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Inhibitory Effect of *Carthamus tinctorius* L. Seed Extracts on Bone Resorption Mediated by Tyrosine Kinase, COX-2 (cyclooxygenase) and PG (prostaglandin) E_2

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Abstract: Anti-bone resorption properties of the Korean herbal formulation, Honghwain (HHI; *Carthamus tinctorius* L. seed) was biochemically investigated. On processing bone metabolism, PGE_2 accelerated production of IL-1β in fetal mouse osteoblast and stimulated physiological activation substance, IL-1β. The novel class of Src tyrosine kinase inhibitors, Herbimycin A (HERB) and HHI reduced COX-2 mRNA levels as well as PGE_2 production induced by IL-1β, TNF-α and IL-6. HHI inhibited in vitro and in vivo bone resorption by inhibition of phosphorylation of peptide substrates. HHI dose-dependently reduced the hypercalcemia induced in mice by IL-1β and partly prevented bone loss and microarchitectural changes in young ovariectomized rats, showing that the protective effect on bone was exerted via the inhibition of bone resorption. These results indicate that the synergy between IL-β, TNF-α, IL-6 on PGE_2 production is due to an enhanced gene expression of COX-2 and that tyrosine kinase (s) are involved in the signal transduction of COX-2 in mouse calvarial osteoblasts. Thus, HHI as a possible Src family kinase inhibitor may be useful for the treatment of diseases associated with elevated bone loss.

Keywords: *Carthamus tinctorius*; Bone Resorption; Tyrosine Kinase; Cox-2; Prostaglandin E.

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Introduction

Natural products of plant origin are still a major part of traditional medicinal systems in many countries. It is well known that Korean herbal medicine, Honghwain (HHI), *Carthamus tinctorius* L. seed extract, is effective for the treatment of gynecological diseases such as osteoporosis and bone resorption. According to the ancient Chinese and Korean medicinal and herbal literature (Shi, 1983), the HHI has been used in Korea and China as an effective biological response modifier for augmenting host homeostasis of body circulation (Shi, 1983). In addition, the HHI has anti-inflammatory properties (Kim *et al.*, 1999). It is used in the treatment of Yin deficiency of liver and kidney, hectic fever, night sweat and dizziness (Hong, 1998). Thus, it still occupies an important place in traditional oriental medicine.

There is also a resurgence of interest in herbal medicines in Western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis (Phillipson and Anderson, 1989). A literature survey on the plant, *Carthamus tinctorius* L. seed, revealed that there is no scientific evidence of its usefulness in the treatment of rheumatoid arthritis and osteoporosis. The need for safer and effective anti-inflammatory drugs and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

The cytokine interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), which are produced mainly by activated monocytes or macrophages, stimulate bone resorption as well as enhance PGE_2 production in several type of cells including calvarial osteoblasts (Canalis and Rydziel, 1996). The IL-6, a potent mitogenic polypeptide, stimulates cell proliferation in a various type of cells (Stein and Sutherland, 1998; Mundyln, 1995). Most conditions that lead to osteoporosis (estrogen deficiency in post-menopausal women, hyperparathyroidism, hyperthyroidism and corticosteroid treatment) are associated with increased bone resorption, which is determined by the number and activity of bone-resorbing cells or osteoclasts (Rodan *et al*., 1996; Roodman, 1996; Baron *et al*., 1993).

Numerous studies have provided compelling evidence that the protein tyrosine kinase Src plays a unique and crucial role in osteoclastic function. The original report from Soriano *et al.* demonstrated that mutant mice with a disrupted *src* gene develop osteopetrosis, a disease characterized by a lack of osteoclastic activity. These Src-deficient mice had normal numbers of osteoclasts, but failed to polarize and form ruffled borders, indicating that Src is not required for osteoclast formation but, rather, for their function (Soriano *et al*., 1991; Boyce, 1992; Lowe *et al*., 1992). These findings are consistent with the high expression of Src in osteoclasts, comparable to that in brain and platelets (Lowe *et al*., 1992; Horne *et al*., 1992; Tanaka *et al*., 1992; Thomas and Brugge, 1997). Therefore, we assessed the activity of the potent non-specific Src inhibitor HERB (Herbimycin A) and HHI on bone resorption *in vitro*, as well as in animal models of hypercalcemia and osteoporosis.

