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17β-HYDROXYWORTMANNIN: A POTENT INHIBITOR OF BONE RESORPTION AND PHOSPHATIDYLINOSITOL–3–KINASE

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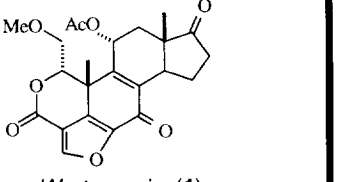
Abstract: Structure–function studies on the natural product wortmannin have identified a 17β-hydroxy derivative as a potent inhibitor of osteoclast function in both cell and animal models. Mechanistic studies indicate osteoclast differentiation is dramatically affected by this class of compounds. Interestingly, comparable potency trends for resorption and phosphatidylinositol-3-kinase inhibition were also observed.

The generalized loss of bone associated with type I osteoporosis is largely due to the decline of ovarian estrogen production in post-menopausal women.[1] In particular, estrogen deficiency increases bone turnover, with bone resorption occurring more rapidly than formation, resulting in a net loss of bone mineral density.[2] Thus, inhibiting the bone resorption activity of osteoclasts represents a cellular target for therapeutic intervention which is best exemplified by the bisphosphonate class of resorption inhibitors.[3] While the beneficial clinical efficacy of bisphosphonates is well established, the relatively long skeletal half-life[3] of this class of compounds prompted us to look for alternative inhibitors of osteoclastic bone resorption. Herein, we communicate our findings on a novel class of extremely potent anti-resorptives based on the natural product wortmannin.

Given the important role of hormones, such as estrogen, in obstructing osteoclastic bone resorption in post-menopausal women, we initiated a screening program to evaluate a diverse number of steroidal frameworks for their anti-resorptive properties. Toward this end, a cell based screen was employed using an avian osteoclast differentiation model.[4] Specifically, osteoclast precursors isolated from the long bones of egg-laying hens maintained on a calcium deficient diet were induced to differentiate into bone resorbing, osteoclast-like cells in the absence and presence of steroid-like compound. Bone resorbing activity was measured by quantitating the tritium release into the media from pre-labeled bone particles co-incubated with the differentiating osteoclast precursors. Of the various compounds examined, the natural product wortmannin (**1**) demonstrated a remarkable ability to inhibit osteoclasts from resorbing bone with a half maximal inhibitory concentration (IC_{50}) of 100 nM. The unique combination of anti-resorptive potency and structural novelty prompted us to investigate structure/function aspects of wortmannin in more detail.

