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원저

Inhibitor activity of *Ulmus davidiana Planch*(UD) Herbal Acupuncture Solution on Cathepsin having bone resorption activity

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Abstract

유근피 약침액이 골재흡수 중 Cathepsin에 대한 억제 작용

김근삼 · 조현석 · 황민섭 · 김갑성 · 이승덕

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유근피[Ulmus davidiana Planch (UD)]는 전통적으로 항 염증질환의 효과와 손상된 피부를 보호해주는 기능, 그리고 또 다른 기능 중 골과 관련한 기능을 지닌것으로 알려져 왔다. 그러므로 한의학에서는 골다공증을 치료하기 위해서 유근피를 사용할 수 있다. 이를 증명하기위해 한의학에서는 유근피 약침액이 실험용 쥐들의 골수 손상증상에 어떤 영향을 미치는지 조사하였다. 유근피의 ethanol 추출 약침액(EE-UD)과 수욕 약침액(WE-UD)은 cathepsin K 와 L의 우수한 억제물로 밝혀졌다. WE-UD는 IC50 수치가 5.32 ㎍/ml일때 cathepsin K를 억제하였고 6.34 ㎍/ml일때 cathepsin L을 억제하였다. 그러나 EE-UD는 cathepsin K와 L을 1.45 ㎍/ml와 2.43 ㎍/ml 수준에서 억제 활동을 보여 WE-UD보다 많은 유의성을 보였다. EE-UD는 0.8 ㎍/ml의 Ki 수치로 cathepsin K에 대하여 우수한 억제물임을 관찰할 수 있었다. 이러한 활동은 분석실험에서도 pH 7.0의 glutathione와 같이 작용하였을때 10배로 늘어났다. 또한 이는 GSH thiolate 음이온의 조합을 지원하므로서 이러한 유효성의 증가는 아마도 효소의 활동 장소로 향한 약침액 배합들의 향상된 화학 작용으로 인한 것으로 사료되었다. WE-UD는 시간 의존적 억제성을 보임으로서 실험과정 중에 불변의 cathepsin K의 분열과 합성 속도를 알 수 있게 해주었다. 마지막으로 EE-UD는 실험용 쥐의 파골세포와 설치류의 골이 관련된 실험에서 골 재흡수성을 억제함이 입증되었다. WE-UD는 cathepsin K 와 L, 그리고 골의 collagen에서의 단백질 분해를 억제하는 작용이 있음

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을 증명하였다. 이와 같은 결과들은 cathepsin K로 인하여 유발된 골 손상의 진행을 예방해주는데 효과적인 것임을 강력히 시사하였으며 또한 골수세포들의 골 재흡수 활동에 효과적인 것이라는 결론을 얻었다.

Key words : *Ulmus davidiana* Planch (Ulmaceae) Korean, bone resorption, bone cells, cathepsin, protease inhibitor

I. Introduction

Since a large decrease in bone mass occurs in the postmenopause state, women are vulnerable to the osteoporosis known as postmenopausal osteoporosis[1]. Ulmus davidiana Planch (Ulmaceae) (UD), is effective for the treatment of inflammation, hyperlipemia, arteriosclerosis, and gynecological diseases such as osteophoresis and bone resorption. According to the ancient Chinese and Korean medicinal and herbal literature[2]. Because natural products of plant originare still a major part of traditional medicinal systems, 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[3]. The need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study. To treat the osteoporosis, a herbal formula containing UD is used in oriental medicine[4].

Plants usedin 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[2-3]. During our field studies, we have coincided following Oriental and herbal remedy claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. The aim of this study is to investigate the antibone resorption activity of UD by using an in vitro screening method based on the inhibitory effects on cathepsin activity. A literature survey on the plant UD revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. Therefore, the need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

Ulmus davidiana Planch (Ulmaceae) is a deciduous tree, which is widely distributed in Korea. The barks of the stem and the root of this plant have been used in oriental traditional medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[5-6]. As a part of our search for new biologically active substances from traditional medicines, we evaluated whether extracts of U. davidiana stem barks (UD) could modulate the induction of RA in mice. UD water extract has been developed on the basis of the known function of the herb, as described in the literature of traditional Chinese and Korean medicine[7-8]. UD is known for their functions in maintaining or assisting blood circulation. UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue[9]. UD has long been used by Korean traditional medicine physicians in Korea. However, little is still known about the mode of action of this traditional medication on bone metabolism.

