Effects of KCA-012 on Bone Metabolism in Organ Culture

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ABSTRACT—3,9-Dihydroxy-5H-benzofuro[3,2-c]quinoline-6-one (KCA-012), the chemical structure of which is closely similar to that of the phytoestrogen coumestrol, inhibited parathyroid hormone-, 1α,25-dihydroxyvitamin D3- and prostaglandin E2-induced bone resorption of cultured fetal rat bones. KCA-012 also increased the calcium content of 9-day chick embryonic femur cultured in vitro. KCA-012 did not show any estrogenic activity as determined by an increase in the uterine weight of ovariectomized rats, whereas coumestrol did. These results indicate that KCA-012 has no estrogenic activity and has unique effects of inhibiting bone resorption and stimulating bone mineralization.

Keywords: KCA-012, Bone resorption, Bone mineralization

Many agents such as calcium (1), estrogen (1, 2), calcitomin (3), vitamin D₃ derivatives (4), bisphosphonates (5), fluoride compounds (6) and an isoflavone derivative (7) have been used for the treatment of osteoporosis. Estrogenic hormones are often used for the treatment of postmenopausal osteoporosis. However, several side effects of estrogens, such as abnormal vaginal bleeding (8) and carcinogenicity (9), have been reported. Therefore, we have been searching for compounds that stimulate bone formation without having estrogenic activity.

Coumestrol (Fig. 1) is a representative naturally produced weak phytoestrogen. Therefore, we synthesized several compounds with structural similarity to coumestrol to eliminate the estrogenic activity and investigated the effect of these compounds on bone metabolism. Here we report that among these compounds 3,9-dihydroxy-5H-benzofuro[3,2-c]quinoline-6-one (which designated as KCA-012, shown in Fig. 1), a serial derivative of benzoquinolines that we previously reported (10), has the unique effects of inhibiting bone resorption of fetal rat long bone in culture and stimulating the mineralization of the femur of 9-day chick embryos in organ culture.

Agents used were as follows: 3,9-dihydroxy-5H-benzofuro[3,2-c]quinoline-6-one (KCA-012), Kissei Pharmaceutical Co., Ltd., Matsumoto; coumestrol, Eastman Kodak, Rochester, NY, USA; estradiol benzoate (Fig. 1), Teikoku Hormone Manufacturing Co., Osaka; synthetic human parathyroid hormone (fragment 1–34, PTH), Peptide Institute, Inc., Minoh; prostaglandin E₂ (PGE₂), Funakoshi, Tokyo; 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂D₃], Wako Pure Chemical Co., Osaka; BGJb-HW2 (phenol red free, ref. 10), Nissui Pharmaceutical Co., Tokyo; and ⁴²CaCl₂, Du Pont/NEN Research Products, Boston, MA, USA.

Fig. 1. Chemical structures of KCA-012, coumestrol and estradiol benzoate.
Bone-resorbing activity was determined by the method of Raisz (11) and was expressed as a percentage of the $^{45}$Ca released into the medium from $^{45}$Ca-labeled fetal rat bone during culture.

To determine the bone mineralizing activity, the femora isolated from 9-day-old chick embryos were cultured by the roller tube method (12) for 6 days in the presence or absence of KCA-012. Then the calcium content of the femora was determined.

A concentrated stock solution of KCA-012 was prepared in DMSO, and an appropriate amount of this solution was added to the medium to obtain the desired concentration. The final concentration of DMSO was restricted to 0.1%.

To estimate the estrogenic activity of the compounds, the increase of uterine weight of ovariectomized rat was determined. Bilateral ovariectomy or a sham operation of female Wistar rats (Japan SLC Inc., Hamamatsu) of 5 weeks of age was carried out under ether anesthesia. Test compounds suspended in saline were administered subcutaneously twice a day (9:00 AM and 6:00 PM) for 3 days from the day after the operation. The rats were sacrificed under ether anesthesia at 4 hr after the final injection for determination of their uterine weight.

