Pharmacological Actions of a Novel, Potent, Tissue-Selective Benzopyran Estrogen

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ABSTRACT

We have identified a new benzopyran derivative, 3-(4-methoxy)phenyl-4-[[4-[2-(1-piperidinyl)ethoxy]phenyl][methyl]-2H-1-benzopyran-7-ol hydrochloride (CHF 4227), with improved in vivo estrogen agonist/antagonist effects. CHF 4227 binds with high affinity to the human estrogen receptor-α and -β (dissociation constant $K_d = 0.017$ and 0.099 nM, respectively). In immature rats, oral administration of CHF 4227 for 3 days inhibited the uterotrophic action of 17α-ethynyl estradiol (EE2) ($ED_{50} = 0.016$ mg/kg · day); raloxifene was 25 times less potent as estrogen agonist ($ED_{50} = 0.39$ mg/kg · day), whereas both compounds were found to be devoid of uterotrophic activity. In line with its estrogen antagonist effect, CHF 4227 significantly prevented the development of dimethylbenz[a]anthracene (DMBA)-induced mammary tumors, the incidence being reduced from 87.5 to 26.3% 6 months after DMBA administration. In ovariectomized (OVX) rats treated orally for 4 weeks, CHF 4227 completely inhibited OVX effects on bone density ($ED_{50} = 0.003$ mg/kg · day) and on serum osteocalcin levels. The protective effects on bone were comparable with those achieved with EE2, whereas raloxifene was less efficacious and 100 times less potent. CHF 4227 reduced serum cholesterol ($ED_{50} = 0.007$ mg/kg · day) and had little to no stimulatory effects on uterine weight, uterine peroxidase activity, and endometrium epithelial thickness. In conclusion, CHF 4227 compares favorably in efficacy and potency with raloxifene in preventing bone loss and in antagonizing EE2 stimulation of the uterus. This profile along with the minimal uterine stimulation suggests a therapeutic advantage to CHF 4227 over EE2 or raloxifene for the treatment of postmenopausal women.

Compounds that can bind to and activate the estrogen receptors (ERs) but cause differential estrogenic or antiestrogenic responses in specific tissue are currently being investigated as alternatives to estrogens for the prevention and treatment of chronic postmenopausal pathologies.

Estrogen replacement therapy has been used primarily to prevent perimenopausal symptoms in addition to preventing and treating osteoporosis (Kiel et al., 1987). Although many other beneficial activities of estrogens have been described, including improvements in cognitive functions and decreases in the risk of coronary disease through their effect on lipids profile (Cumming, 1991; Stampfer and Colditz, 1991), there are several undesirable side effects associated with chronic estrogen therapy that create difficulties in compliance.

In particular, the return of withdrawal bleeding is one of the major reasons for a woman stopping estrogen therapy. In addition, estrogens when administered without progesterin, 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).

These adverse effects of estrogens have led to an increased interest in drugs thought to maintain estrogen protective effects on bone and serum lipid profile, whereas being characterized by antagonist activity or no activity in reproductive tissues. In addition to an improved side effect profile, compared with estrogens, a tissue-selective estrogen agonist/antagonist may have potential utility in the treatment and prevention of estrogen-dependent cancer, because of its antiestrogen effects on reproductive tissues (Eppenberger et al., 1991).

The well known antiestrogen tamoxifen maintains bone mass and lower blood lipids in rats (Jordan et al., 1987) and postmenopausal women (Love et al., 1994). On the other hand, the estrogenic effects in the endometrium in women can result in an increased incidence of endometrial cancer coincident with prolonged tamoxifen therapy (Kedar et al., 1994), preventing its use to mimic some of the helpful actions of estrogens after the menopause. Subsequent work has 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 compounds with this possible spectrum of activities (Sato et al., 1999; Miller et al., 2001). However, the development of many of these compounds as drugs has failed because of their unacceptable uterine changes (Sato et al., 1996; Greese et al., 1997). Moreover, although in humans raloxifene increased bone mineral density, showing a significant reduction in vertebral fractures, it is less effective than other inhibitors of bone resorption at the skeleton (Khovidhunkit and Shoback, 1999). In fact, raloxifene increases bone mineral density at the spine by 1.5 to 2% (Delmas et al., 1997) compared with 4 to 5% for estrogen replacement therapy and 7 to 9% for bisphosphonates (Liberman et al., 1995). There is increasing evidence that some compounds with a benzopyran structure can initiate estrogen agonist/antagonist actions when applied to biological systems (Sato et al., 1999). We demonstrated that some benzopyran derivatives (i.e., 3-phenyl-4-piperidinyl-ethoxy-benzyl-benzopyrans and, in particular, 3-phenyl-4-[(4-[(2-1-piperidinyl)ethoxy]phenyl)methyl]-2H-1-benzopyran-7-ol, CHF 4056) are SERMs that produce beneficial effects on bone and cholesterol levels similar to those previously reported for raloxifene, whereas they maintain antagonist effects on the uterus (Galbiati et al., 1999, 2002). These compounds diverged dramatically from EE2 and from others SERMs characterized by a benzopyran structure such as levormeloxifene, in its lack of significant estrogenic effects on uterine tissue.

