52	2,4-difluoro	4.5

Table 5. Influence of Inner Ring Substitution on Drug Potency against J774 Macrophages



compd	R_1	R_2	IC_{50} (μM)
16	unsubstituted	unsubstituted	18 ± 3
19	4-fluoro	unsubstituted	8 ± 1
26	2,4-difluoro	3-methoxy	33 ± 3
50	4-trifluoromethyl	unsubstituted	5 ± 1
52	2,4-difluoro	unsubstituted	4.5 ± 1.5
53	2,4-difluoro	3-chloro	3.5 ± 0.5
54	2,4-difluoro	3-trifluoromethoxy	1 ± 0.3
55	2,4-difluoro	2-methyl	0.8 ± 0.1
56	4-fluoro	2-methyl	2.5 ± 0.25
57	2,4-difluoro	3-ethyl	0.15 ± 0.05
58	unsubstituted	3-trifluoromethyl	4.5 ± 1
59	2,4-difluoro	3-trifluoromethyl	0.7 ± 0.1
60	4-trifluoromethyl	3-fluoro	5.5 ± 1.5
61	4-trifluoromethyl	2-fluoro	5 ± 1

contrast to the ester derivatives, structural variations of sulfonamide derivatives can lead to a dramatic increase in potency but never resulted in a substantial decrease. All the less potent sulfonamide derivatives have an IC_{50} between 30 and 35 μM, as shown in Table 3. This suggests a nonspecific activity that is unaffected by structural variations on the aromatic ring.

The presence of nonspecific activity is supported when considering the 3′-derivatives as shown in Table 4, which also shows a minimum potency of around 30 μM. The 3′-derivatives were significantly less potent than their 2′ or 4′ counterparts, which are of similar potency. The reduced potency of the 3′ derivatives is more likely to be due to electronic rather than steric effects, as fluorine in particular has very low steric demands.

We next investigated the effects of adding substituents onto the inner ring and found that further increases in potency could be obtained (Table 5). The 2-methyl, 3-trifluoromethyl, and 3-trifluoromethoxy substitutions increased potency 3- to 5 fold, while addition of 2-fluoro, 3-fluoro, or 3-chloro groups had very little effect on the potency and a 3-methoxy group caused a 5-fold reduction in potency (Table 5). These results are not easily explained because trifluoromethyl and chloro groups are often regarded as being interchangeable[22] and have similar electronic, hydrophobic, and steric characteristics.

Simple QSAR models were generated using the ChemDraw8 add-on for Excel. This allows a regression analysis to be performed on a range of physicochemical parameters[25] with respect to the observed activity. For the outer ring, we selected Hansch (π) for the hydrophobic parameter, molar refractivity

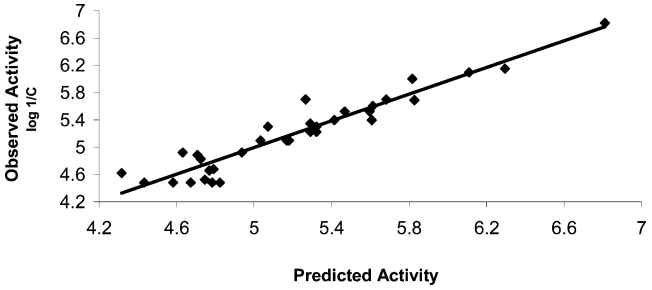


Figure 2. QSAR plot for drug activity in J774 macrophages. The graph shows observed vs predicted activity using linear regression, for a range of ortho and para aromatic substituents on biphenylsulfonamide derivatives. The equation for the regression curve is $0.26\pi^{\text{ring a}} + 0.59F^{\text{ring a}}(\text{o/p}) - 0.027\ \text{MR}^{\text{ring a}} + 3.06\pi^{\text{ring b}} - 3.03\sigma^{\text{ring b}} - 0.23\ \text{MR}^{\text{ring b}} + 4.75$, where π is the hydrophobicity constant, F is the inductive constant, σ is the Hammett constant, and MR is the molar refractivity. $R^2 = 0.91$; $F = 42.4$.