Materials and Methods

Materials

HHI (Honghwain), *Carthamus tinctorius* L. seed's aqueous extract was mass-produced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University

College of Oriental Medicine (Kyungju, Korea). A total of 42 g of *Carthamus tinctorius* L. seed was added to 500 ml of distilled water and boiled for 2 hours, filtered and then concentrated to 10 ml. The extracts were lyophilized and aliquots (50 mg) were separately stored at –20°C for next experiments

Radiochemicals were from Amersham International Co. (Seoul, Korea). All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Seoul, Korea).

Human recombinant IL-1β was our deposit (Park *et al.*, 1999). TNF-α and IL-6 were purchased from R&D Systems (Funakoshi, Co., Ltd., Tokyo, Japan). Herbimycin A was from Sigma Chemical Co. (St. Louis, MO, USA). Radioimmunoassay kits for PGE_2 were from Ammersham. It was stored in aliquots of 100 mg dissolved in 91 mL of 100 mmol/L Tris buffer (pH 7.8) containing 2 mmol/L sodium azide, and diluted with phosphate-buffered saline (PBS) prior to use.

Methods

Calvarial Osteoblast Cultures

Cultures of osteoblast cells were established from calvaria obtained from healthy 10 two-month-old mice. Minced pieces of the tissue were explanted to 25 cm^2 Falcon tissue culture flasks containing 5 ml of Eagle's basal medium (BME). The osteoblasts were obtained by trypsinisation of the primary outgrowth of cells as previously described (Kuroki *et al.*, 1992). Osteoblasts were seeded and grown in BME supplemented with 5% fetal calf serum, L-glutamine, penicillin-streptomycin and HEPS for 24 hours. The cell layers were then rinsed three times with serum-free BME medium and incubated in the absence or presence of IL-1β, TNF-α, IL-6 and HHI alone or in combinations at the concentrations indicated in the legends to figures and tables. At the end of the incubation period, indicated in the legends to figures and tables, the monolayer was frozen for isolation of total RNA. The cells used for the experiments proliferated in the logarithmic phase between the 6th and the 12th passage.

The cultures of C3H10T1/2 cells [supplied from American Culture Type Collection (ACTC), NIH, MD, USA] reached confluency after 3 days and were subsequently deprived of growth factors by reducing the serum concentration in the medium to 0.2% for 1 day (this treatment is subsequently referred to as serum starvation). MC3T3-E1 cells were maintained as previously described (Ua *et al.*, 1997). The test compounds were added for the indicated time periods during serum starvation. A 2-hour treatment with compound was chosen for routine analysis. They were dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L and diluted in starvation medium. The final DMSO concentration did not exceed 0.1% and did not affect tyrosine phosphorylation.

Quantification of mRNA for COX-1 and COX-2, and Prostaglandin E_2 Determination

The cells were scraped in 2 ml of 10 mM EDTA (pH 8.0), 0.5% SDS and RNA was isolated according to Sambrook *et al.* (1988). The levels of mRNA for COX-1 and COX-2 were

determined by southern hybridization which was carried out by using RNA probes, synthesized *in vitro* using a RNA transcription kit, and labeled with 35S-rCTP (Songyang *et al*., 1995). The amount of PGE_2 was determined in the medium by using commercially available radioimmunoassay kits with [^{125}I]- PGE_2 antiserum. The PGE_2 antiserum has 30% cross-reactivity with in PGE_2.

Protein Kinase and Cellular Src Assays

The cells were maintained and deprived of growth factors as described above. Test compounds were added to the cells for 2 hours, followed by inspection of cell morphology and cell extraction as described above. Equal amounts of protein (15 µg) were resolved by SDS-PAGE, and protein phosphorylation was detected by antiphosphotyrosine Western blotting.

