Effects of 3-Phenyl-4-[[4-[2-(1-piperidinyl)ethoxy]phenyl]methyl]-2H-1-benzopyran-7-ol (CHF 4056), a Novel Nonsteroidal Estrogen Agonist/Antagonist, on Reproductive and Nonreproductive Tissue

ELISABETTA GALBIATI, PAOLA LORENZA CARUSO, GABRIELE AMARI, ELISABETTA ARMANI, SILVIA GHIRARDI, MAURIZIO DELCANALE, and MAURIZIO CIVELLI

Departments of Pharmacology (E.G., P.L.C., M.C.) and Chemistry (G.A., E.A., S.G., M.D.), Chiesi Pharmaceuticals S.p.A., Parma, Italy

Received July 20, 2001; accepted November 15, 2001  This article is available online at http://jpet.aspetjournals.org

ABSTRACT

We have discovered a new, nonsteroidal, estrogen agonist/antagonist, 3-phenyl-4-[[4-[2-(1-piperidinyl)ethoxy]phenyl]methyl]-2H-1-benzopyran-7-ol (CHF 4056). The aim of this study was to determine the effects of CHF 4056 on a series of parameters (body weight, uteri, serum cholesterol, and bones) that were previously shown to be sensitive to estrogens and to selective estrogen receptor modulators (SERMs). CHF 4056 is a benzopyran derivative that binds with high affinity to the human estrogen receptors α and β (dissociation constant Kᵢ of 0.041 and 0.157 nM, respectively). In immature rats, CHF 4056 diverged dramatically from EE2 and levormeloxifene treated animals, compared with vehicle-treated OVX rats. In line with the results observed in immature rats, also in OVX rats CHF 4056 diverged dramatically from EE2 and levormeloxifene in its lack of significant estrogenic effects on uterine tissue. In conclusion, CHF 4056 is a new SERM that produces beneficial effects on bone and cholesterol levels, while maintaining antagonist effects on the uterus.

The decreased production of ovarian steroids, which occurs after the climacteric, has been linked to a number of postmenopausal pathologies, in particular osteoporosis and coronary heart disease (Lindsay, 1988; Ross et al., 1990). Estrogen replacement therapy is effective in reducing the risks associated with these pathologies (Cumming, 1991). However, there are several undesirable side effects associated with chronic estrogen therapy that create difficulties in compliance. In this respect, estrogens, when administered without progestin, substantially increase the incidence but not the mortality of endometrial cancer (Ziel and Finkle, 1975; Vesey, 1984); furthermore, concerns about the increased risk of breast cancer associated with estrogen replacement therapy have been raised (Cauley et al., 1999; Jacobs, 2000). Accordingly, an ideal therapy should prevent bone loss and improve the serum lipid profile as estrogen does, without inducing proliferative effects on reproductive tissues. It is this need that has provided the impetus to the pharmaceutical industry to search for new estrogens that may modulate estrogen receptors in a tissue-selective manner.

Tamoxifen was the first estrogen agonist/antagonist shown to inhibit bone loss in castrate female rats (Jordan et al., 1987) and in postmenopausal women (Love et al., 1994). However, its estrogen-like effect on human endometrial carcinoma (Satyaswaroop et al., 1984; Kedar et al., 1994) and its strong ephatocarcinogenic effects in rats (Greaves et al., 1993) prevented the chronic use of tamoxifen to mimic some of the helpful actions of estrogen after the menopause. Subsequent endeavors have seen the first approval of a selective estrogen receptor modulator (SERM) for the prevention and treatment of osteoporosis (raloxifene; Black et al., 1994) and the emergence of several new synthetic compounds, including triphenylethylene, naphthalene, and benzopyrans derivatives, with this possible spectrum of activities (Sato et al., 1999). However, the development of many of these compounds as drugs is problematic because of their excessive

ABBREVIATIONS: SERM, selective estrogen receptor modulator; DEXA, dual energy x-ray absorptiometry; ER, estrogen receptor; sham, sham ovariectomized controls; OVX, ovariectomized controls; BMD, bone mineral density; CHF 4056, 3-phenyl-4-[[4-[2-(1-piperidinyl)ethoxy]phenyl]methyl]-2H-1-benzopyran-7-ol; EE2, 17α-ethynyl estradiol; UWR, uterine weight/body weight ratios.
stimulation of uterine tissue (Sato et al., 1996; Grese et al., 1997).

With this in mind, we wanted to design a series of novel SERMs demonstrating significant protective effects on non-reeproductive tissue (bone and cholesterol levels) and antagonistic effects associated with a low degree of intrinsic estrogenicity on reproductive tissue. A compound characterized by this pharmacological profile may have potential utility in the prevention and treatment of a number of postmenopausal pathologies such as osteoporosis, coronary heart disease, and estrogen-dependent human cancer.