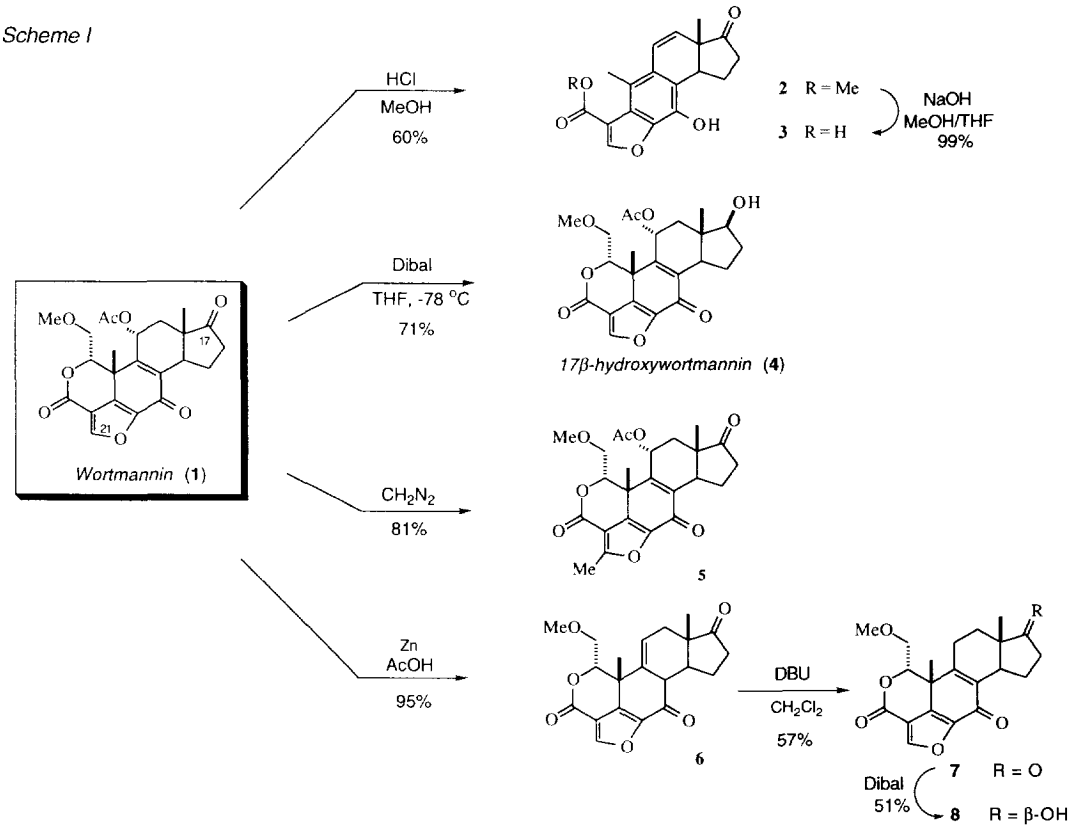


Wortmannin (**1**)

While originally isolated for its anti-fungal characteristics,[5] wortmannin has since demonstrated potent anti-inflammatory properties.[6] Additionally, it has been shown to inhibit superoxide formation, phospholipase D activation,[7] and myosin light chain kinase.[8] More recently, wortmannin has been shown to be a highly potent and selective inhibitor of phosphatidylinositol-3-kinase (PI-3-kinase).[9] Of the various structural motifs present in wortmannin, the furan moiety appears to play a major role in dictating biological activity, particularly in the anti-inflammatory[10] and kinase inhibitory responses.[8,9] In fact, covalent attachment of wortmannin to myosin light chain kinase, via conjugate addition of a native cysteine thiol to the furan ring, has been proposed as one possible inhibitory mode of action.[8,9a]

Given these precedents, we were interested in preserving the integrity of the furan functionality while systematically altering the remainder of the nucleus. Specifically, we felt aromatization of the cyclohexadienone moiety would dramatically lower the susceptibility of the furan moiety to nucleophilic attack[11] without significantly perturbing steric parameters. We were also interested in determining the relative importance of the acetoxy group, as well as the 17-keto functionality. The pioneering synthetic studies on wortmannin provided an

Scheme I



ideal starting point for structural tools to address these issues.[6] Thus, treatment of wortmannin[12] (Scheme I) with acidic methanol (HCl) provided benzofuran **2** in which aromatization of the cyclohexanedienone moiety is accompanied by (a) lactone hydrolysis, (b) loss of methoxyacetaldehyde (presumably via a vinylogous retro-aldol reaction), and (c) elimination of the acetoxy functionality.[13] Saponification of the methyl ester gave a quantitative yield of the corresponding carboxylic acid (**3**). Removal of the C-11 acetoxy group was accomplished via zinc reduction of wortmannin to give alkene **6** which was converted to 11-desacetoxy-wortmannin (**7**) by DBU isomerization. Chemoselective reduction of the 17-keto moiety to the corresponding alcohol proved somewhat problematic. Previous studies by Haefliger and coworkers reported moderate yields of the desired product when diborane was employed as the reducing agent.[6b] In an effort to improve upon this process, a variety of protocols were screened (lithium aluminum hydride, sodium borohydride, lithium triethylborohydride, lithium tri-sec-butylborohydride) all of which resulted in significant over-reduction and/or decomposition. However, we found reaction of **1** with di-isobutylaluminum hydride in THF under strict temperature controlled conditions (-78 °C) selectively reduced the 17-keto functionality in the presence of the lactone, cylohexadienone, acetoxy, and furan moieties. This remarkably chemoselective reaction allowed access to cyclopentanone ring analogs **4** and **8** in good yield. Lastly, point modification of the furan ring was accomplished by reaction of wortmannin with diazomethane to provide 21-methylwortmannin (**5**). This reaction presumably occurs via a 1,3-dipolar cycloaddition to provide a pyrazoline intermediate which subsequently undergoes loss of nitrogen.[14]

