Bone Resorption Inhibitors from Hop Extract

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We searched hop extract for active component(s) that inhibited bone resorption in the pit formation assay, and isolated xanthohumol and humulone from hop extract. Especially humulone had extraordinarily strong inhibitory activity and the IC₅₀ (concentration of 50% inhibition) value was 5.9 × 10⁻⁶ M.

Key words: bone resorption inhibitor; hop extract; humulone; xanthohumol

It is believed that the cause of osteoporosis is an imbalance between bone formation and bone resorption.1 In the western countries, estrogen is the first-choice therapeutic drug for postmenopausal osteoporosis. Estrogen derivatives are used in hormone replacement therapy (HRT) to improve 'quality of life' of women.

Some scientific papers suggest that hop (Humulus lupulus L.) extract for beer brewing might contain estrogens. Although no firm conclusion has been drawn as to the estrogenic activity in their results.3 Therefore we speculated that there was a possibility of finding bone resorption inhibitors in hop extracts using the pit formation assay.4 In brief, dentine slices (6 mm diameter, 0.15 mm thickness) were placed in each well of a 96-well culture plate (Falcon, Becton Dickinson and Company, Lincoln Park, NJ). One hundred microliters of 2-MEM (minimum essential medium) containing 5% fetal bovine serum (Flow Laboratories, Scotland) and synthetic rat parathyroid hormone (residues 1–34, Bachem, Inc., CA) were added to each well and each slice was overlaid with 100 μl of 1 × 10⁶ mouse bone cells suspension. The cells were then incubated for 3 days at 37°C in a humidified atmosphere of 10% CO₂ and 90% air. After incubation, the cells on a slice were removed, and resorbed pits were stained with Coomasie brilliant blue. Bone resorption was assessed by counting the number of pits under a light microscope.

To isolate active compounds efficiently in the purification process, two color tests, 1% FeCl₃ and molybdophosphoric acid alkaline reagents,5 were used. These two reagents reacted with the phenolic hydroxyl groups of the active compounds. Silica gel 60 F₃₄₄ (Merck) precoated TLC plates were used and the solvent system was EtOAc: MeOH = 30:1. We checked the bone resorption inhibitory activity in two different kinds of hop extract (SKW East Asia Limited, Tokyo, Japan). One was 'hop paste' and the other was 'hop cake'. We found that the two hop extracts had very strong inhibitory activity and started to isolate the active compounds from the hop extracts.

The alpha acid content (30%) of the hop paste was enriched by CO₂ extraction and beta acid in hop paste was not detected on TLC. The hop paste (20 g) was dissolved in 87% MeOH (200 ml) and washed with hexane (70 ml × 3). The aqueous MeOH layer was concentrated under reduced pressure to an oily residue (6.48 g). From this oily residue, we isolated crude crystals of active compound according to the procedure of Woeffler.6 In brief, Pb(CH₃COO)₃Pb(OH)₂ (8 g) was added to the MeOH solution (50 ml) of the active compound and ether extraction (200 ml × 3) was done. The benzene solution (25 ml) of the residue from ether extraction was added to a benzene solution (10 ml) of o-phenylenediamine. Yellow needles (2.97 g) were obtained from the benzene solution and the needles were resolved in the mixture of 20% HCl (7 ml) and ether (200 ml). After the evaporation of the ether layer, yellow crystals (2 g) were obtained. On repeated recrystallization from 75% AcOH, this afforded humulone (0.76 g) with MW: 362 (C₂₁H₂₉O₂), mp 63°C, [x]₀ = 206° (c 0.38, MeOH), FAB-MS m/z: 361 [M−H]⁻ and 292 [M−C₆H₅−H]−. All the above physico-chemical properties, ¹H-NMR and ¹³C-NMR spectra of humulone were identical to those reported in the literature.³,7,1²