Our chemical plan involved surveying the various structural classes known to interact with the estrogen receptor, including in particular the benzopyrans exemplified by levormeloxifene, which is an estrogen agonist/antagonist that has been shown to inhibit bone loss in postmenopausal women (Bjarnason et al., 1997) and in the ovariectomized rat model (Novak et al., 1997). However, the partial estrogenic effects on uteri in women raised issues of safety of levormeloxifene as a compound for use in the prevention and treatment of postmenopausal women. Its development as an antiestrogenic drug was discontinued when the compound was in advanced clinical studies (Mitlak and Cohen, 1999).

We performed a wide variety of synthetic modifications of the benzopyran moiety at various sites to obtain a SERM characterized by a lower estrogen agonist effect on reproductive tissue as compared with levormeloxifene. Several candidate compounds were prepared and tested for the following activities: 1) binding affinity to human ER-α and -β; 2) antiestrogenic and estrogenic effect on uterine growth in an immature female rat model, which is widely accepted as the model for studying in vivo agonist and antagonist estrogenic effects (Epfenberger et al., 1991); 3) estrogenic effects on bone, total serum cholesterol, and uterus in an OVX rat model of postmenopausal bone loss (Kalu, 1991). As a result of our screening, we have discovered a new, orally active, potent estrogen agonist/antagonist named CHF 4056 (Fig. 1) with potential advantages over estrogen in the uterus. Herein, the pharmacological properties of CHF 4056 observed in the above-mentioned experimental models are reported. In addition, its in vivo effects are compared with those of EE2 and levormeloxifene.

Materials and Methods

All animals studies were performed in strict accordance with the decree Legislativo sulla sperimentazione animale (Italian law on rules for animal experimentation, Decree 116, January 27, 1992) and the “European Directive for the protection of vertebrate animals used for experimental and other scientific purposes” (European Union Directive 86/609/CEE).

Chemicals. CHF 4056 (3-phenyl-4-[(4-[2-(1-piperidinyl)ethoxy]phenyl)methyl]-2H-1-benzopyran-7-ol), $M_r = 441.59$; levormeloxifene (1-[2-[(3$\beta$R,4R)-3,4-dihydro-7-methoxy-2,2-dimethyl-3-phenyl-2H-1-benzopyran-4-yl]phenoxyl][ethyl]pyrrolidine hydrochloride), $M_r = 494.08$; and raloxifene ([6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl]-4-[2-(1-piperidinyl)ethoxy]phenyl]-methanone hydrochloride), $M_r = 510.06$, were synthesized by Chiesi Farmaceutici S.p.A. (Parma, Italy). EE2, $M_r = 286.4$; 17$\beta$-estradiol, $M_r = 272.4$; and diethylstilbestrol, $M_r = 268.4$ were obtained from Sigma (St. Louis, MO). Kits for radioimmunoassay of osteocalcin were supplied by Biomedical Technologies Inc. (Stoughton, MA). All other reagents were purchased from Sigma.

Human ER-α and ER-β Binding. ER-α and -β binding analysis was performed as previously described (Obourn et al., 1993). Briefly, the standard assay was performed in a volume of 100 μl, containing a final concentration of 0.5 nM 3H-estradiol (New England Nuclear, Boston, MA), increasing concentration of unlabeled CHF 4056 or reference compounds (0.01–100 nM), 5 μl of diluted (1:100 in binding buffer) human recombinant ER-α or -β (insect Sf9 cells), and 95 μl of binding buffer (10 mM Tris, pH 7.5, 10% glycerol, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin). The incubation was carried out at room temperature for 3 h. After incubation, 100 μl of 50% hydroxyapatite slurry (equilibrated in 50 mM Tris, pH 7.4, and 1 mM EDTA) was added to each tube and vortexed three times over 15 min. One milliliter of wash buffer (40 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, and 100 mM KCl) was added to each reaction, the reactions were centrifuged at 10,000 g for 5 min, and the supernatant was aspirated. The wash step was repeated two additional times, and then the hydroxyapatite pellet was resuspended in 400 μl of ethanol, transferred to a scintillation vial, and counted. Non-specific binding was defined as that which occurred in the presence of 1 μM diethylstilbestrol and represented 10 to 15% of the total binding. $K_i$ values were calculated using the equation of Cheng and Prusoff (1973) using the observed half-maximal inhibition concentration of the tested compound, the concentration of radioligand employed in the assay, and the dissociation constant value of the ligand. The data were also fitted by an iterative program (RECEPT) for nonlinear regression analysis (Benfenati and Guardabasso, 1984) both to a one-site and to a two-site model. The one-site model was then chosen when it yielded the best correlation coefficient and when the improvement of goodness-of-fit for the two-site model was not statistically significant ($P < 0.05$) according to the F test on the sums of squared errors.