With the requisite derivatives in hand, effects on bone resorption activity were determined employing the avian osteoclast differentiation model.[4] Examination of the cellular responses to this series of compounds revealed several distinct trends (Table 1). For example, aromatization of the cyclohexadienone moiety (with concomitant lactone opening) resulted in total lack of inhibition. Removal of the acetoxy group (**7**, IC_{50} = 506 nM) also diminished activity relative to wortmannin as did functionalization of the furan ring (**5**). In contrast, dramatic increases in potency were observed upon reduction of the 17-keto moiety. For example, 17β-hydroxywortmannin (**4**, IC_{50} = 10 nM) was an order of magnitude more potent than wortmannin (IC_{50} = 98 nM). By comparison, two well-established bisphosphonates were also examined in this assay, including etidronate (1-hydroxyethane-1,1-diphosphonic acid, EHDP), and alendronate (4-amino-1-hydroxybutylidene-1,1- bisphosphonic acid, ABP). EHDP inhibited bone resorption activity with IC_{50} = 100 μM, while the potent third generation bisphosphonate, ABP, was three orders of magnitude more potent with IC_{50} = 0.1 μM. Thus, wortmannin and **4** compared favorably with ABP in ability to block the resorption activity of isolated osteoclasts.

Table 1. Effects of wortmannin and derivatives on bone resorption and PI 3-kinase activity

cmpd	*Osteoclast Resorption* IC_{50} (nM)	*PI 3-Kinase* IC_{50} (nM)
3	> 1000	> 32,000
2	>1000	4600
5	>1000	>500
6	–	54
7	506	16.7
8	103	–
1	98	4.2
4	10	0.5
EHDP	100000	>50,000
ABP	100	>50,000

The striking *in vitro* potency of **4** prompted us to examine its bone pharmacology in a well established model for post-menopausal osteoporosis. Specifically, an ovariectomized (OVX) rat assay was used in which the well-documented bone loss associated with ovariectomy can be prevented by anti–resorptives such as estrogen[15] and bisphosphonates.[2] Thus, 6–month old OVX animals were dosed daily (oral) for 5-weeks with **4** or estrogen (Figure 1). Ovariectomy produced a marked osteopenia in the distal metaphysis of the femur which was prevented by ethynyl estradiol (EE_2, 100 μg/kg/day).[15] 17β-hydroxywortmannin (**4**) produced a similar level of protection from bone loss in this assay, although the effect was detected at a considerably lower dose (0.1 μg/kg/day). Interestingly, and despite its steroidal structure, **4** did not produce an estrogen-like effect on the uterus. For example, OVX animals given 100 μg/kg/day EE_2 had a 250 % increase in uterine weight, while animals given **4** showed no significant change in uterine weight as compared to OVX controls. In contrast to wortmannin,[6] toxic effects were not observed in the OVX rat for 17β-hydroxywortmannin.