In the present study we characterized the pharmacological properties of a novel benzopyran derivative CHF 4227 (4-methoxy derivative of CHF 4056, Fig. 1), which was found in our search for tissue-selective estrogen characterized by an improved estrogen agonist/antagonist activity compared with raloxifene in bone, serum lipids, and in uterine tissue. A molecule characterized by this pharmacological profile may have therapeutic advantages over the marketed raloxifene for the treatment of postmenopausal pathologies, such as osteoporosis and estrogen-dependent cancer.

We investigated the effect of CHF 4227 for the following activities: 1) binding affinity to human ER-α and β; 2) anti-estrogenic and estrogenic effect on uterine growth in an immature female rat model (Eppenberger et al., 1991); 3) estrogenic effects on bone, serum cholesterol, and uterus in an O VX rat model of postmenopausal bone loss (Kalu, 1991); and 4) prevention of development of dimethylbenz[a]anthracene (DMBA)-induced mammary carcinoma in rats (Huggins et al., 1961).

Herein, the pharmacological properties of CHF 4227 observed in the above-mentioned experimental models are reported. In addition, its in vivo effects were compared with those of EE2 and raloxifene.

Materials and Methods

All animals studies were performed in strict accordance with the Decreto 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 #86/606/CEE).

**Chemicals.** 3-[4-Methoxyphenyl-4-[[4-[[2-1-piperidinyl]ethoxy]phenyl][methyl]-2H-1-benzopyran-7-ol hydrochloride (mol.wt. = 508.06; CHF 4227) and [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl]-[4-[[2-1-piperidinyl]ethoxy]phenyl]methane hydrochloride (mol.wt. = 510.6; raloxifene) were synthesized by Chiesi Farmaceutici S.p.A. (Parma, Italy). EE2 (mol. wt. = 296.4) and diethylstilbestrol (mol. wt. = 288.4) were obtained from Sigma-Aldrich (St. Louis, MO). Kits for radioimmunoassay of osteocalcin were supplied by Biomedical Technologies (Stoughton, MA). All other reagents were purchased from Sigma-Aldrich.

**Human ER-α and ER-β Binding.** ER-α and β binding analysis was performed as described previously (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 (PerkinElmer Life Sciences, Boston, MA), increasing concentration of unlabeled CHF 4227 (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% hydroxylapatite 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 and was centrifuged at 10,000g for 5 min, and the supernatant was aspirated. The wash step was repeated two more times and then the hydroxylapatite pellet was resuspended in 400 μl of ethanol, transferred to a scintillation vial, and counted. Nonspecific binding was defined as that which occurred in the presence of 1 μM diethylstilbestrol and represented 10 to 15% of the total binding. Kᵢ values were calculated using the equation of Cheng and Prusoff (1973) using the observed half-maximal inhibition concentration (IC₅₀) of the tested compound, the concentration of radioligand used 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.** Female Sprague-Dawley rats (21 days old), weighing approximately 40 to 50 g (Charles River Italica, Calco, Italy) were treated by oral gavage with either vehicle (0.5% methylcellulose, 3 ml/kg), CHF 4227 (0.001–10 mg/kg · day), raloxifene (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 (UWRs) were calculated for each animal. The inhibition percentage
of the estrogen-induced response was then calculated by the following formula: % inhibition = 100 × \frac{(UWR_{EE2} - UWR_{test\ agent})}{(UWR_{EE2} - UWR_{control})}.