Immature Female Rat Study. Twenty-one-day-old female Sprague-Dawley rats, weighing approximately 40 to 50 g (Charles River, Calco Italy) were treated by oral gavage with either vehicle (0.5% methylcellulose, 3 ml/kg), CHF 4056 (0.01–10 mg/kg/day), levormeloxifene (0.01–10 mg/kg/day), or EE2 at 0.05 mg/kg/day for 3 days. The compounds under investigation were also administered 15 min before the EE2 gavage, used as estrogenic stimulus to increase uterine weight. Nonestrogenic controls were given vehicle alone.

Animals were fasted overnight after the final dose. The rats were autopsied 24 h after the final dose. At autopsy, the uterine wet weight was determined, and uterine weight/body weight ratios (UWR) were calculated for each animal. The inhibition percentage of the estrogen-induced response was then calculated by the following formula: % inhibition = 100 × ([UWR<sub>EE2</sub> – UWR<sub>post agent</sub>]/UWR<sub>EE2</sub> – UWR<sub>control</sub>).

Four-Day OVX Rat Study. Virgin Sprague-Dawley rats (90 days old) were obtained from Harlan Nossan (Correzzana, Italy) and group housed on a 12-h light/dark cycle. The animals had ad libitum access to both food and tap water. Animals were randomized into experimental treatment groups, with six animals per treatment group. Compound administration was initiated 14 days after ovariectomy, to insure clearance of endogenous estrogen and to allow for

![Fig. 1. Chemical structure for CHF 4056. CHF 4056 is a new benzopyran derivative that is structurally related to the well known SERM levormeloxifene.](image-url)
acclimatization to the home cage. Compounds were dissolved in 0.5% methylcellulose and given by daily oral gavage in a volume of 3 ml/kg of body weight. Animals were dosed for 4 consecutive days and fasted in the evening after the final dose. On the next morning the animals were sacrificed by exsanguination from the abdominal aorta under anesthesia with ketamine and xilazine. The uterus were collected and weighed. Blood samples were allowed to clot at 4°C for 2 h and then centrifuged at 2000g for 10 min. Serum samples were collected and stored at −80°C; serum cholesterol was assayed using a high performance colorimetric assay (Roche, Mannheim, Germany). One horn of the uterus was removed, weighed, and transferred into a Tris buffer for analysis of eosinophil uterine peroxidase activity (see below).

Four-Week O VX Rat Study. Nine- to 10-month-old virgin Sprague-Dawley rats, weighing approximately 280 to 300 g (Harlan Nossan) were used in this study. The animals were acclimatized to the local vivarium conditions (22 ± 2°C; 12 h light/dark cycle) for 2 weeks and housed individually during the experimental period.

Bilateral ovariectomies were performed under ketamine hydrochloride (80 mg/kg) and xilazine hydrochloride (12 mg/kg) (Sigma) anesthesia except on sham-ovariectomized controls (sham). Upon recovery from anesthesia, animals were sorted into experimental groups (seven to nine rats per group, per experiment): sham, O VX, O VX plus 0.1 mg/kg EE2, O VX plus 0.01 to 10 mg/kg CHF 4056, and O VX plus 0.01 to 10 mg/kg levormeloxifene. Compound administration began 1 day postsurgery. Test compounds and vehicle (0.5% methylcellulose) were given by daily oral gavage in a volume of 3 ml/1000 g of body weight. Food (Teklad 9609 diet, 0.6% calcium, 0.4% phosphorus, and 1 IU/g vitamin D3; Teklad, Madison, WI) was available ad libitum to the sham-operated control rats. The food consumption of O VX rats was restricted to the same amount as that of sham rats to minimize the increase in body weight associated with ovariectomy. After 4 weeks of treatment, the rats were sacrificed by exsanguination from the abdominal aorta under anesthesia with ketamine and xilazine. Blood samples were allowed to clot at 4°C for 2 h and then centrifuged at 2000g for 10 min. Serum samples were collected and stored at −80°C; serum cholesterol was assayed using a high performance colorimetric assay (Roche), serum osteocalcin was determined by radioimmunoassay (Price and Nishimoto, 1980). At sacrifice uteri were removed and wet weight was determined on a Mettler balance to evaluate ovariectomy. From each animal, one uterine horn was used for histological evaluation, whereas the second horn was weighed and transferred into a Tris buffer for analysis of uterine eosinophil peroxidase activity (see below).