Cysteine proteases comprise a group of proteolytic enzymes involved in many

physiological processes and which could potentially play important roles in a number of pathological situations. Cathepsins are defined as lysosomal proteases, and the majority are members of the papain-like cysteine protease family. Eleven different sequences of human cysteinyl cathepsins (B, H, L, S, C, K, O, F, V, X, and W) have been identified to date[10]. Cysteine proteases contain a cysteine and histidine pair at the active site which forms a stable thiolate-imidazolium ion pair and is required for enzyme activity[11]. The role of cathepsins is in intracellular protein degradation. Cathepsins are broadly distributed to tissues and cathepsins L and B are highly expressed in lysosomes, and secreted outside lysosomes during diseases such as cancer and Alzheimer's disease[12]. Recently, two cathepsins, cathepsins K and S, which are members of the cathepsin L subfamily, have been described as being more tissue-specific and were found to be involved in specialized cellular processes. Cathepsin S has a specific function in antigen processing[13] and represents a potential target for the treatment of autoimune diseases. Cathepsin K is almost exclusively expressed in bone-resorbing osteoclasts[14]. Thus, development of specific inhibitors for this enzyme could potentially be used as treatment for diseases involving excessive bone resorption. Moreover, recent work has demonstrated that nonselective inhibitors of cysteine proteases,which are active against cathepsin K significantly reduce bone resorption in in vitro and in vivo models[15-16].

Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using these animal models from non steroidal sources, such as bisphosphonates[17], calcitonin[17], calcium products[17-18] and ipriflavone[19] are clinically employed as effective medications. Traditional medicines have been reevaluated by clinicians[20], because these medicines have fewer side effects and because they are more suitable for long-term use as compared to chemically synthesized medicines. About forty kinds of oriental medicines are claimed to be effective for gynecological diseases such as climacteric psychosis, feeling of cold, menstrual disorders, dysmenorrhea, and low back pain. It has been suggested that the effectiveness of oriental medicines on low back pain seems to correspond to their efficacy in curing osteoporosis[21-22].

In the present report, we describe a UD extract with some degree of the inhibition of cathepsins K and L. The extracts were shown to be potent inhibitors of the degradation of denaturated collagen by cathepsin K and of bone resorption in an in vitro model. Treatment and pretreatmentof the medicinal extracts of UD to cat K strongly inhibited cat K activity and bone resorption activity. These results clearly suggested that cat K involves the osteoporosis pathway. This result suggested that the UD extracts is effective for bone resorptive action in bone cells.

Ⅱ. Materials and methods

1. Chemicals

A 17beta-estradiol was purchased from Sigma Chemicals (St. Louis, MO). All other reagents were purchased from Wako Chemicals (Osaka, Japan). Tissue culture media and reagents, Fetal bovine serum (FBS) were from Gibco (Chagrin Falls, OH).

2. Plant material

Ulmus davidiana Planch (Ulmaceae)extracts

was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University (Kyungju, Korea). For new substance, the stem barks of U. davidiana were collected from Mt. Phal-gong, Kyungbuk Province, South Korea in May 2002, and identified by Professor Kap-Sung Kim, College of Oriental Medicine, Dongguk University, South Korea. Fresh stems were dried in a dark, well ventilated place. The voucher specimen (No. UD-W-57) is deposited in the Herbarium of this college.

3. Preparation of herb extract and fractions

UD was purchased from a market specializing in herbs (Kyungju herb market, Kyungju, Korea). The herb had a moisture content of <10% by weight, and was air-dried. Air-dried barks (totalling 70 g dry weight) were mixed, minced with a grinder, and extracted by storing in 1 litre of boiling water for 3 hours. The supernatant was filtered with 10 μm cartridge paper and ethanol was removed by rotary evaporation (Eyela, Tokyo, Japan), and concentrated extracts were freeze-dried. This process generally produced 15 g of brown powder. A voucher specimen has been deposited at the Kyungju Oriental Medical Hospital, Dongguk University, Kyungju city, Kyungbuk, Korea under acquisition number UD-13.

4. Biochemical experinents on herbal preparation

Both water and ethanol extracts were tested against cathepsins K, L, andB. Purified human recombinant cathepsins K and L were obtained from Axys Pharmaceuticals Inc., (Richmond, CA, USA) and purified human cathepsin B was obtained from Sigma Co. (St. Louis, MO, USA).