**Four-Week OVX Rat Study.** Virgin Sprague-Dawley rats (9–10 month old), weighing approximately 280 to 300 g (Harlan Nossan, Correzzana, Italy) 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 ovarioctomies were performed under ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (12 mg/kg) (Sigma-Aldrich) anesthesia except on sham-ovariectomized controls (sham). Upon recovery from anesthesia, animals were sorted into experimental groups (7–9 rats/group/experiment): sham, OVX, OVX plus 0.1 mg/kg EE2, OVX plus 0.001 to 10 mg/kg CHF 4227, and OVX plus 0.1 to 10 mg/kg raloxifene. Compound administration began 1 day post-surgery. 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 (0.6% calcium, 0.4% phosphorus, and 1 IU/g vitamin D3, Teklad 9609 diet; Madison, WI) was available ad libitum to the sham-operated control rats. The food consumption of OVX rats was restricted to the same amount as that of sham rats to minimize the increase in body weight associated with ovarioctomy. After 4 weeks of treatment, the rats were sacrificed by exsanguination from the abdominal aorta under anesthesia with ketamine and xylazine. 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 colorimeter assay (Roche Applied Science, Mannheim, Germany), 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 ovarioctomy. 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 ultra high-resolution mode (line spacing 0.0254 mm and resolution 0.0127 mm) 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 after ovarioctomy (baseline scan) and 4 weeks after surgery. The anatomical region examined was the lumbar spine L1 to L4. All animals were anesthetized before scanning with a mixture of ketamine and xylazine. For each scan a rat was placed in a supine position with the spine parallel to the long axis of the densitometry table. The lumbar spine was scanned using the pelvic bones as landmark; analysis of this site was accomplished by dividing vertebral and intervertebral spaces with subregional 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 every day. Percentage of protection was calculated by the following formula: % protection = \frac{(\% \text{ change } BMD_{\text{test compound}} - \% \text{ change } BMD_{\text{OVX control}})/(\% \text{ change } BMD_{\text{sham control}} - \% \text{ change } BMD_{\text{OVX control}})}{100} × 100.

**Uterine Histology.** Formalin-fixed uteri were processed for conventional paraffin embedding. Sections of about 5 µm in 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 Ibas20 computerized imaging system run on a 386 personal computer (Kontron Instruments, Watford, Herts, UK). The images were acquired with a black-and-white camera (JVC, Yokohama, Japan) fitted with a 50-mm macro lens (for myometrial thickness) or an Axioscope microscope (for endometrium epithelia). A black-and-white camera was used because 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 (original 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 were expressed in pixel ± S.E.M. The effects of the test compounds on the endometrium epithelia and myometrial thickness were also measured as percentage of increase relative to OVX, vehicle-treated controls, with sham control values defined as 100% and OVX controls defined as 0% (increase = \frac{\text{pixel}_{\text{test agent}} - \text{pixel}_{\text{OVX}}}{\text{pixel}_{\text{sham}} - \text{pixel}_{\text{OVX}}} × 100).

**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 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 Kinematics, 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 centrifuge (J2-MI; Beckman Coulter, Fullerton, 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.

**Prevention of Development of DMBA-Induced Mammary Tumors.** Mammary carcinomas were induced in rats by a single intragastric administration of 20 mg of DMBA (Sigma-Aldrich) in 1 ml of corn oil at 50 days of age. Forty days later, tumor measurement (the two largest perpendicular diameters of each tumor) was performed with calipers biweekly. The animals were treated for 6 months with 0.5% methylcellulose (n = 40) or CHF 4227 2 mg/kg · day (n = 19), commencing 3 days before the oral administration of DMBA. Some of the control animals died or were killed by cervical dislocation under anesthesia before the end of the experiment because their tumors grew too large. The tumors size and number in these rats at death together with those measured at later time from the surviving animals were used for the analysis of the incidence of tumors, average tumor number per rat, and average tumor size per tumor-bearing animal. All rats were killed 6 months after DMBA administration.

**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 post hoc multiple comparisons. Probability values of <0.05 were considered to be statistically significant. Analysis of the incidence of development of mammary tumors was performed using Fisher’s exact test. Analysis of the tumor number per rat was carried out using the Mann-Whitney U test.