Bone Densitometry. Bone mineral density (BMD) was measured by dual energy X-ray absorptiometry (DEXA) using a Hologic QDR-1000 plus instrument equipped with dedicated software for small animal measurements. An ultrahigh-resolution mode (0.0254-cm line spacing and 0.0127-cm resolution) was used with a collimator of 0.63-mm diameter. This technique provides an integrated measure of both cortical and trabecular bone. In vivo DEXA measurements were carried out immediately before ovariectomy (baseline scan) and 4 weeks after surgery. The anatomic region examined was the lumbar spine L1–4. All animals were anesthetized before scanning with a mixture of ketamine and xilazine. For each scan a rat was placed in a supine position with the spine parallel to the long axis of the densitometer table. The lumbar spine was scanned using the pelvic bones as landmark; analysis of this site was accomplished by dividing vertebra and intervertebral spaces with sub-regional high resolution software and including only target vertebra in the global region of interest. The stability of the instrument was controlled by scanning a phantom each day. Percent protection was calculated by the following formula: % protection = (% change BMDtest compound − % chance BMDoxVx control)/(% chance BMDsham control − % chance BMDoxVx control) × 100.

Uterine Histology. Formalin-fixed uterus were processed for conventional paraffin embedding. Sections of about 5-μm thickness were obtained from each block. Slides were stained with hematoxylin and eosin before undergoing image analysis for the measurement of endometrium epithelia and myometrial thickness. The measurements were performed using an Ibacs20 computerized imaging system (Kontron/Zeiss, Welwyn Garden City, UK) run on a Kontron 386 personal computer. The images were acquired with a JVC (Yokohama, Japan) black and white camera fitted with a 50-mm macro lens (for myometrial thickness) or an Axioskop microscope (for endometrium epithelia). A black and white camera was used as it is more sensitive than a color one.

The dedicated software consists of the following steps: 1) image acquisition: the shading was previously corrected to eliminate defects/artifacts due to nonhomogeneous illumination of the measurement field. The samples were then placed on a transilluminator (myometrium) and on the microscope (magnification, 20×; endometrium epithelia); 2) image improvement: the quality of the image was improved by using special algorithms to show up the areas occupied by the myometrium and epithelium, respectively; and 3) field measurement: each area was measured and the mean thickness was calculated for each parameter.

For each parameter, the data was expressed in pixels ± S.E.M. The effects of the test compounds on the endometrium epithelia and myometrial thickness were also measured as percent increase relative to O VX, vehicle-treated controls, with sham control values defined as 100% and O VX controls defined as 0 (% increase = 100 × (pixeltest compound − pixeloxVx)/pixeloxVx).

Uterine Eosinophil Peroxidase Activity. The test protocol was based on the method described by White et al. (1991). The assay is based on the oxidation of o-phenylenediamine by uterine eosinophil peroxidase in the presence of hydrogen peroxide (H2O2).

In brief, after the removal of uterus and recording of whole uterine weight, the uterine horns were bisected. One horn from each animal was weighed and homogenized (Polytron Kinematica, Luzern, Switzerland) on ice in 50 mM Tris buffer, pH 8.0 (200 μl/mg of tissue) containing 0.05% (v/v) Triton X-100. Samples were centrifuged at 3000 rpm for 10 min at 4°C in a Beckman centrifuge J2-MI (Palo Alto, CA). The resulting supernatant was filtered through a 45-μm filter. Duplicate 200-μl aliquots of the filtered supernatant (equivalent to 1 mg of tissue) were added to a spectrophotometric cuvette. The reaction was initiated with the addition of 800 μl of substrate solution containing 3.5 mM o-phenylenediamine 2HCl and 0.0005% H2O2 in 50 mM Tris buffer pH 8. The apparent maximal velocity (mOD/min) was determined by continuous recording of the absorbance at 490 nM at room temperature.

Statistical Analysis. Results are expressed as mean ± S.E.M. Significance was determined by analysis of variance and, when analysis of variance was significant, by the Newman-Keuls test for posthoc multiple comparisons. Probability values of < 0.05 were considered to be statistically significant.

Results

Human ER-α and ER-β Binding Effects. CHF 4056 binds with high affinity to the human ER-α and ER-β. Binding Ki were 0.041 ± 0.011 and 0.157 ± 0.028 nM, respectively (Tab 1). This competitive binding assay showed that CHF 4056 competes for a single binding site on both ER-α and ER-β. For comparison, Ki values for some SERMs, 17β estradiol, and diethylstilbestrol were evaluated and reported in Table 1.