To determine inhibitory potency, extracts were preincubated 30 min with the enzyme, prior to the addition of the substrate Z-Phe-Arg-pNA (25 M). All assays were performed in 96-wells plates at compound concentrations ranging from 0.01 g(10 ng) to 1000 g (1.0 mg).

IC50 values were determined by fitting experimental values to a four-parameter logistic model. To determine pre-steady-state kinetic parameters, enzymatic activity was measured at room temperature in stirred cells using a fluorometer (PTI fluorescence system) and Z-Phe-Arg-AMC as substrate.

5. Osteoblasts isolation and culture

Mouse calvarial osteoblasts were isolated from neonatal BALBc mice by enzymatic digestion, as described for rat osteoblasts[23]. The population released during the last three digestions was highly enriched in cells expressing two markers of the osteoblast lineage, alkaline phosphatase and osteocalcin[24]. Cells released by collagenase digestions were washed and grown to confluent in 75㎠ culture flasks (Falcon) in Dulbesso's modification of Eagle's medium (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS; Gibco, BRL, Bethesda, MD, USA). Incubations were carried out at 37C in a humidified atmosphere of 5% CO2/95% air; the medium was changed every 2-3 days. Cells were grown to confluence at 37C and cultured in duplicate or triplicate wells for an additional 24 h in serum-free medium supplemented with Polymixin B sulfate to prevent endotoxin effects prior to treatment.

6. Enzyme inhibition

To measure enzyme activity, enzymatic assays were carried out in 50 mM MES pH 5.5 containing 2.5 mM DTT, 2.5 mM EDTA and 10% DMSO. IC50values of UD were determined for cathepsin K, L, and B using 25 M Z-Phe-Arg-pNA as substrate. Prior to the addition of substrate, differnt concentraions of the inhibitor ranging from 1000 g down to 20 ng were preincubated for 30 min with the enzyme (2-4 nM) to allow the establishment of the enzyme-inhibitor complex. Substrate was then added and the enzyme activity measured from the increase of OD at 405nm. The final volume of the reaction was 300 l. Assays were performed in 96-well plate format and the plates read using a Vmax (molecular devices) plate reader. The percent inhibition of the reaction was calculated from a control reaction containing only the vehicle.

IC50curves were generated by fitting percentage inhibition values to a four-parameter logistic model using a data analysis computer program (SOFTmax PTO, Molecualr Devices). The inhibition of human cathepsin K gelatinase activity was measured using a fluorescent assay based on the release of quenched fluorescein from DQ gelatin (Molecular Probes), Assays were performed under the same conditions as for the colorimetric assay. The final concentration of DQ gelatin was 0.25 mg/ml and the reaction was measured using excitation and emission wavelengths of 495 and 515 nm, respectively[18].

7. Kinetic Analysis

The measurement of enzyme activity to determine kinetic parameters was performed in 50 mM MES pH 5.5 containing 2.5 mM DTT, 2.5 mM EDTA and 10% DMSO. Z-Phe-Arg-AMC was used as substrate and enzymatic activity was measured at room temperature in 1 mL stirred cells using a spectrofluorometer (Shimazu Co., Kyoto, Japan) with excitation and emission wavelengths of 355 nm of 460 nm, respectively. Product formation was measured at different inhibitor concentrations following initiation of the reaction by the addition of the enzyme (0.1 nM final concentration). The Km value for Z-Phe-Arg-AMC was determined (30 M) for cathepsin K, and inhibition assays were performed at a substrate concentration 10-fold below the Km value. The formation of product (P) with time for an enzyme inhibited by a slow-binding inhibitor is described by the following equation24): P = vst - (vs-vo)(1 - e-kobs)/kobs

where vs is the rate of the reaction at steady-state, vo is the initial velocity of the reaction, and kobs is the apparent first-order rate constant characterizing the establishment of the steady-state velocity. Experimental values were fitted by nonlinear regression and fitted parameters vs, vo, and kobs were used to estimate pre-steady-state kinetic parameters kon and koff and the dissociation constant ki using the following relationships[24].