**Results.** Human ER-α and ER-β Binding Effects. CHF 4227 binds with high affinity to purified recombinant human ER-α (Kd = 0.017 ± 0.002 nM) and ER-β (Kd = 0.099 ± 0.005 nM). Compared with the well known SERM raloxifene (Kd for...
ER-β = 1.62 ± 0.348 and for ER-α = 0.071 ± 0.008 nM), the affinity for ER-α and ER-β was 4- and 16-fold higher, respectively. This competitive binding assay showed that CHF 4227 competes for a single binding site on both ER-α and ER-β.

**Immature Female Rat Assay.** In immature female rats, treatment with EE2 at 0.05 mg/kg p.o. for 3 days significantly increased uterine wet weight (~170%) compared with vehicle-treated controls. This concentration of EE2 was the lowest producing near-maximal effect and was chosen on the basis of preliminary dose-response experiments.

CHF 4227 administered orally before estrogen stimulus inhibited the uterotrophic action of EE2 in a dose-related manner, and total antagonism was observed at 0.1 to 1 mg/kg · day (Fig. 2A). The dose-response relationship suggested an oral half-maximal antagonism, ED50, of 0.016 mg/kg · day. In the same experimental conditions, raloxifene (ED50 = 0.39 mg/kg · day) was about 25 times less potent than CHF 4227 as estrogen antagonist. CHF 4227, like raloxifene, when administered alone did not increase uterine weight compared with vehicle-treated control rats (Fig. 2B).

**OVX Rat Assay.** CHF 4227 effects on a number of estrogen target tissues (bone, uterus, and serum cholesterol) were evaluated in 9- to 10-month-old OVX rats that were dosed for 4 weeks post-surgery and compared with OVX and sham controls.

Despite pair-feeding, at the end of the study, body weight gain in OVX rats (38 g) was higher than that in sham controls (5 g). Body weight gain in 0.1 mg/kg · day EE2-treated OVX rats (~15 g) was significantly lower than in OVX and sham controls. These effects of ovariectomy and EE2 on body weight were previously shown to reflect changes in amount of adipose tissue (Sato et al., 1996). Although to a lesser extent, CHF 4227, like raloxifene, mimicked EE2 in preventing gain of body weight in this model (body weight gain over 4 weeks was 8 and 4 g at 0.1 and 1 mg/kg · day, respectively).

Four weeks after surgery, ovariectomy induced a significant osteopenic effect in the lumbar spine L1 to L4 as measured by DEXA (7.23% reduction in BMD in OVX rats compared with sham rats was observed; Fig. 3). CHF 4227 completely inhibited ovariectomy effects on BMD with ED50 of about 0.003 mg/kg · day (100% protection against ovariectomy-induced bone loss at 1 mg/kg · day). This effect was comparable with that achieved with EE2, whereas raloxifene was less efficacious (maximal protection: 60% at 1–10 mg/kg · day) and about 100 times less potent (Fig. 3).

Serum osteocalcin, a well known biochemical marker of bone turnover, was significantly higher in OVX rats (47.16 ± 2.06 ng/ml), compared with sham controls (28.30 ± 2.86 ng/ml). This increase was fully prevented by treatment with 0.1 to 1 mg/kg · day CHF 4227 or by treatment with 0.1 mg/kg · day EE2.

The OVX rats had a tendency toward higher serum cholesterol levels compared with the sham group, although the significance varied from experiment to experiment. The 0.1 mg/kg · day EE2 significantly decreased total serum cholesterol levels, compared with both sham and OVX controls (Fig. 4). Similarly, CHF 4227 dose dependently lowered cholesterol in OVX rats, with half-maximal efficacy ED50 of 0.007 mg/kg; the maximally effective hypocholesterolemic dose was observed at 0.1 mg/kg · day (mean serum cholesterol 74% lower than OVX control). CHF 4227 was about 50 times more
potent than raloxifene (ED50 = 0.33 mg/kg · day) in lowering serum cholesterol levels compared with controls in this model.

Uterine weight in OVX rats was significantly decreased compared with that in sham control, and EE2 (0.1 mg/kg · day) treatment maintained uterine weight in OVX rats to a level of sham control (Fig. 5A). At 1 mg/kg · day CHF 4227 had no effect on uterine weight compared with OVX controls. In OVX rats treated with CHF 4227 at 0.001, 0.01, 0.1, and 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). CHF 4227 uterine weight effects were not significantly different from raloxifene effects, suggesting marginal effects on the uterus.