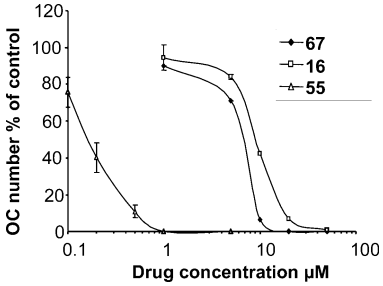


Figure 3. Effects of compounds **16**, **55**, and **67** on osteoclast number. **16**, **55**, and **67** were tested in murine osteoclast cultures. The graph indicates the number of osteoclasts expressed as percentage of the vehicle control group. The graph represents the average of three independent experiments, with $n = 5$ in each experiment.

(MR) as the steric parameter, and the Swain−Lupton inductive constant (F) as the electronic parameter. The last was selected over the more commonly used Hammett constant (σ) because it better explained the high potency of the fluoro derivatives, for which the Hammett value would have given a very small contribution. Conversely for the inner ring, we found that the Hammett constant gave a better correlation. We feel this is justified, as the resonance contribution will have a stronger influence on the sulfonamide moiety with inner ring substituents and therefore is a much more important factor than for outer ring substituents.

Figure 2 shows that there is a very strong correlation between the nature of the ring substituents and the inhibitory effect on macrophage survival. We found a highly significant relationship between an increase in potency and substituents on the outer ring with increasing electron-withdrawing capacity (F) ($p < 0.001$), increasing hydrophobicity (π) of the substituent ($p < 0.0001$), and reduced size (MR) of the substituent ($p < 0.01$). In spite of the smaller number of derivatives studied, we also found a strong relationship between substituents on the inner ring with increased hydrophobicity (π) of the substituent ($p < 0.0001$), reduced electron-withdrawing capacity (σ) of the substituent ($p < 0.0001$), and reduced size (MR) of the substituent ($p < 0.0001$) with an increase in potency. However, we recognize that the values for the inner ring constants are likely to be unreliable because of the small number of derivatives made and variations in literatures values. The main outlier in this model is the 2′-nitro derivative, for which the high potency clearly is not adequately described, even when using $\pi = 0.24$ rather than -0.28.[21−23] As described earlier, this suggests a further enhancement to the electronic properties of the ring. The model suggests a balance between hydrophobic properties, which increase potency, and electron-withdrawing properties, which decrease potency; groups such as trifluoromethyl or trifluoromethoxy may have sufficient hydrophobicity to overcome the electron-withdrawing properties, whereas halogens do not.

QSAR models using these compounds suggested a pattern with potent compounds requiring an electron-donating, hydrophobic substituent. Using the equation generated, we were able to predict a potency of 130 nM for the 3-ethyl derivative **57**; synthesis and testing gave an actual value of 150 nM, demonstrating that the model can be successfully used to design highly potent compounds. Our model suggests that further addition of hydrophobic substituents will further increase potency; however, these derivatives are likely to prove difficult to use in aqueous medium and may break the Lipinsky rules[26] with a ClogP value of more than 5, suggesting they are too lipophilic to be suitable as oral drugs.

Our results suggest a biological target with a lipophilic electron-rich binding pocket, which interacts with the outer ring; a hydrogen bond donating residue, which interacts with one of the sulfonamide oxygens; and a hydrogen bond donor or acceptor, which interacts with the terminal hydroxyl group. The inner ring may also bind to a lipophilic target, but it is uncertain whether the electronic properties are more important for regulating this interaction or for modulating the hydrogen-bonding properties of the sulfonamide. There may also be a binding role for the sulfonamide nitrogen, which may explain the large increase in potency seen in certain derivatives relative to their ester bioisosteres. The lack of significant effect on potency by N-alkylation indicates that the nitrogen is not required as an H-bond donor but may still be an H-bond acceptor. We have not observed a link between biphenyl conformation and potency.