8. Bone-Resorption Assay

The long bones were aseptically isolated from a 10-day-old mouse hare and the soft tissue removed. The bones were minced into ~1-mm pieces with scissors in 10 mL of -MEM (Gibco BRL, Gaithersburg, MD, USA) containing penicillin/streptomycin, pH 7.1. The volume was brought to 25 ml and the tissue transferred to a 50-mltube. The tube was rocked gently for 60 cycles, the tissue allowed to settle for 1 min and the supernatant withdrawn with a pipet and retained. 25 ml of

medium was added back to the tissue and rocked again. The second supernatant was combined with the first. Cells were diluted 1:10 in 2% acetic acid in PBS, counted by hemacytometer and diluted to 5 x 106 cells/ml in -MEM containing 2% FBS. 200 l aliquots were plated onto 6-mm diameter x 200 m bovine bone slices. After 2 h, test compounds were diluted in -MEM containing 2% FBS, 10 nM 1,25(OH)2D3, the plating medium removed and 200 lof test media was added to triplicate wells. The cultures were incubated for 3 days at 37C in a 5% CO2atmosphere. The medium was then removed from the cultures and collagen fragments released into the medium measured by the ELISA assay (Molecular Devices, CA, USA).

9. Statistics

Data were obtained from 3-5 measurements and were expressed as the means standard deviations. Statistical comparisons were made by ANOVA and Scheffe's tests using a statistic software program. The difference was considered significant when $P < 0.05$.

Ⅲ. Result

1. Inhibition of Human Cathepsins K, L, and B by water extract and ethanol extract of Ulmus davidiana Planch (Ulmaceae)

As shown in Table 1, the original water extract of Ulmus davidiana Planch (Ulmaceae) (UD-WE), taken through screening of the herbal sample collection, inhibits human cathepsins K and L with IC50 values of 5.32±0.43 and 6.34±0.53 g, respectively. Ethanol extraction process afforded potent inhibitors of cathepsins K and L, indicating that ethanol extract of Ulmus davidiana Planch (Ulmaceae) (UD-EE) contained potent inhibitors of cathepsins K and L. By ethanol extraction, the inhibitions of cathepsin K and L increased to 1.45±0.13and 2.43±0.32 g/ml, respectively, as compared to UD-WE. The UD-EE proved to be the most potent and selective (cathepsins K and L versus B) extracts. Moreover, UD-EE inhibits both rat and human cathepsin K with similar IC50 values (Table 1).

On the other hand, the UD-WE and UD-EE were also shown to be active in the gelatinase and bone-resorption assays (Table 1). For example, the UD-WE was equipotent in the gelatinase and human cathepsin K assays (4.43±0.52 and 5.23±0.43 g/ml, respectively). The more potent UD-EE (1.22g/ml for cathepsin K) was also very potent in the gelatinase assay with IC50 values of 0.23 g/ml. In the functional in vitro bone-resorption assay, UD-EE was shown to inhibit bone resorption with IC50 values of 1.21g/ml.

Table 1. Inhibition of human cathepsins K, L, and B by water-extract (WE) and ethanol extract (EE) of Ulmus davidiana Planch (UD)

	IC50 (g/ml)				
Extract	Cat K	Cat L	Cat B	Cat K	
	Gelatinase assay		Bone resorptionassay		
UD-WE	5.32±0.43	6.34±0.53	15.32±1.32	4.43±0.52	5.23±0.43
	1.22±0.13	2.54±0.32	12.43±1.33	0.23±0.20	1.21±0.25

Cat : Cathepsins, UD-WE : Water extract of Ulmus davidiana Planch (UD). UD-EE : Ethanol extract of Ulmus davidiana Planch (UD).

2. Time-dependent inhibition and reversibilty of cathepsins by UD-WE and UD-EE by kinetic experiements

Inhibition of cathepsins K and L by UD-WE and UD-EE was found to be time-dependent and fully reversible. Fig. 1 and

2 show the reaction progress curves for the onset of inhibition of human cathepsin K by UD-WE and UD-EE (Fig. 1 and 2). The curves show that for an equal final concentration of inhibitor, the same steady-state velocity is obtained either when the reaction is initiated by the addition of enzyme or when the enzyme and the inhibitor are preincubated and then diluted into the assay mixture containing substrate. These kinetics are consistent with a reversible time-dependent mechanism of inhibition. Generally, the possible mechanisms can describe reversible time-dependent inhibitors. The mechanism characterizes the extracts where the initial velocity of the enzymatic reaction is independent of the inhibitor concentration. This mechanism is often referred to as an apparent single step binding process[13].