Immature Female Rat Assay. In immature female rats the ovaries do not produce estradiol; however, the uterus is fully responsive to exogenous estrogen and hence this model permits a ready measure of an agonist or antagonist action. In these rats, treatment with EE2 at 0.05 mg/kg p.o. for 3 days significantly increased uterine wet weight (150–200%) compared with vehicle-treated controls. This concentration of EE2 was the lowest producing near-maximal effect and was
CHF 4056: A New Nonsteroidal Estrogen Agonist/Antagonist

Fig. 2. A, Effect of CHF 4056 (■) and levormeloxifene (▲) on EE2-induced uterine weight increase in immature rats. B, Effect of CHF 4056 (■), levormeloxifene (▲) and EE2 (▲) on uterine weight in immature rats. The estrogenic and antiestrogenic activity was expressed as uterine weight/body weight ratios. Each point is the mean ± S.E.M. of one to three experiments. For each experiment n = 8. Statistical significance relative to the EE2-treated control (A) or vehicle-treated control (B) is denoted by * (P < 0.05) and ** (P < 0.01).

TABLE 1
Human ER-α and ER-β binding affinity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Estrogen Receptor Affinity (K&lt;sub&gt;i&lt;/sub&gt;)</th>
<th>nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ER-α (K&lt;sub&gt;i&lt;/sub&gt;)</td>
<td>ER-β (K&lt;sub&gt;i&lt;/sub&gt;)</td>
</tr>
<tr>
<td>CHF 4056</td>
<td>0.041 ± 0.011</td>
<td>0.157 ± 0.028</td>
</tr>
<tr>
<td>Roloxifene</td>
<td>0.071 ± 0.008</td>
<td>1.62 ± 0.348</td>
</tr>
<tr>
<td>Levormeloxifene</td>
<td>0.269 ± 0.022</td>
<td>1.83 ± 0.522</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>0.513 ± 0.044</td>
<td>0.624 ± 0.046</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>0.018 ± 0.009</td>
<td>0.015 ± 0.008</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>0.193 ± 0.039</td>
<td>0.496 ± 0.173</td>
</tr>
</tbody>
</table>

CHF 4056, administered daily p.o. before estrogen stimulus, completely antagonized EE2 stimulation of the uterus in a dose-dependent manner down to vehicle control levels (Fig. 2A). The dose-response relationship suggested an oral half-maximal antagonism, ED<sub>50</sub>, of 0.33 mg/kg-day with full antagonism at 1 to 10 mg/kg-day. In the same experimental conditions, levormeloxifene only partially antagonized EE2 stimulation of the uterus (maximal effect ~40% inhibition at 10 mg/kg-day) (Fig. 2A). CHF 4056 when administered alone did not increase uterine weight compared with vehicle-treated control rats, whereas levormeloxifene significantly increased this parameter in a dose-dependent way and a maximal agonist activity of ~65% that of EE2 was apparent at 1 mg/kg (Fig. 2B).

Four-Day OVX Rat Assay. Differential tissue selectivities of CHF 4056 have been demonstrated in OVX rats treated for 4 days with endpoints of serum cholesterol lowering and uterine stimulation (uterine weight and uterine peroxidase activity).

Specifically, 90-day-old OVX rats were dosed by oral gavage, for 4 consecutive days, commencing 2 weeks after ovariectomy to insure clearance of endogenous estrogen and to allow for acclimation to the home cage. A vehicle-treated sham and OVX control group was included in each experiment along with OVX rats given p.o. either EE2 or levormeloxifene as internal standards. Cholesterol levels were lowered significantly below vehicle-treated OVX rats by 0.01, 0.1, and 1 mg/kg CHF 4056 (Table 2). This protective effect was similar to the one observed with 1 mg/kg levormeloxifene and 0.1 mg/kg EE2.

In the same animal model, CHF 4056 had no significant effects on uterine wet weight and uterine peroxidase activity, which is a useful marker for estrogen-effected growth responses (Lyttle and DeSombre, 1977), whereas EE2 and levormeloxifene significantly stimulates these parameters versus OVX control (Tab 2).

Four-Week OVX Rat Assay. CHF 4056 effects were evaluated in 9- to 10-month-old OVX rats that were dosed for 4 weeks postsurgery and compared with OVX and sham controls.

Tissue-specific estrogen agonist effects were examined using uterine weight, uterine histology, uterine eosinophil peroxidase activity, BMD, serum osteocalcin, and serum cholesterol levels as endpoints.

Despite pair-feeding, at the end of the study, body weight gain in OVX controls (+36.5 g) was greater than that in sham controls (+7.5 g), whereas body weight gain in 0.1 mg/kg CHF 4056-treated OVX rats (−24 g) was significantly lower than in OVX and sham controls. Although to a lesser extent than EE2, 0.1 to 10 mg/kg CHF 4056, like levormeloxifene, lowered body weight gain (+4.6 and −3.8 g at 1 and 10 mg/kg/day, respectively) to significantly below OVX in a dose-related manner. The effects of ovariectomy, estrogens, and SERMs such as tamoxifen and nafoxidine on body weight were previously shown to reflect changes in amount of adipose tissue (Sato et al., 1996). Accordingly, we can hypothesize that also CHF 4056 may affect OVX-stimulated accumulation of adipose tissue.