Data are expressed as the means ± S.E.M. Statistical significance was determined by Student's t-test for the comparison between two groups or by one-way analysis of variance followed by Dunnett's test for multiple group comparison.

KCA-012 inhibited PTH (1.8 x 10^{-8} M)-, 1α,25(OH)_{2}D_{3} (10^{-9} M)- and PGE_{2} (10^{-6} M)-induced bone resorption of fetal rat limb bone in culture, whereas it did not inhibit basal bone resorption (Fig. 2). The inhibitory effect of KCA-012 on the bone resorption seemed to be not caused
by toxicity of the drug because at the highest concentration used (10^{-3} M), it did not influence basal bone resorption (Fig. 2A). Furthermore, histologically, there was no evidence of cellular damage in the cultures of 9-day embryonic chick femora (data not shown). Our findings indicate that KCA-012 is a good inhibitor of bone resorption.

Since bone tissue has both bone-mineralizing and -resorbing activity occurring simultaneously, quantitative separation of bone-forming activity from bone-resorbing activity is difficult in organ culture. The femora of 9-day chick embryos are actively growing. Therefore, bone-forming activity largely exceeds bone-resorbing activity. Thus, an increase in calcium accumulation in vitro reflects bone-mineralizing activity. In fact, Endo et al. showed a direct stimulative effect of 1α,25(OH)2D3, 24,25(OH)2D3 and PTH on bone mineralization by cultured 9-day chick embryonic femora (12). KCA-012 (2 × 10^{-6} M), an effective concentration to inhibit bone resorption, also increased the calcium content of 9-day chick embryonic femora from 29 ± 1 μg (control, n = 6) to 36 ± 2 μg (KCA-012, n = 6) (P < 0.05) during the culture, indicating that it stimulates bone mineralization. Therefore, KCA-012 is unique substance in inhibiting bone resorption and, at the same time, stimulating bone mineralization, because its effective concentrations for the two effects were about the same.

Subcutaneous administration of coumestrol (100 mg/kg/twice a day) for 3 days resulted in a marked increase in the uterine weight of ovariectomized rats. Administration of estradiol benzoate also increased the uterine weight. On the contrary, KCA-012 (10 or 100 mg/kg/twice a day) was ineffective, indicating that it has no estrogenic activity (Table 1). Separation of bone metabolizing activity and estrogenic activity is important for the treatment of osteoporosis, because administration of estrogen sometimes results in side effects such as vaginal bleeding (8) and tumors in the breast and sexual organs (9).

Many kinds of drugs that inhibit bone resorption are used clinically for the treatment of osteoporosis. In contrast to these drugs, KCA-012, which is structurally similar to coumestrol, is unique in having no estrogenic activity and in causing not only inhibition of bone resorption but also stimulation of bone mineralization at about the same concentration. KCA-012 seems to be a good tool for the study of bone metabolism.

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REFERENCES


Table 1. Effects of KCA-012, coumestrol and estradiol benzoate on uterine weight of ovariectomized rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Uterine weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (ovariectomized, vehicle)</td>
<td></td>
<td>116.3± 5.9</td>
</tr>
<tr>
<td>Sham-operated (vehicle)</td>
<td></td>
<td>187.2± 16.0*</td>
</tr>
<tr>
<td>KCA-012</td>
<td>10 mg/kg</td>
<td>97.5± 6.2</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>101.5± 7.8</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>100 mg/kg</td>
<td>448.0±47.4**</td>
</tr>
<tr>
<td>Estradiol benzoate</td>
<td>0.05 μg/kg</td>
<td>100.5±7.5</td>
</tr>
<tr>
<td></td>
<td>0.15 μg/kg</td>
<td>126.5±14.5</td>
</tr>
<tr>
<td></td>
<td>2.0 μg/kg</td>
<td>329.3±18.1**</td>
</tr>
</tbody>
</table>

Data are means±S.E.M. for 6 rats. Each test drug or saline (vehicle) was administered subcutaneously twice a day for 3 days after ovariectomy. *P < 0.05 and **P < 0.01 vs the control value.