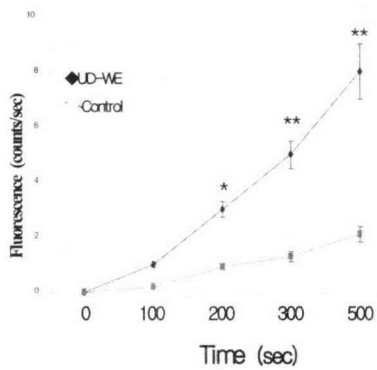

Fig. 1. Time-dependent inhibition and reversibility of cathepsins by water extract of Ulmus davidiana Planch (UD-WE)

Kinetic curves were obtained when the reaction was initiated by the addition of 0.5 nM cathepsin K. For inhibitory studies, cathepsin K and the inhibitor were preincubated for 15 min and they were assayed. Water extract of Ulmus davidiana Planch (Ulmaceae), Fluorescence (counts/sec) was determined with inhibitor [5.32 g/ml UD-WE] or without inhibitor [only 0.5 nM Cat K] as a control.

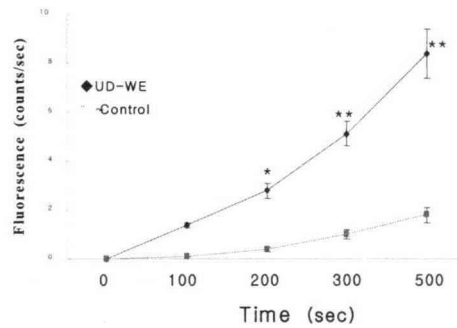

Fig. 2. Time-dependent inhibition and reversibility of cathepsins by ethanol extract of Ulmus davidiana Planch (UD-EE)

Kinetic curves were obtained when the reaction was initiated by the addition of 0.5 nM cathepsin K. For inhibitory studies, cathepsin K and the inhibitor were preincubated for 15 min and they were assayed. Ethanol extract of Ulmus davidiana Planch (UD). Fluorescence (counts/sec) was determined with inhibitor [1.11 g/ml UD-EE] or without inhibitor (only 0.5 nM Cat K) as a control.

3. Kinetics of inhibition of human cathepsin K by UD-WE and UD-EE

The results for both the UD-WE and UD-EE showed that the rate of association of cathepsin K and the inhibitors is linearly dependent on inhibitor concentration. The results were therefore, consistent with an apparent one-step binding mechanism of the inhibitor to the enzyme.

The data presented in Table 2 showed more effective inhibitions by UD-EE. It is unlikely that the high IC50in UD-WE is due to a slow rate of diffusion into the enzyme active site. As hypothesized for other low-molecular-weight time-dependent inhibitors[25], it is possible that one or more transient enzyme-inhibitor

(EI)complexes occur before the formation of the final EI complex.

Table 2. Inhibition of human cathepsin K by water extract and ethanol extract of Ulmus davidiana Planch (UD)

Extract	Ki (g/ml) ± SE	IC50 (g/ml)
UD-WE	4.3 ± 0.35	4.1
UD-EE	0.8 ± 0.04	0.6

UD-WE : Water extract of Ulmus davidiana Planch (UD), UD-EE : Ethanol extract of Ulmus davidiana Planch (UD).

4. Possible reactivity of the herbal extract with thiols in amino aicd of cathepsin K enzymatic activite site

The results, shown in Table 1 and Table 2, suggested that UD-WE may contains several cyanamide molecules as pharmacologically active compounds and these inhibit cathepsinK enzyme activity. Therefore, to determine if the enzymatic inhibition observed UD-WE or UD-EE is due to the reactivity of the cyanamide molecules toward of the active site cysteine thiolate anion, IC50values were determined in the presence and absence of glutathione (GSH) at pH 5.5 and 7.0. Increasing the pH to 7.0 will favor the formation of the GSH thiolate anion, which is a better nucleophile and can react with the inhibitor during the preincubation step. Results shown in Table 3 show that when the assay was performed at acidic pH (pH 5.5, i.e. the optimal pH for cathepsins K and L), IC50values were not significantly affected by the presence of GSH. The inhibitory effects of extracts were only slightly affected by the presence of GSH at pH 5.5. However, UD-WE which had an IC50 value of 3.82±0.42 g/ml for cathepsin K had an higher IC50 by a factor of 34 in the presence of GSH at pH 7.0.