^{32}P-based tyrosine kinase assays with human Src enzyme [Upstate Biotechnology, Inc. (UBI) Lake Placid, NY] were performed with a kinase buffer containing 20 mmol/L Tris, pH 7.4, 10 mmol/L $MgCl_2$, 0.1 mmol/L sodium vanadate, and 1 mmol/L dithiothreitol (DTT), substrates poly Glu-Tyr (4:1), or optimal Src substrate (OSS) (Songyang *et al*., 1995) peptide at 1 and 0.5 mg/mL; 50 µmol/L adenosine triphosphate (ATP), and 0.5–2 uCi of ^{33}P-rATP per assay. Incubations were performed for 15 minutes at room temperature. The reactions were stopped with 33 mmol/L ethylenediaminetetraacetic acid (EDTA) and spotted onto phosphocellulose paper squares (Whatman P81, Maidstone, England). The paper squares were washed three times with 0.5% phosphoric acid, once with absolute ethanol, and air-dried, and the paper-bound radioactivity was quantified in a liquid scintillation counter. Chicken Src was expressed and purified as described before (Park *et al*., 1999a); the assay was similar to that with human Src except that 6 µmol/L ATP was used for routine screening, and the phosphorylated poly Glu-Tyr was retained on an Immobilon-P membrane (Millipore, Volketswil, Switzerland).

Cell Viability Assessment

MC3T3-E1 cells were seeded in six-well plates at 6×10^4 cells/well and grown to confluency for 3 days. Cells in duplicate wells were deprived of serum for 1 day and subsequently treated with increasing concentrations of DPP or corresponding concentrations of DMSO for 3 days. The cells were examined under the microscope every day and photographs were taken. After 3 days, cells were trypsinized, stained with Trypan blue, and counted with a hemocytometer.

Cell Extraction

Cells in a six-well plate were washed twice with PBS (without calcium and magnesium) and lysed in 250 µL of Nonidet P-40 (NP-40) lysis buffer (25 mmol/L Tris, pH 7.4, 10% glycerol, 1% NP-40, 50 mmol/L NaF, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10 µg/mL aprotinin, leupeptin and pepstatin

A). Lysis was performed on ice for 15–30 minutes. The lysates were collected and clarified by centrifugation at 14,000 rpm for 5 minutes at 4°C in a microcentrifuge. The protein concentration was determined in the extracts (Bradford micro method; BioRad, Glattbrugg, Switzerland) and the samples were prepared for SDS-PAGE analysis by mixing with 1/6 volume of 6× concentrated SDS sample buffer and incubating for 4 minutes at 95°C.

IC_{50} Calculation

The IC_{50} values were determined graphically as the concentration of inhibitor which reduced the signal of untreated control cells by 50%. The exposures that had an experimental 50% control value (equals the signal of one-half of the protein analyzed) approaching the calculated 50% signal (equals calculated half of the control value) were taken as being in the linear range for the enhanced chemiluminescence (ECL) reaction and X-ray film response.

In Vitro Bone Resorption Assay

Fetal rat long bones were prepared and cultured as described by Park *et al.* (1999b). In brief, timed pregnant Sprague-Dawley rats were injected with radiolabeled ^{45}Ca subcutaneously (s.c.) (100 µCi) on the 18th day of gestation. The following day, radii and ulnae were dissected and then pre-cultured in 0.5 mL of BGJ medium supplemented with 1 mg/mL of bovine serum albumin (BSA) in 24-well tissue culture plates in a CO_2 incubator at 37°C for 24 hours. The bone explants were then cultured in the presence or absence of the agents to be tested for 2 days. The medium was removed and replaced with fresh medium supplemented with the test agents, and culture was continued for another 3 days before terminating the experiment. Aliquots of conditioned medium of days 2 and 5 and the acid extract [trichloroacetic acid (TCA), 5% (w/v)] of the bone explants were counted for ^{45}Ca by liquid scintillation. Bone resorption was assessed as the percentage of total ^{45}Ca that was released into the medium.

Animals for *In Vivo* Experiments and IL-1β-Induced Hypercalcemia in Mice

Male mice (Tif: MAGf; Novartis Animal Farm) of 25–30 g body weight were divided into five groups of eight and fed a standard maintenance diet containing 0.75% calcium. Eight-week-old (175–209 g) female rats of the Sprague-Dawley-derived strain Tif:RAlf (Novartis Animal Farm) were divided into groups of eight and fed *ad libitum* a breeding diet containing 1.2% calcium. All animals were kept at a temperature of 24°C with a 12:12 hours light/dark cycle. The method of Sabatini *et al*. (1988), and Serban and Rodorf-Adam (1986) was used to induce hypercalcemia in mice.