The degree of eosinophilic peroxidase activity induction in the uterus may be a useful marker for estrogen-effected growth responses (Lyttle and DeSombre, 1977). In our experimental conditions, eosinophilic peroxidase activity was about 100-fold greater in sham than in OVX uteri. EE2 (0.1 mg/kg · day) treatment significantly elevated eosinophilic peroxidase activity up to sham levels (Fig. 5B). In contrast, CHF 4227 at 0.001 to 10 mg/kg · day and raloxifene at 0.1 to 10 mg/kg · day had no significant effect on eosinophilic peroxidase activity compared with OVX uteri.

To clarify possible stimulatory effects of CHF 4227 on the endometrium, uteri were evaluated at higher resolution by histological techniques. Uterine epithelial thickness was significantly decreased in OVX control rats compared with sham controls (63%). EE2 treatment significantly increased the thickness of the endometrium epithelia (Fig. 6), maintaining uterine histology at the levels of sham controls, whereas CHF 4227 and raloxifene had minimal insignificant effects (P > 0.05). CHF 4227 and raloxifene had not significant effects also in the myometrium (data not shown).

Prevention of Development of DMBA-Induced Mammary Tumors. Mammary carcinoma induced by DMBA in rats is a widely used model to study the factors that control hormone-sensitive breast cancer in women. In fact, the development and growth of these tumors are particularly sensitive to the stimulatory action of estrogens and prolactin (Asselin et al., 1977).

As shown in Fig. 7, 6 months after DMBA administration, 87.5% of controls animals (35 of 40) had developed at least one palpable mammary carcinoma. In contrast, treatment with 2 mg/kg · day CHF 4227 caused a significant inhibition of tumor development, the incidence being reduced to 26.3% on day 180 of the study (5 of 19 rats developed single mammary tumor). It is of interest to note that mean tumor number per animal was markedly decreased from 2.40 ± 0.29 in controls animals; in the CHF 4227 group one tumor had a large area (4550 mm²), about 2 times the largest tumors present in the control group, whereas the other four tumors were much smaller (4, 56, 64, and 90 mm², respec-
Fig. 6. Effect of CHF 4227 (■), raloxifene (▼), and EE2 (▲) on endometrium epithelia thickness in OVX rats treated for 4 weeks. Data are expressed as pixel ± S.E.M (n = 7 to 8). Statistical significance relative to vehicle-treated OVX rats is denoted by *, P < 0.01.

Fig. 7. Effect of daily oral administration of 2 mg/kg CHF 4227 (■) or vehicle (▲) on the number of animals who developed palpable mammary carcinoma induced by DMBA throughout the 180-day observation period. Data are expressed as percentage of the total number of animals in each group. Each point is the mean ± S.E.M. of one experiment. The inset represents the effect on average tumor number per animal (mean ± S.E.M. of one experiment. The inset)

Discussion

Long-term use of estrogens is effective in reducing the risks associated with the decreased production of ovarian steroid after the climacteric (Kiel et al., 1987). Unfortunately, concerns regarding the increased incidence of breast and endometrial cancer and some other undesirable side effects, including breakthrough bleeding, are considerable drawbacks to the initiation and long-term use of estrogens (Vesey, 1984; Jacobs, 2000). Accordingly, a therapeutic agent that can mimic the protective effects of estrogen on nonreproductive tissues without inducing significant proliferative effect on the uterus and breast, would be highly desirable for postmenopausal women.

CHF 4227 is a novel orally active nonsteroideal estrogen agonist/antagonist. In vitro binding studies have demonstrated that CHF 4227 displays high affinity to the human ER-α and ER-β (Kᵢ values of 0.017 and 0.099 nM, respectively). Differential tissue selectivities of CHF 4227 have been studied in OVX rats treated for 4 weeks with endpoints of body weight, serum cholesterol lowering, bone tissue, and uterine stimulation.