Peptidic nitriles are known to be potent and reversible inhibitors of cysteine proteases of the papain family26). Unlike aldehydes, which are inhibitors of both serine and cysteine proteases, nitriles seem to be more specific for cysteine proteases27). Although EE-UD is more potent for cathepsin K (Table 1), it is also relatively potent for the inhibition of papain with an IC50 value of 2.7 g/ml at pH 5.5.

Table 3. Effect of GSH on the inhibitory activity of water extract and ethanol extract of Ulmus davidiana Planch (UD-WE & UD-EE) at pH 5.5 and 7.0 toward cathepsin Ka

Extract	IC50 (g/ml)			
	no GSH		+10 mM GSH	
	pH 5.5	pH 7.0	pH 5.5	pH 7.0
UD-WE	3.82±0.42	2.87±0.34	4.32±0.41	76.64±6.5***
UD-EE	0.56±0.04	0.53±0.06	0.70±0.07	2.11±0.22***

Extracts were preincubated 30 min with GSH prior to the addition of the enzyme. UD-WE: Water extract of Ulmus davidiana Planch (UD), UD-EE: Ethanol extract of Ulmus davidiana Planch (UD). *** P <0.001; Significantly different from those at pH 5.5.

5. Pharmacokinetic profile of extracts of Ulmus davidiana Planch (UD)

From the above results, it was assumed that some cyanamide-containing compounds were present as effector components in the extracts (Table 3). Also, it was suggested that at neutral pH, the cyanamide-containing compounds might form stable isothiourea ester bonds with the thiol-containing molecules such as GSH or protein cystein. To determine if the reactivity of the cyanamide toward thiols could be responsible for possessing the suitable pharmacokinetic profile, in vivo dosing studies were performed with UD-WE and UD-EE.

Furthermore, 1-cyanopyrrolidinyl compound, a well known cyanamide molecule was also

examined as a model. The results of this study are presented in Fig. 3. When UD-WE was administered orally in rats at a dose of 20 mg/kg in a 1% methocel suspension, the UD-WE was well-absorbed with a bioavailability of 40%. The half-life of UD-EE was determined after oral administration at a dose of 5 mg/kg and found to be 2.0 h in rats.

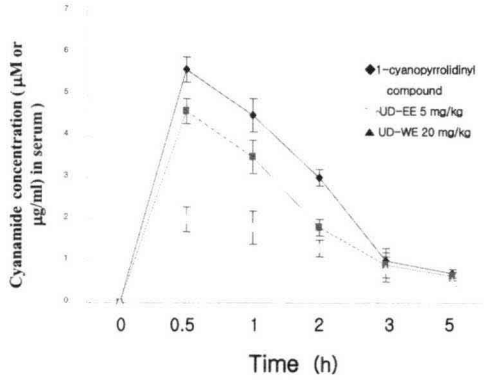

Fig. 3. Mean plasma levels after oral administration of water extract and ethanol extract of Ulmus davidiana Planch (UD-WE & UD-EE) in mice.

Dosing volumes of UD-EE and UD-WE for oral administration were 5 and 20 ml/kg, respectively. UD-WE: Water extract of Ulmus davidiana Planch (Ulmaceae), UD-EE: Ethanol extract of Ulmus davidiana Planch (Ulmaceae). 1-cyanopyrrolidinyl compound, WE-UD Oral-20mg/kg (n=3), EE-UD Oral-5 mg/kg(n=3).

Ⅳ. Conclusion

Korean traditional medicines, which have been developed over some 3000 years[20] and are known to have low toxicity, may offer advantages over the longer term over synthetic agent medication. Although the preventive mechanism of these agents remains to be explained, this initial study does show that korean traditional medicines that have traditionally been effective for the gynecological diseases[21-22] may also be administered for the prevention of osteoporosis.

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[28-29]. During our field studies, we have coincided following Oriental and herbal remedy claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. The aim of this study is to investigate the antibone resorption activity of UD by using an in vitro screening method.. A literature survey on the plant UD revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. Therefore, the need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

UD is an extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published work that is well accepted by the traditional oriental medicine community, UD was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. The UD have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support[30]. Although the barks of UD stem and root have been used in oriental traditional medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs anti-

inflammatory action in vivo. To evaluate the role of UD on inflammatory diseases, we studied the effect of the water extract of UDon the production of collagen-induced RA in rats in vivo. Our results showed that the UD clearly reduced this inflammatory disease in a dose-dependent manner.