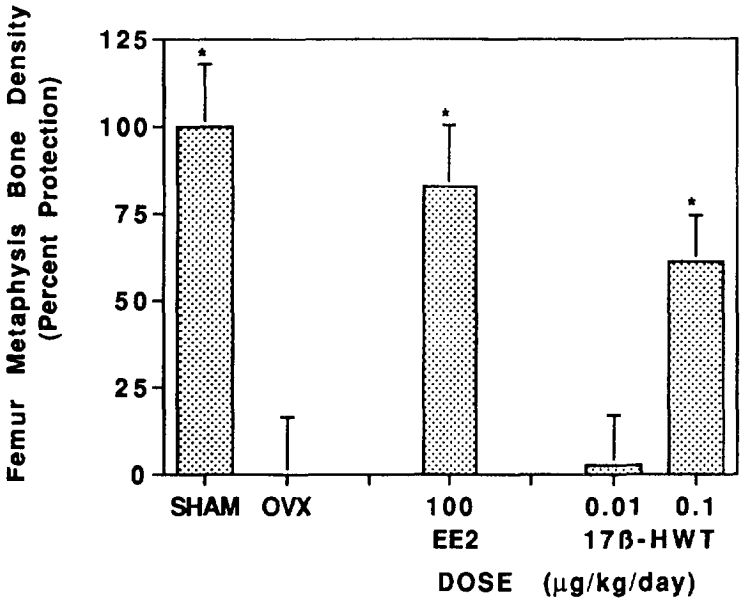


Figure 1. Sparing of ovariectomy induced bone loss by **4** in the OVX rat model. Percent protection was calculated for individual animals by the following formula: % protection = $[(BMD_{test}-BMD_{ovx}/(BMD_{sham}-BMD_{ovx}) \times 100$. Astericks indicate groups significantly distinct from the OVX control at $P \leq 0.05$ with one way anaysis of variance and subsequent Fisher's PLSD range test. .

Owing to the pronounced *in vitro* and *in vivo* effects of 17β-hydroxywortmannin, we became interested in exploring potential mechanisms of action for this class of compounds at the cell and/or molecular level. At the molecular level, reports from our own labs, as well as others, have demonstrated the potent inhibitory effects of wortmannin on PI-3-kinase.[9a] Significantly, this important signaling enzyme has recently been identified in the ruffled border of osteoclasts[16] and inhibitors of PI-3-kinase, such as wortmannin, have been postulated to play a major role in fusion of the ruffled border of osteoclast with apical membrane.[17] Given this data, we were interested in determining if a correlation existed between inhibition of osteoclastic function and PI-3-kinase

activity for wortmannin and its derivatives. Thus, inhibition of PI-3-kinase activity was determined using purified bovine enzyme.[9a] In this assay, 17β-hydroxywortmannin exhibited subnanomolar inhibition (IC_{50} = 0.4 nM) of this enzyme which was followed most closely in potency by wortmannin (IC_{50} = 4.2 nM). Comparison of the inhibition of PI-3-kinase and osteoclast resorption (Table 1) shows the most effective agents at inhibiting PI-3-kinase were also the most effective at inhibiting osteoclastic bone resorption. In addition, overall potency trends in these two activities mirrored each other for all analogs mutually examined. While this observation adds to the growing body of evidence that PI-3-kinase is involved in osteoclast function, further studies are underway to examine this apparent correlation in more detail. Interestingly, the anti-resorptive bisphosphonates EDHP and ABP had no effect on PI-3-kinase.

In summary, a novel class of anti-resorptives based on wortmannin has been identified. Modification of the steroid-like nucleus of this natural product shows that while aromatization of the cyclohexadienone moiety, or alkyl functionality on the furan (C-21), has little effect on osteoclast function, reduction of the 17-keto group dramatically improves inhibition of the bone resorptive process. The presence of the 11-acetoxy group also appears important for optimal activity. In animal models, 17β-hydroxywortmannin proves efficacious in inhibiting the bone loss associated with estrogen deficiency in an OVX rat model. From a mechanistic standpoint, striking similarities in potency for inhibition of resorption activity and PI-3-kinase suggest a role for this enzyme in osteoclast differentiation.

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