Four weeks after surgery, a significantly higher bone loss from baseline in OVX rats as compared with sham rats was observed in the lumbar spine L1−4 (percentage change in BMD was −9.39 ± 0.60 and −0.11 ± 0.75%, respectively; P < 0.01). The effect of CHF 4056 on BMD as measured by DEXA is shown in Fig. 3A. At doses of 0.1 to 1 mg/kg/day, CHF 4056 significantly attenuated ovariectomy effects on BMD, with maximal efficacy observed at 1 mg/kg (50% protection).
protection appeared to be similar to the one of 0.1 mg/kg/day EE2 or levormeloxifene 3 mg/kg/day.

Serum osteocalcin, a well-known biochemical marker of bone turnover, was significantly increased (69%) in OVX rats compared with sham controls (Fig. 3B). This increase was fully prevented by treatment with CHF 4056 or by treatment with levormeloxifene and EE2.

The OVX group had a tendency toward higher serum cholesterol levels compared with the sham group, although the significance varied from experiment to experiment. 0.1 mg/kg-day EE2 or 0.1 to 10 mg/kg-day levormeloxifene significantly decreased total serum cholesterol levels, compared with both sham and OVX controls (Fig. 4). Similarly, 0.1 to 10 mg/kg-day CHF 4056 lowered cholesterol to below OVX and sham in a dose-dependent manner. The minimally effective hypocholesterolemic dose of CHF 4056 was 0.1 mg/kg-day (mean serum cholesterol 38% lower than OVX control, \( P < 0.01 \)). Maximal lowering of cholesterol (69% relative to the OVX control) occurred at 10 mg/kg-day and the ED50 (half-maximal efficacy) over this dosage range was 0.12 mg/kg-day.

As expected, uterine weight in OVX rats was significantly decreased compared with that in sham control, and EE2 (0.1 mg/kg) treatment maintained uterine weight in OVX rats to a level of sham control (Fig. 5A). At 0.01 mg/kg-day CHF 4056 had no effect on uterine weight compared with OVX controls. In OVX rats treated with CHF 4056 at 0.1 to 10 mg/kg/day, uterine weight was significantly lower than both sham controls and EE2-treated OVX rats, whereas it increased slightly but significantly compared with OVX controls (Fig. 5A).

---

**TABLE 2**

Four-day OVX rat study: effect of CHF 4056 on serum cholesterol, uterine weight, and uterine peroxidase activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Serum Cholesterol Levels (mg/dl)</th>
<th>Uterine Weight (mg/g body weight)</th>
<th>Uterine Peroxidase Activity (mOD/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.1</td>
<td>119.5 ± 4.97</td>
<td>0.39 ± 0.01</td>
<td>1.24 ± 0.21</td>
</tr>
<tr>
<td>OVX</td>
<td>0.1</td>
<td>20.88 ± 3.59**</td>
<td>0.93 ± 0.02</td>
<td>1.05 ± 0.26</td>
</tr>
<tr>
<td>OVX + EE2</td>
<td>0.01</td>
<td>65.93 ± 6.47**</td>
<td>0.42 ± 0.02</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>OVX + CHF 4056</td>
<td>0.1</td>
<td>56.14 ± 7.13**</td>
<td>0.45 ± 0.03</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>26.21 ± 3.91**</td>
<td>0.42 ± 0.03</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td>OVX + levormeloxifene</td>
<td>0.1</td>
<td>62.55 ± 11.61</td>
<td>0.58 ± 0.03</td>
<td>0.73 ± 0.02**</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>27.67 ± 6.22**</td>
<td>0.72 ± 0.02**</td>
<td>0.73 ± 0.02**</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of CHF 4056 (□), levormeloxifene (▼), and EE2 (▲) on bone mineral density of lumbar vertebrae L1–4 (A) and serum osteocalcin (B) in OVX rats treated for 4 weeks. Bone mineral density values are reported as percent change in BMD from the baseline. Each point is the mean ± S.E.M. of two experiments. Statistical significance relative to vehicle-treated OVX rats is denoted by * (\( P < 0.05 \)) and ** (\( P < 0.01 \)). For each experiment \( n = 7–9 \).

**Fig. 4.** Effect of CHF 4056 (□), levormeloxifene (▼), and EE2 (▲) on serum cholesterol levels in OVX rats treated for 4 weeks. Each point is the mean ± S.E.M. of two experiments. Statistical significance relative to vehicle-treated OVX rats is denoted by * (\( P < 0.05 \)) and ** (\( P < 0.01 \)). For each experiment \( n = 7–9 \).