In the present report, we have shown that cathepsins K and L can be inhibited by WE-UD andEE-UD. Peptidic nitriles are known to be potent and reversible inhibitors of cysteine proteases of the papain family[26]. Unlike aldehydes, which are inhibitors of both serine and cysteine proteases, nitriles seem to be more specific for cysteine proteases[27]. Although EE-UD is more potent for cathepsin K, it is also relatively potent for the inhibition of papain with an IC50value of 0.5 g/ml at pH 5.5.

These were probably postulated to be contained cyanamide-based compounds, represent a new class of nonpeptidic cysteine protease inhibitors. They inhibit cathepsins K and L in a time-dependent manner and form a possible reversible isothiourea ester link with the active site cysteine of the enzyme. EE-UD, a potent inhibitor of cathepsins K and L,which was also active in an in vitro model of bone resorption. Moreover, these inhibitors proved to have good pharmacokinetic properties in rats and thus would be suitable for animal studies where the roles of cathepsins K and L could be studied. In conclusion, the Hanbang medicine, UD, could prevent the development of bone loss induced by protease. This result strongly suggests that these Hanbang medicines are useful for preventing postmenopausal osteoporosis and osteoporosis associated with both the ovary function failure.

Overall, our results suggest that the effect of UD in the inhibition of inflammatory diseases may be partially associated with the down-regulation of IL-1. Our results indicate that UD has great potential as an alternative to these treatments, and has no adverse effects. UD can be given orally, and it inhibits disease progression by both controlling inflammatory proteins and protecting cartilage. Its cost is also estimated to be substantially lower than that of recombinant proteins. The data presented in this study show that UD warrants further investigation, including preclinical and clinical studies. We are now in progress to isolate active molecules, as have tried[31].

V. References

1. Ettinger, B.; Genant, H.K.; Cann, C.E.Postmenopausal bone loss is prevented by treatment with low-dosage estrogen with calcium. Ann. Intern. Med. 1987, 106, 40-45.
2. Shi, X.C. Chinese-English terminology of traditional chinese medicine (1983) Hunan Science Publishing Co. pp71-98. China.
3. Phillipson, J.D. Anderson, L.A. Ethnopharmacology and western medicine. J. Ethnopharmacol. 1989, 25, 61-72.
4. Hong, S.N. Ph. D. Thesis, Dongguk University, Kyungju, Korea, 2001.
5. Lee, S.J., 1996. Korean Folk Medicine, Monographs Series No. 3. Publishing Center of Seoul National University, Seoul, South Korea, 39 pp.
6. Jun CD, Pae HO, Kim YC, Jeong SJ, Yoo JC, Lee EJ, Choi BM, Chae SW, Park RK, Chung HT. 1998. Inhibition of nitric oxide synthesis by butanol fraction of the methanol extract of Ulmus davidiana in murine macrophages. J Ethnopharmacol. 62, 129-135.
7. Shizhen L. (1590 A.D.). Bon Cho Gang Mok