Bone Loss in Ovariectomized Rats

Four groups of eight rats and mouse were ovariectomized (ovx) by electrotomy via the

dorsal route in anesthesia. One additional group of rats served as the intact control group. Immediately after ovariectomy, two ovx groups were given 10 and 50 mg/kg of HHI by oral gavage twice daily on weekdays and once a day at weekends for 6 weeks. The intact control group and one ovx control group were similarly treated with 0.9% (w/v) saline. At the end of the experiment, the animals were killed and the uterus was dissected out and weighed to confirm that ovariectomy had been successful (data not shown). The first lumbar vertebra (LV-1) and both femurs were removed for biochemical and morphometric analyses. Calcium and hydroxyproline (HP) in femoral trabecular bone were determined by chemical analysis according to the method of Gunness-Hey and Hock (1984). Briefly, the femurs were cut in half at the middle of diaphysis and the proximal halves were discarded. The epiphysis of the distal half was cut off and the bone was split into saggital halves, then the marrow was flushed out with water. With a dental curette, the trabecular bone was scraped out of both cortical shells, combined and put into 5% trichloroacetic acid (TCA). After standing 16 hours at room temperature, the TCA extract was removed and its calcium content determined colorimetrically with *o*-cresolphthalein complexone. The remaining demineralized matrix was successively washed with ethanol and methylenechloride and dried under vacuum prior to determining its hydroxyproline content. The dried matrix was hydrolyzed with 6 mol/L HCl at 120°C for 5 hours and the hydroxyproline content was determined colorimetrically (Jamall *et al*., 1981).

Statistics Analysis

Results of the above animal studies are given as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) and by Tukey's multiple comparison test against the control group.

Results

Inhibitory Activity of HHI on COX-2 Expression and PGE_2 Production Mediated by Cytokines (TNF-α, IL-1β and IL-6) Alone or in Combination

The cytokine IL-1β and to a lesser extent IL-6 (≥10 ng/ml), stimulated PGE_2 production in osteoblasts. Furthermore, the increase of COX-2 mRNA levels stimulated by the combination of IL-1β and IL-6 were accompanied by a synergistic increase of PGE_2 production which was dependent on the concentration of IL-6. HHI treatment with 100 ng/ml of IL-6 in the presence or absence of IL-1β and TNF-α showed the significant inhibition of PGE_2 production which was dependent on the concentration of IL-6. Neither IL-1β, TNF-α, and IL-6 not the combination of the three cytokines increased COX-1 mRNA levels in mouse osteoblasts (Table 1). Furthermore, when TNF-α or IL-6 in 6 hours of cultures on COX-1 and COX-2 mRNA were assayed in osteoblasts, in the group in which HHI was present, the COX-2 levels were significantly decreased compared to the group in which HHI was absent.

Time Course Effect of HHI on IL-1β, TNF-α and/or IL-6 and Combination-Induced COX-2 mRNA Level.

The COX-2 mRNA levels induced by IL-1β, TNF-α or IL-6 or the combination reached a maximum within 6 hours of stimulation and thereafter declined in 12-hour cultures (Table 2) When time course effect of IL-1β (10 ng/ml), TNF-α (10 U/ml), IL-6 (10 ng/ml) and the combination with HHI were examined on expression of COX-2 mRNA level in mouse osteoblasts, combination with HHI significantly decreased the expression of COX-2 mRNA levels ($p < 0.01$).

Table 1. Effect of IL-1β, TNF-α or IL-6 in the Absence or Presence of HHI (100 µg/ml) in 6 Hours of Cultures, on COX-1 and COX-2 mRNA Level in Osteoblasts (n = 6)

Treatment	COX-1 cpm/10 µg RNA		COX-2 cpm/10 µg RNA	
	–	+ HHI	–	+ HHI
Control	858 ± 79	865 ± 87	380 ± 43	383 ± 34
IL-1β	922 ± 98	911 ± 98	930 ± 87	632 ± 65
TNF-α	1012 ± 103	998 ± 98	780 ± 90	455 ± 45*
IL-6	979 ± 98	943 ± 76	840 ± 85	365 ± 34 +
IL-1β + IL-6	900 ± 87	954 ± 8	955 ± 54	476 ± 45*
TNF-α + IL-6	1025 ± 123	999 ± 1	899 ± 65	366 ± 35 +

*$p < 0.05$; **+$p < 0.01$ against the control group without HHI; n = 6.