**Fig. 5.** Effect of CHF 4056 (□), levormeloxifene (▼), and EE2 (▲) on uterine weight in OVX rats treated for 4 weeks. Each point is the mean ± S.E.M. of two experiments. Statistical significance relative to vehicle-treated OVX rats is denoted by * (\( P < 0.05 \)) and ** (\( P < 0.01 \)). For each experiment \( n = 7–9 \).

**** Statistical significance relative to vehicle-treated OVX rats (\( P < 0.01 \)).
5A). Levormeloxifene’s effect on uterine weight was slightly more pronounced compared with the one of CHF 4056.

In our experimental conditions, eosinophilic peroxidase activity was ~165-fold greater in sham than in OVX uteri. 0.1 mg/kg/day EE2 treatment significantly elevated eosinophilic peroxidase activity up to sham level (Fig. 5B). In contrast, CHF 4056 at 0.01 to 10 mg/kg/day had no significant effect on eosinophilic peroxidase activity compared with OVX uteri, whereas levormeloxifene significantly increases this parameter (Fig. 5B).

To better clarify possible stimulatory effects of CHF 4056 on the endometrium, uteri were evaluated at higher resolution by histological techniques. Uterine epithelial and myometrial thickness were significantly decreased in OVX control rats compared with sham controls (73 and 40%, respectively). EE2 and levormeloxifene treatment significantly increased the thickness of the endometrial epithelium (120 and 124%, respectively, Fig. 6A), whereas CHF 4056 had minimal insignificant effects. In the myometrium, EE2 significantly increased the thickness; instead, CHF 4056 and levormeloxifene had insignificant effects (Fig. 6B).

To sum up, uterine histology parameters showed that marked atrophy of uterus involving the epithelium and muscularis due to ovariectomy was not significantly decreased by oral treatment with CHF 4056 (0.01–10 mg/kg/day). Instead, EE2 and levormeloxifene, which caused full disappearance of epithelial atrophy, maintained endometrium histology at the levels of sham controls.

**Discussion**

Estrogens can inhibit bone resorption and consequently they can be used for the treatment and prevention of postmenopausal osteoporosis; in this respect, it is well established that long-term hormone replacement therapy reduced fractures rate in postmenopausal women (Lindsay et al., 1980; Weiss et al., 1980). Moreover, estrogens have beneficial activities in the cardiovascular system (Zumoff, 1993; Grodstein et al., 1997) and may also have beneficial effects in the central nervous system, as several studies have linked them to improvements in cognitive function and in delaying the onset of Alzheimer’s disease (Grady et al., 1992).

Despite the overall health benefits of estrogens, relatively few women actually considered hormone replacement therapy or the therapy was discontinued within a year (Lobo, 1995). This reduced compliance with therapy is because of
the potential link between estrogen replacement therapy and breast/endometrial cancer and some other side effects including breakthrough bleeding (Jacobs, 2000; Evans and Turner, 1995). Thus, a therapeutic agent that has estrogen-agonist effects on the skeleton and cardiovascular system and estrogen-antagonist activities on the uterus and breast would be highly desirable for postmenopausal women.

CHF 4056 is a new nonsteroidal estrogen agonist/antagonist that binds with high affinity to the human ER-α and ER-β (Kᵢ were 0.041 and 0.157 nM, respectively). Compared with raloxifene—the only SERM currently approved for the prevention and treatment of postmenopausal osteoporosis with possible additional protective effects on breast cancer, cardiovascular diseases, and cognitive functions—the ER-α affinity of CHF 4056 was similar, whereas the affinity for ER-β was 10-fold higher (Tab 1). It appears quite clear today that ER-β has biological roles that are distinct from those of ER-α (Gustafsson, 1999). Knock-out mice deficient in ER-β show a distinct phenotype when compared with that of ER-α −/− mice. In this respect, because it appears that ER-β ligands could prove to be useful therapeutically in targeting the neuroprotective (Kuiper et al., 1998) and cardioprotective actions of oestrogens (Mahela et al., 1999), the higher affinity for this receptor may qualify CHF 4056 as a SERM with more potential on brain and/or cardiovascular system than raloxifene.

However, it should be noted that in light of recent studies the action of estrogens on cardiovascular system is still a matter of debate (Clemett and Spencer, 2000) and that to date we have no experimental evidence that CHF 4056 could have a different pharmacological profile compared with raloxifene either in the central nervous system (e.g., cognitive function, hot flushes) or in the cardiovascular apparatus.

In immature rats, the compound antagonized estrogen stimulation of the uterus down to the level of vehicle-treated controls with no estrogenicity. In the same experimental conditions, the structurally related compound levormeloxifene only partially blocked estrogen-induced uterine weight gain (~ 40% inhibition) because of its evident agonist activity (~65% that of EE2). Thus, although CHF 4056 and levormeloxifene share the same benzopyran core, the pharmacological profile of CHF 4056 in immature rats appears strictly different. Studies are in progress to clarify the structural features of CHF 4056 that are relevant in reducing the uterine-stimulating effects associated with levormeloxifene in immature rats. Preliminary data indicate the importance of the 4-methylenic hinge between the aminoalchoxyphenyl side chain and the benzopyran moiety of CHF 4056, the deletion of which results in increases in estrogenic activity (data not shown).