We selected compound **55** from the studies in J774 macrophages as the most promising agent for further investigation and studied its effects on survival of authentic osteoclasts in vitro and ovariectomy induced bone loss in vivo. The effects of **55** on survival of mouse osteoclasts were compared with those of **67** and the unsubstituted sulfonamide **16** (Figure 3). This showed that **55** was about 60 times more potent at inhibiting osteoclast survival than the unmodified sulfonamide derivative **16** and **67**. Thus, **16** had a potency similar to that of the ester **67** (IC_{50} of 8 μM versus 9 μM) whereas the substituted compound, **55**, had an IC_{50} of approximately 150 nM. In addition, all three compounds were considerably more potent in osteoclast cultures than in J774 macrophage cultures. Because NFκB activation is crucial for osteoclast formation and survival but not for macrophages, this supports our original observation[18] that these compounds inhibit RANKL- and TNF-induced NFκB signaling.

Ovariectomy resulted in a 35% loss in trabecular bone volume because of a decrease in trabecular thickness and trabecular number (Figure 4), and **67** (5 (mg/kg)/day) only partially prevented this bone loss. Compound **55**, however, completely prevented the ovariectomy-induced bone loss at the same dose, indicating that the observed increase in potency in vitro also translates into increased in vivo potency.

Conclusion

We have previously shown that **67** represents a novel class of osteoclast inhibitors. However, the potentially labile ester bond present in **67**, its lack of potency, and the limited scope to improve upon this led us to develop more potent and stable bioisosteres of this compound. Our results show that, using

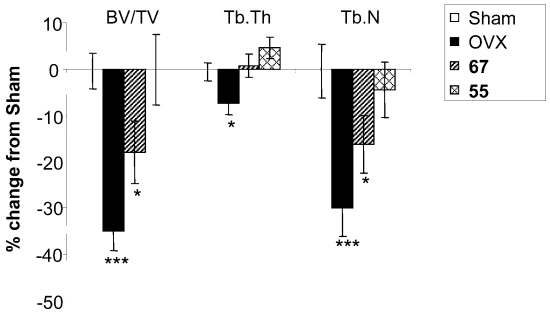


Figure 4. Effects of **67** and compound **55** on ovariectomy-induced bone loss. Both **67** and **55** were administered at 5 (mg/kg)/day by intraperitoneal injection. Data are expressed as percentage change from sham operated control animals ± SEM, $n = 6$: BV/TV, bone volume as percentage of total tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number. Statistical significance of the difference to sham control (ANOVA, Dunnet's post-test) is indicated by asterisks: (∗) $p < 0.05$; (∗∗∗) $p < 0.001$.

QSAR-aided drug design, we have been able to achieve a 100-fold increase in potency of our antiresorptive drugs. Furthermore, one of the optimized compounds completely prevented ovariectomy-induced bone loss in vivo. In conclusion, we have developed a novel class of compounds with sufficient antiresorptive potency to be considered clinical candidates for the treatment of osteoporosis and other bone disorders characterized by increased osteoclast activity.

Experimental Section

General Methods for Synthesis of Compounds. Method A. General Method for Suzuki Coupling. 4-Bromophenylsulfonic acid (4-hydroxybutyl)amide (1 g) was dissolved in a mixture of toluene (8 mL) and ethanol (8 mL). 2,4-Difluorophenylboronic acid (1 g) was added followed by 2 M Na_2CO_3 (8 mL). The mixture was stirred vigorously under N_2, and $(PPh_3)_4Pd$ (0.2 g) was added. The mixture was refluxed with stirring for 3 h under an atmosphere of N_2. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate and washed with water and saturated NaCl solution. After the mixture was dried (Na_2SO_4), the solvent was evaporated and the resultant oil purified by column chromatography to give a white powder or clear oil.

Method B. General Method Sulfonamide Formation. Biphenyl-4-sulfonyl chloride (1 g) and 4-aminobutanol (1 g) were dissolved in dichloromethane (30 mL). Pyridine (1 mL) was added, and the mixture was stirred for 2 h at room temperature. Water (30 mL) was added, and the organic phase was separated. The aqueous portion was washed with ethyl acetate (30 mL), and the organic phases were combined and dried (Na_2SO_4). The solvents were evaporated, and the title compound was obtained as a white solid following trituration with ether or following column chromatography (ethyl acetate/petroleum spirit) if required.