[Compendium of materia medica]. Peking: ChungKuK ChungEuiHak, 1978.
8. Hocheol Kim. Herbalogy. Seoul: Young Lim Sa, 2000.
9. Heo Jun. (1613 A.D.). Dong Ui Bo Gam [The precious mirror of oriental medicine]. Seoul: Namsangdang, 1999.
10. Turk, B. Turk, D.Turk, V. Lysosomal cysteine proteases: more than scavengers. Biochim. Biophys. Acta 2000, 1477, 98-111.
11. Storer, A.C. Menard, R. Catalytic mechanism in papain family of cysteine peptidases. Methods Enzymol. 1994, 244, 486-500.
12. Littlewood-Evans, A.J. Bilbe, G.; Bowler, W.B. Farley, D.; Wlodarski, B. Kokubo, T. Inaoka, T. Sloane, J. Evans, D.B. Gallagher, J.A. The osteoclast-associated protease cathepsin K is expressed in human breast carcinoma. Cancer Res. 1997, 57, 5386-5390.
13. Riese, R.J. Mitchell, R.N. Villadangos, J.A. Shi, G.P. Palmer, J.T. Karp, E.R. De Sanctis, G.T. Ploegh, H.L. Chapman, H.A. Cathepsin S activity regulates antigen presentation and immunity. J. Clin.
14. Li, Y.P. Alexander, M. Wucherpfennig, A.L. Yelick, P. Chen, W. Stashenko, P. Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. J. Bone Miner. Res. 1995, 10, 1197-2202.
15. Everts, V. Delaisse, J.M. Korper, W.Beertsen, W. Cysteine proteinases and matrix metalloproteinases play distinct roles in the subosteoclastic resorption zone. J. Bone Miner. Res. 1998, 13, 1420-1430.
16. Falgueyret, J.P.; Oballa, R.M.; Okamoto, O.;Wesolowski, G.; Aubin, Y.; Rydzewski, R.M.; Rasit, P.; Riendeau, D.; Rodan, S.B.; Percival, M.D. Novel, nonpeptidic cyanamides as potentand reversible inhibitors of human cathepsin K and L. J. Med. Chem. 2001, 44, 94-104.
17. Kalu, D.N. The ovariectomized rat model of postmenopausal bone loss. Bone Miner. 1991, 15, 175-192.
18. Morris, H.A.; Need, A.G.; Horowitz, M.;O'Leughlin, P.D.; Nordin, B.E.C. Calcium adsorption in normal and osteoporotic postmenopausal women. Calcif. Tissue Int. 1991, 48, 240-243.
19. Agnusdei, D.; Comporeaale, A.; Zacchei, F.; Gennari, C.; Baroni, M.; Costi, D.; Biondi, M.; Passeri, M.; Ciacca, A.; Sbrenna, C.; Falsettini, E.; Ventura, A. Effects of ipriflavone on bone mass and bone remodeling in patients with established postmenopausal osteoporosis. Curr. Ther. Res. 1992, 51, 82-91.
20. Xiu, R.J. Microcirculation and traditional Chinese medicine. JAMA 1988, 269, 1755-1757.
21. Koyama, T.Osteoporosis with Kampo treatment (in Japanese). Sanfujinka Chiryo 1991, 63, 203-207.
22. Terasawa, K. Kampo: Japanese-oriental medicine, K. K. Standard McIntyre, Tokyo, Japan. 1993.
23. McCarthy, T.L. Centrella, M. Raisz, L.G. Canalis, E. Phosphoglandin E2 stimulates insulin-like growth factor I synthesis in osteoblast- enriched cultures from fetal rat bone. Endocrinology 1991, 128 2895-2900.
24. Izquierdo-Martin, M. Stein, R.L. Transition-state structural features for the association of metalloproteases with phosphorus-containing inhibitors. J. Am. Chem. Soc. 1992, 114, 1527-1528.
25. Duggleby, R.G. Attwood, P.V. Wallace, J.C. Keech, D.B. Avidin is a slow-binding inhibitor of pyruvate carboxylase. Biochemistry 1982, 21, 3364-3370.
26. Hanzlik, R.P. Zygmunt, J. Moon, J.B.

Reversible covalent binding of peptide nitriles to papain. Biochim. Biophys. Acta 1990, 1035, 62-70.
27. MacLeod, A.M. Baker, R. Freedman, S.B. Patel, S. Merchant, K.J. Roe, M. Saunders, H.Synthesis and muscarinic activities of 1,2,4-thiadiazoles. J. Med. Chem. 1990, 33, 2052-2059.
28. Kim HM, An C.-S, Jung K-Y, Choo Y-K, Park J-K, Nam S-Y. Rehmannia Glutinosa inhibits tumor necrosis factor-alpha and interleukin-1 secretion from mouse astrocytes. Pharmacol. Res. 1999; 40: 171-6.
29. Hong H.T Effects of Yukmijihangtang-Jahage extracts on cellular regulation of bone cell and function. Thesis of Ph.D. Dongguk University, Kyungju, Korea. 1998.
30. Won KCEH. Chinese medicine encyclopedia. Seoul: DaeSung,1992.
31. Kim JP, Kim WG, Koshino H, Jung J, Yoo ID. 1996. Sesquiterpene O-naphthoquinones from the root bark of Ulmus davidiana. Phytochemistry. 43, 425-430.