Differential tissue selectivities of CHF 4056 have been studied in OVX rats treated for 4 days/weeks with endpoints of body weight, serum cholesterol lowering, bone tissue, and uterine stimulation. Ovariectomy resulted in significant osteopenic responses after 4 weeks in the lumbar spine L1–4 as measured by DEXA densitometric techniques. CHF 4056 was able to reduce the decreases in BMD of L1–4 lumbar vertebrae at doses of 0.1 to 1 mg/kg/day; maximal efficacy (50% protection at 1 mg/kg/day) was indistinguishable from that of 0.1 mg/kg/day EE2. Moreover, the OVX-induced serum osteocalcin gain was completely prevented by treatment with 1 mg/kg-day CHF 4056. These results showed that CHF 4056 maintained lumbar spine BMD and bone turnover at levels comparable with those seen with EE2. Whereas the results of these studies show that CHF 4056 will provide protection against OVX-induced bone loss after 4 weeks, a longer term study will be performed to show that these effects will be maintained and that CHF 4056 are not simply delaying the eventual loss of BMD due to estrogen deficiency.

EE2 produced a marked hypcholesterolemic effect in OVX rats; this effect is attributed to up-regulation of hepatic LDL receptors, resulting in enhanced clearance of circulating LDL (Brown and Goldstein, 1980). Under the same experimental conditions, CHF 4056 significantly decreased total serum cholesterol in a dose-dependent manner, indicating that the compound acts as an estrogen receptor agonist on serum cholesterol in rat models of postmenopausal osteoporosis. This inhibitory effect on serum cholesterol levels was similar after 4 days and 4 weeks of treatment (Table 2; Fig. 4), indicating that no tolerance occurs toward CHF 4056’s estrogen agonist effect on cholesterol metabolism.

In line with the results observed in the immature rats assay, also in OVX rats CHF 4056 has minimal stimulatory effects on the uterus. In fact, after 4 weeks of treatment, histological analysis of the uterine tissue showed that CHF 4056 has nonsignificant effects on endometrium epithelia thickness. In contrast, EE2 and levormeloxifene increased epithelial thickness (120 and 124%, respectively), demonstrating significant uterine hypertrophic effects. This trend was reproduced in analysis of uterine eosinophil peroxidase activity to show that CHF 4056 is less stimulatory in the uterus than estrogen or levormeloxifene. CHF 4056 caused, after 4 weeks of treatment, a statistically significant increase in uterine weight relative to the OVX controls, although it was much less pronounced than that observed in EE2-treated animals. However, this marginal effect on uterine weight was coupled with the lack of a stimulatory activity on endometrium epithelia and uterine peroxidase, indicating that it may not be clinically relevant. In favor of this consideration, CHF 4056’s profile on uterine tissue in OVX rats is superimposable to the one previously observed for raloxifene (Black et al., 1994), which, in clinical studies with postmenopausal women, did not show stimulatory effects on the uterus (Delmas et al., 1997). In contrast, the clinical development of levormeloxifene, which significantly affected the epithelia thickness (Fig 6A) and the uterine eosinophil peroxidase activity (Fig 6B), was discontinued after reports of endometrial thickening side effects in postmenopausal women. Thus, CHF 4056 is expected to have a better tissue selectivity profile in postmenopausal women compared with the structurally related compound levormeloxifene. The fact that this tissue selectivity is not simply the result of selective tissue distribution is confirmed by the ability of CHF 4056 to completely antagonize the effects of estrogen on the immature rat uterus.

In conclusion, the new benzopyran derivative CHF 4056 has promise as an agent with beneficial bone and cardiovascular effects. Our data showed that CHF 4056, EE2, and levormeloxifene have overlapping patterns of effects on bone, cholesterol, and adipose tissue but distinct activities on uteri. The minimal uterine stimulation suggests a potential therapeutic advantage of CHF 4056 over EE2. Moreover CHF 4056, being characterized by marked estrogen antagonist activity, may be of interest in the prevention and treatment of estrogen-dependent human tumors. Concerning this, in-
vestigations in in vitro assays (MCF-7 proliferation) and in animal model of postmenopausal breast cancer with CHF 4056 are ongoing.

References
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (Io) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.

Address correspondence to: Dr. Maurizio Civelli, Department of Pharmacology, Chiesi Pharmaceuticals S.p.A., Via Palermo 28/A, 43100 Parma, Italy. E-mail: m.civelli@chiesigroup.com