Biphenyl-4-sulfonic acid (4-hydroxybutyl)amide (16) was prepared as a white powder from biphenyl-4-sulfonyl chloride and 4-aminobutanol using method B. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

2′,4′-Difluoro-2-methylbiphenyl-4-sulfonic acid (4-hydroxybutyl)amide (55) was prepared as a white solid from 4-bromo-3-methylphenylsulfonic acid (4-hydroxybutyl)amide **31** and 2,4-difluorophenylboronic acid, using method A. Anal. ($C_{17}H_{19}F_2NO_3S$) C, H, N.

Biphenyl-4-carboxylic acid 4-hydroxybutyl ester (67) was prepared as a white solid from biphenylcarbonyl chloride and 1,4-butanediol. Anal. ($C_{17}H_{18}O_3$) C, H, N.

Alamar Blue Viability Assay. J774 cells or osteoblasts were plated in 96-well plates at 10^4 cells per well in 100 μL of αMEM supplemented with 10% FCS and penicillin and streptomycin and grown overnight. The next day drugs were added to the cultures, and culture was continued for another 48 h. At the end of the culture period cell survival was determined by adding 10 μL of Alamar Blue reagent (Biosource) per well, incubating the cells for a further 3 h, and measuring viability as fluorescence (excitation at 530 nm, emission at 590 nm) using a Labtech FL600 plate fluorimeter. Each compound was tested in at least three independent experiments, with $n = 5$ in each experiment. The IC_{50} was calculated using Graphpad Prism software.

Murine Osteoclast Generation. Bone marrow was flushed out of the femora and tibiae of adult mice using Hank's balanced salt solution (HBSS). The resulting cell suspension was spun down at 300 g for 3 min, resuspended in 10 mL of culture medium (α-MEM supplemented with 10% FCS and penicillin/streptomycin), and cultured in one 10 cm Petri dish per mouse for 3 days at 37 °C in the presence of 100 ng/mL M-CSF. Subsequently, the Petri dish was washed three times with PBS, and the adhered cells were harvested using trypsin digestion. The cells were resuspended in culture medium supplemented with 100 ng/mL RANKL and 25 ng/mL M-CSF, at a density of 4×10^4 cells/mL, and 125 μL per well of this suspension was seeded onto dentine slices in a 96-well plate. The cells were cultured at 37 °C in 5% CO_2 for 7 more days, with medium changes after 2 and 4 days. Compounds to be tested were added during the second medium change and present for 72 h.

Tartrate-Resistant Acid Phosphatase (TRAcP) Staining. Osteoclasts were identified by staining for TRAcP essentially as described by van't Hof et al.[27] Briefly, at the end of the culture period dentine slices with adherent cells were fixed in 4% paraformaldehyde, washed with PBS, and incubated with naphthol−ASBI−phosphate, pararosanilin, and sodium tartrate in acetate buffer (30 mM) at 37 °C for 45 min. TRAcP positive cells with three or more nuclei were considered to be osteoclasts.

Ovariectomy-Induced Bone Loss. Ovariectomy or sham ovariectomy was performed in 9-week old adult female C57/BL6 mice obtained from Harlan Laboratories (U.K.) as previously described.[28] Treatment with compounds was commenced 2 days after ovariectomy or sham ovariectomy by intraperitoneal administration of the drug in corn oil in a dose of 5 (mg/kg)/day. Controls received oil alone. The treatment was continued for 21 days, and the experiment was terminated on day 23. Bone mineral density and content was measured at the right tibial metaphysis by μCT using a Skyscan 1072, at a resolution of 5 μm. The images were reconstructed using the Skyscan ConeRec program and analyzed using Skyscan CTAN software. The measurements were taken from 200 slices directly distal of the growth plate.

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Supporting Information Available: Details of compound synthesis, spectroscopic data (^1H and ^{13}C NMR spectra), mass spectrometry data, and elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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