The hop cake (250 g) was also extracted with acetone (2 liter) to give a syrup (50 g) after the removal of the solvent, which inhibited the pit formation. One-third of the extract syrup (17.5 g) was put on an anion exchanger Dowex-1 (X4, 200–400 mesh, CH₃COO⁻ type) column (5 cm diameter X 27.5 cm length).⁵ The three active fractions, 1, 2, and 3, were eluted by step-wise elution with 0.5%, 5%, and 20% acetic acid in 80% methanol, respectively. Each fraction was pooled, separately, and evaporated under reduced pressure, but the beta acid in frac. 1 was unstable in the evaporation process. The dry weight of frac. 3 was 80 mg with MW: 354 (C₂₁H₂₂O₂), mp 167–169°C, and MS (El) m/z: 354 (M⁺). On the basis of all these spectral data including ¹H-NMR and ¹³C-NMR spectra, the structure of the compound was found to be xanthohumol.⁷ A part (42 mg) of frac. 2 was put on a reversed phase column (Chromatorex-ODS DM1020T) with 80% MeOH as the solvent. The most active portion showed a single peak in the HPLC analysis, but its ¹H-NMR and ¹³C-NMR spectra indicated that it was a mixture of two alpha acids, humulone and adhumulone (3:1).⁹ Adhumulone was synthe-

Fig. Structure of Humulone and Xanthohumol.

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sized chemically because it is very difficult and time-consuming to purify and isolate. The bone resorption inhibitory activity of the compound was almost equal to that of humulone in the concentration of $1 \times 10^{-6}$ M. The compound in the other portion was identified as pure cohumulone on the basis of its spectral data. Cohumulone showed almost no inhibitory activity in the pit formation assay.

The results of the inhibitory activity in pit formation assay of the two lead compounds, xanthohumol and humulone, are shown in Table. Xanthohumol inhibited the pit formation in a dose-dependent manner. Significant inhibition was observed at or above the concentration of $10^{-6}$ M (p < 0.01). Xanthohumol showed no cytotoxic or anti-bacterial activity at the concentration $10^{-4}$ to $10^{-6}$ M. The bone resorption inhibitory activity of humulone was very strong. The IC50 value was estimated to be $5.9 \times 10^{-9}$ M. We did not observe cytotoxicity of humulone at the IC50 concentration. This is the first finding that xanthohumol and humulone are strong inhibitors of bone resorption.

The mechanism of the inhibition by xanthohumol is still not clear but xanthohumol is speculated to be a precursor of an estrogenic compound, because demethylxanthohumol is a 'proestrogen' and is metabolized to the active estrogenic compound prenylnaringenin by cyclization in vivo.45 Naringenin, lacking a prenyl group, is also reported to be an agonist to estrogen.122 Another possibility for the mechanism of the action of xanthohumol is that the compound may inhibit interleukin-1 (IL-1) release or biosynthesis because xanthohumol is a chalone derivative.113 IL-1 is a cytokine that causes bone resorption, and the biosynthesis of IL-1 is affected by estrogen.21 Therefore we speculate that xanthohumol may inhibit bone resorption through the inhibition of IL-1 biosynthesis directly or indirectly.

The mechanism of action of humulone is also not clear but some recent papers suggest a possible mechanism of action of humulone. One of the papers says that osteoclasts produce reactive oxygen intermediates (ROI) that are involved in the process of bone resorption.13,14 In this paper, oxygen-radical scavengers such as pyrrolidine dithiocarbamate and N-acetyl cysteine inhibited bone resorption. Humulone is also reported to be an antioxidant reagent in another recent paper.5 Therefore humulone may inhibit bone resorption through its oxygen-radical scavenging of ROI of osteoclasts. On the other hand, the recent papers suggest a close relationship between bone resorption and signal transduction. For example, herbimycin A, a pp60csrc tyrosine kinase inhibitor, inhibited osteoclastic bone resorption,16,17 and wortmannin, a specific phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, inhibited bone resorption.18,19 Humulone may inhibit bone resorption by its inhibitory activity on the signal transduction pathway, like wortmannin.

The structure–activity relationship of humulone derivatives in the pit formation assay is now being studied by the systematic chemical synthesis of various compounds. Because it is time-consuming to obtain pure humulone derivatives in large amounts, including beta acids from hop extracts. In the preliminary experimental result, lupulon, a beta acid, was synthesized and was a strong inhibitor in the pit formation assay. The details of the structure–activity relationship of humulone derivatives including isohumulone derivatives will be published in the near future.

The carcinogenesis of xanthohumol and humulone has not yet been reported so far. We think that xanthohumol and humulone may be candidates for the therapeutic drugs for osteoporosis.

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References