Table 2. Time Course Effect of IL-1β (10 ng/ml), TNF-α (10 U/ml), IL-6 (10 ng/ml) and the Combination with HHI on Expression of COX-2 mRNA Level in Mouse Calvarial Osteoblast

Treatment	Time (Hours)			
	2	4	6	12
Control	120	230	330	360
IL-1β	387	530	880	890
TNF-α	320	480	760	732
IL-6	365	470	840	720
IL-1β + HHI	187*	280*	434*	460*
TNF-α + HHI	197*	240*	355*	410*

Mean ± SD of triplicate experiments representing one of three separate experiments with similar results.
*Significantly different from untreated control cells ($p < 0.01$); (n = 6).

Comparative Effect of HHI and Tyrosine Kinase Inhibitor on COX-2 mRNA Expression and PGE_2 Production in Mouse Calvarial Osteoblasts

To compare the biological activity of HHI, the effects of tyrosine kinase inhibitors, herbimycin A (HERB), on COX-2 mRNA level and PGE_2 production was also investigated (Table 3). The specific tyrosine kinase inhibitor, HERB and HHI strongly reduced COX-2 mRNA levels induced by IL-1β, TNF-α or IL-6 or the combination of them. However, HERB and HHI did not affect COX-1 mRNA level induced by the cytokines (data not

shown). Also, the specific tyrosine kinase inhibitor, HERB, did not affect COX-1 mRNA level in osteoblasts. On effect of HHI on HERB treatment, PGE_2 production induced by TNF-α, IL-6, IL-1β + IL-6 and TNF-α + IL-6 was significantly decreased compared to the group in which HHI was absent or only HERB treatment. These indicate that HHI plays a role in inhibiting tyrosine kinase activities of the bone resorbing cells.

In Vitro Activity of HERB and HHI on Src Inhibition by Means of IC_{50} Value

HERB has been identified as potent inhibitors of Src in enzymatic assays. The finding that HERB exhibit potent tyrosine kinase inhibition is novel. Table 4 shows *in vitro* activity of HERB. The HERB compound inhibited chicken Src with an IC_{50} value of 0.1 µmol/L, was selective against the serine/threonine kinase Cdc2. In conclusion, HERB increased the potency of Src inhibition *in vitro* and chose HERB for further characterization *in vitro* and *in vivo*. Furthermore, inhibitory IC_{50} values of HHI (µg/ml for HHI) were calculated for the inhibition of purified protein kinase activity in enzymatic assays. When chicken recombinant Src enzyme was used in these assays, HHI inhibited chicken Src with IC_{50} value of 16 µg/ml.

Table 3. Effect of IL-1β, TNF-α or IL-6 in the Absence or Presence of HERB (1.0 µM) and HHI in 6 Hours of Cultures, on PGE_2 Production in Osteoblasts (n = 6)

	PGE_2 Production (pg/10^2 cells)		
Treatment	**–**	**+ HERB**	**+ HERB + HHI**
Control	1.8 ± 0.1	1.9 ± 0.2	1.5 ± 0.4
L-1β	13.3 ± 1.9	10.1 ± 0.9	9.2 ± 0.6
TNF-α	12.2 ± 1.8	7.8 ± 0.8	7.0 ± 0.5*
IL-6	12.0 ± 1.7	8.4 ± 0.8	7.3 ± 0.6*
IL-1β + IL-6	16.9 ± 1.3	7.0 ± 1.3	7.7 ± 0.8*
TNF-α + IL-6	15.5 ± 1.4	10.4 ± 1.6	8.2 ± 1.1*

*$p < 0.05$ against the control group without HHI.

Table 4. Potency of Inhibitor, HERB and HHI Toward Protein Kinase *In Vitro*

	Herbimycin A (HERB)		HHI	
	Src of Enzyme (µM)	**Cellular Src (µM)**	**Src of Enzyme (µg/ml)**	**Cellular Src (µg/ml)**
IC_{50} value (µM or µg/ml)	0.2	2.3	16.8	33.4

IC_{50} values (µmol/L for HERB or µg/ml for HHI) were calculated for the inhibition of purified protein kinase activity in enzymatic assays. Chicken recombinant Src enzyme was used in these assays. For a description of enzymes and assays, see the "Materials and Methods" section.

HHI Inhibits Human Src Enzyme In Vitro

We used chicken Src and poly-Glu-Tyr as a substrate. We tested whether HHI inhibits the same potency toward human Src using a more relevant Src-specific substrate, the OSS peptide (Rueggsegger *et al.*, 1996). HHI dose-dependently inhibited phosphorylation of poly-Glu-Tyr with an IC_{50} value of 5.5 nmol/L, and of the OSS (optimal Src substrate)

peptide with an IC_{50} value of 12.2 µg/ml (Table 5). Thus, HHI potently inhibits human Src independently of the type of the peptide substrate.

Table 5. HHI Inhibits Peptide Phosphorylation by Recombinant Human Src

	HHI Concentration (µg/ml)				
	0.1	**1**	**1**	**50**	**100**
OSS Peptide [IC^{50} = (12.2 µg/ml)]	3	34	70	79	85
Poly Glu-Tyr [IC^{50} = (5.5 µg/ml)]	3	24	48	72	93

The kinase activity of human Src (UBI) was measured with the peptide substrates poly-Glu-Tyr and OSS peptide using the phosphocellulose paper method as described in the "Materials and Methods" section. The results are presented as a mean of two experiments performed in duplicate.

Concentration of HHI Which Half-Maximally Inhibits Src is Not Cytotoxic

It is important to note that HHI did not inhibit cellular protein phosphorylation via a cytotoxic effect. Compound concentrations much higher than the IC_{50} values for the inhibition of phosphorylation did not influence cell morphology, did not damage the cell monolayer, and did not influence cell viability for up to 3 days of treatment (Table 6). Confluent, serum-deprived cultures of MC3T3-E1 cells in six-well plates were treated with HHI or with corresponding concentrations of DMSO for 3 days. In comparison to day 0, the cell number did not change (Table 6).

Table 6. Viability of MC3T3-E1 Cells After Treatment with HHI for 3 Days

DMSO (%)	Cell No. ($X10^6$)	%	HHI (µg/ml)	Cell No. ($X10^6$)	%
0	2.3 ± 0.01	100	0	2.3 ± 0.21	100
0.01	2.1 ± 0.06	99	50	2.4 ± 0.20	99
0.1	2.0 ± 0.12	96	100	2.2 ± 0.21	97

Confluent, serum-deprived cultures of MC3T3-E1 cells in six-well plates were treated with HHI or with correspnding concentrations of DMSO for 3 days. In comparison to day 0, the cell number did not change. Cells were observed every day and no morphological changes or damage to the cell monolayer were noted. After 3 days, the triplicate cultures for each treatment were trypsinized, stained with Trypan blue, and counted. The number of viable cells is expressed as mean ± standard deviation and as a percentage of untreated cultures. Viability under all conditions was about 95%.

HHI Inhibit PTH (Parathyroid Hormone) Induced Bone Resorption In Vitro

The effect of HHI as the possible candidate of Src inhibitor on bone resorption was evaluated *in vitro* in the fetal rat long bone organ culture system described previously (Park *et al.*, 1999c). Fetal rat long bones were cultured in the presence of 10 nmol/L human PTH- (1-34) in the presence or absence of HHI over the concentration range 0.1–100 µg/ml. Bone resorption was assessed as the percentage release of ^{45}Ca into the culture medium at day 5 of culture. In this system, bone resorption is stimulated with PTH and measured by the release of ^{45}Ca into the medium from fetal long bones pre-labeled with ^{45}Ca. As the results in Table 7. HHI inhibited the PTH-stimulated release of ^{45}Ca in a concentration-dependent manner with an apparent IC_{50} value of 17 µg/ml. This value is similar to that obtained for

the inhibition of bone resorption with isolated osteoclasts (IC_{50} value of 23 µg/ml in our preliminary data). This potency of inhibition of bone resorption was in the range of inhibition of Src activity in cells (17–34 µg/ml), suggesting that the observed inhibition of bone resorption may be mediated via Src inhibition. We could also show that HHI did not affect signaling by several growth factors in osteoblastic cells. In addition, the HHI did not affect the activity of several serine/threonine protein kinases *in vitro* (data not shown). Thus, HHI was not expected to inhibit the serine/threonine-specific cAMP-dependent protein kinase, a major mediator of PTH action in osteoblasts (Parsons and Parsons, 1997). Furthermore, Src is not required for PTH-mediated adenylate cyclase response in osteoblastic cells (Izbicka *et al*., 1994). Thus, it is likely that the inhibitory effect of HHI in this organ culture system is mediated by Src in osteoclasts.

Table 7. HHI Inhibits Bone Resorption in the Fetal Long Bone Organ Culture System

	HHI Concentration (µg/ml)				
	0.1	**1**	**10**	**50**	**100**
^{45}Calcium Release	65	53	34	24	21*

Fetal rat long bones were cultured in the presence of 10 nmol/L human PTH- (1–34) in the presence or absence of HHI over the concentration range 0.1–100 µg/ml. Bone resorption was assessed as the percentage release of ^{45}Ca into the culture medium at day 5 of culture. Four experiments are done and the results from a representative experiment are shown as percent release over control for six long bones per group (mean ± SEM, n = 6). * Significantly different from untreated control cells ($p < 0.01$).

HHI Partly Prevent Bone Loss and Protect Bone Microarchitectural Features in Young Ovx Rats

The ovx rat is often used as a model of post-menopausal osteoporosis (Uy *et al.,* 1995) Whether HHI partly prevents bone loss in young ovariectomized (ovx) rats, the effect of HHI on bone loss in young ovx rats was measured by the trabecular calcium and hydroxyproline content of the distal femur. HHI was administered twice a day at doses of 10 and 50 mg/kg orally for 6 weeks, beginning immediately after ovariectomy. We used 8-week-old female rats to assess the effect of orally administered HHI on the ovariectomy-induced bone loss. Bone mass was measured by determining the amount of calcium and hydroxyproline in the trabeculae of the distal femur (Table 8). In comparison to the saline-treated intact controls, ovariectomy produced a mean reduction of –63% ($p < 0.05$) in the calcium and hydroxyproline content of the trabeculae of the distal femur. This effect was partly prevented by treatment with the Src inhibitor HHI administered orally. At the highest dose of compound, the loss of calcium and hydroxyproline was only –39%, representing a 37% reduction ($p < 0.05$) in the bone loss induced by ovariectomy.

Together, these data show that we identified an Src inhibitor which is potent and specific in enzymatic and cellular assays, and which can inhibit bone resorption *in vitro* and *in vivo*.

Table 8. HHI Partly Prevents Pone Loss in Young Ovariectomized (ovx) Rats

	Control (PBS)	Control (OVX)	OVX + 10 mg/kg	OVX + 50 mg/kg
Trabecular Calcium (mg/femur)	0.86	0.25	0.35	0.48
Trabecular HP (ug/femur)	19	3.9	7.6*	12.6*

The effect of HHI on bone loss in young ovx rats was measured by the trabecular calcium and hydroxyproline (HP) content of the distal femur. HHI was administered twice a day at doses of 10 and 50 mg/kg orally for 6 weeks, beginning immediately after ovariectomy. *$p < 0.05$ against the ovx control.

Discussion

The involvement of COX-1, COX-2 and tyrosine kinases, on the synergy between TGF and IL-1β on PGE_2 production were shown in human gingival fibroblasts (Yucel-Lindberg *et al*., 1999). TGF-β strongly suppressed the activities of the cytokines IL-1β, TNF-α and IL-6 actions on stimulation of COX-2 mRNA expression and PGE_2 production in osteoblasts. It has been reported that TGF-β inhibit prostaglandin production (data not shown). Here we show that IL-6 induces the expression of COX-2 mRNA as well as potentiates the stimulatory effect of IL-1β and TNF-α on the expression of COX-2 mRNA, which further indicates that the enzyme COX-2 is involved in the action of IL-6 as well as in the synergy between IL-6 and IL-1β or TNF-α on PGE_2 production. COX-1 mRNA levels were not affected by the cytokines, neither alone nor in combination.

In this study, we used the HHI to investigate the involvement of tyrosine kinases in synergy between IL-1β or TNF-α and IL-6 in mouse osteoblasts. The HHI reduced COX-2 mRNA level and PGE_2 production induced by the combination of IL-1β or TNF-α and IL-6. Development of small molecular weight inhibitors of protein tyrosine kinases may be useful for the treatment of a number of diseases, but so far it has been mostly exploited in the field of oncology (Klohs *et al*., 1997). As knowledge on the specific roles of protein kinases in certain tissues increases, possibilities will arise to develop protein kinase inhibitors for the therapy of many other diseases in addition to cancer. Our data show that the selective Src inhibitor HHI inhibits bone resorption in the fetal long bone system. Obviously, it is hard to totally exclude that inhibition of another protein kinase does not contribute to inhibition of bone resorption. In particular, we do not expect broad selectivity against some other Src family members such as Hck, which may also be involved in regulating bone resorption (Boyce *et al*., 1992; Stein *et al*., 1994).

Based on the inhibition of IL-1-induced hypercalcemia with HHI, it was expected that the ovariectomy-induced bone loss would be affected as well, but it was possible that the effect on bone mass could be obscured by unfavorable pharmacokinetics or toxic effects of the compound. We have shown that the loss of the trabecular bone is partly prevented by treatment with the Src inhibitor HERB. It was clearly indicated that HHI extract and HERB treatments were synergistic in inhibiting the Src activity. The synergistic activity was also dose-dependent in *in vivo* and *in vitro* effects. Toxicity of HHI did not seem to contribute to the positive effects on bone, since the animals survived the 6 weeks treatment with relatively

high doses without significant changes in body weight (data not shown). As suggested, the works were also performed using 6-week-old rats, however, the significantly different results were obtained more certainly in case of 8-week-old rats. Therefore, we used 8-week-old female rats to assess the effect of orally administered HHI. In toxicity studies, in ranges of 1 to 300 mg/kg doses, up to 100 mg/kg doses were non-toxic, indicating that the reason why the 10 and 50 mg/kg doses were used.

Plants used in folk medicine have been accepted as one of the main sources of drug discovery and development. In Korea, there is a rich treasury of ethnobotanical knowledge, and over the past decade, we have been widely engaged in the research on this subject. Natural products of plant origin are still a major part of traditional medicinal systems in oriental countries. There is also a resurgence of interest in herbal medicines in Western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis (Phillipson and Anderson, 1989). A literature survey on the plant, *Carthamus tinctorius* L. seed, revealed that there is no scientific evidence of its usefulness in the treatment of rheumatoid arthritis and osteoporosis. The need for safer and effective anti-inflammatory drugs and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

Conclusion

Anti-bone resorption properties of the Korean herbal formulation, Honghwain (HHI; *Carthamus tinctorius* L. seed) was investigated. TGF-β, herbal medicine and their combinations of HHI + TGF-β reduced the COX-2 mRNA level, PGE_2 biosynthesis and bone resorption. The novel class of Src tyrosine kinase inhibitors, Herbimycin A (HERB) and HHI reduced COX-2 mRNA levels as well as PGE_2 production induced by IL-1β, TNF-α and IL-6. HHI inhibited *in vitro* and *in vivo* bone resorption by inhibition of phosphorylation of peptide substrates. HHI dose-dependently reduced the hypercalcemia induced in mice by IL-1β and partly prevented bone loss and microarchitectural changes in young ovariectomized rats, showing that the protective effect on bone was exerted via the inhibition of bone resorption. These results indicate that the synergy between IL-1β, TNF-α, IL-6 on PGE_2 production is due to an enhanced gene expression of COX-2 and that tyrosine kinase (s) are involved in the signal transduction of COX-2 in mouse calvarial osteoblasts. Thus, HHI as a possible Src family kinase inhibitor may be useful for the treatment of diseases associated with elevated bone loss.

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