Bilateral ovariectomy in the rat decreases circulating serum estrogen levels, which results in increased bone turnover. Bone loss in the OVX rat reflects the skeletal changes observed in postmenopausal women: rapid decrease in bone mass, preferential loss of trabecular bone, and responsiveness to estrogen replacement therapy (Kalu, 1991). In our experimental conditions, ovariectomy resulted in significant osteopenic responses after 4 weeks in the lumbar spine L1 to L4 as measured by DEXA densitometric techniques. CHF 4227 prevented the ovariectomy-induced reduction in BMD of L1 to L4 lumbar vertebrae, with an ED₅₀ of about 0.003 mg/kg · day. Total protection against OVX-induced bone loss was observed between 0.1 and 1 mg/kg · day; moreover, at the same doses CHF 4227 fully prevented also the rise in serum osteocalcin levels induced by castration. In line with previously reported data (Black et al., 1994), raloxifene only partially prevented the OVX effects on bone tissue (maximal effect = 60% protection) seeming less effective and about 100 times less potent than CHF 4227. Thus, our data suggest that CHF 4227 might exert in clinical studies an improved benefit in the bone tissue compared with raloxifene, which does not seem to be as efficacious as estrogens and bisphosphonates (Liberman et al., 1995). Although the results of these studies show that CHF 4227 will provide protection against OVX-induced bone loss after 4 weeks, a longer term study is in progress to show that these effects will be maintained and that CHF 4227 are not simply delaying the eventual loss of BMD due to estrogen deficiency.

EE2 produced a marked hypocholesterolemic effect in OVX rats; this effect is attributed to up-regulation of hepatic low-density lipoprotein receptors, resulting in enhanced clearance of circulating low-density lipoprotein (Brown and Goldstein, 1980). Under the same experimental conditions, CHF 4227 significantly decreased total serum cholesterol in a dose-dependent manner with an ED₅₀ of about 0.007 mg/kg · day and maximal cholesterol lowering effect observed at 0.1
mg/kg · day. As in the bone tissue, again CHF 4227 is significantly more potent (about 50 times) than raloxifene in lowering plasma cholesterol levels.

Although the mechanisms for the increased potency of CHF 4227 in vivo, relative to raloxifene, are not fully clear at the moment, the higher binding affinity to the estrogen receptors (4 times for ER-α and 16 times for ER-β) and the higher oral bioavailability of CHF 4227 may be relevant factors. In particular, the oral bioavailability of CHF 4227 in rats is 64% (data not shown), which is a significant improvement over raloxifene (5%: Rosati et al., 1998). The insertion of the 4′-methoxy group seems to be responsible for the higher oral bioavailability of CHF 4227; in line with this hypothesis, the demethoxylated derivative CHF 4056 (Galbiati et al., 2002) is characterized by an oral bioavailability similar to the one of raloxifene. In parallel, also the in vivo potency and efficacy of CHF 4056 and raloxifene are superimposable (Galbiati et al., 2002), underlying the fact that the bioavailability may be an important feature to justify the improved SERM profile of CHF 4227.

Presently, we are investigating the in vivo metabolism of CHF 4227, relative to CHF 4056, to clarify possible biotransformation mechanisms that could account for the higher bioavailability of CHF 4227. In this respect, preliminary data suggest that a minimization of glucuronidation process in position 7 (on phenolic group of the benzopyran moiety) of CHF 4227 might be an important point.

Estrogens play a predominant role in breast cancer development and growth (McGuire et al., 1975). Because the first step in the action of estrogens in target tissue is binding to the estrogen receptor, a logical approach for the prevention and treatment of estrogen-sensitive breast cancer is the use of compounds that block the interaction of estrogens with their specific receptor. Consequently, the use of the antiestrogen tamoxifen is the standard therapy for breast cancer, at all stages of the disease; in addition, raloxifene therapy is associated with a potential decreased risk of developing breast cancer (Clemett and Spencer, 2000). CHF 4227, being characterized by marked estrogen antagonist activity on reproductive tissue, might be of interest in the prevention and treatment of estrogen-dependent tumors. To test this hypothesis, we studied the effect of CHF 4227 on the development of DMBA-induced mammary carcinoma in rats, a model widely used to study the factors that control hormone-sensitive breast cancer in women (Asselin et al., 1977). In this assay, CHF 4227 significantly prevents the development of DMBA-induced mammary tumors, the incidence being reduced from 87.5 to 26.3% 6 months after DMBA administration. Moreover, our data clearly show that CHF 4227 not only significantly reduces the percentage of rats bearing DMBA-induced tumors but also decreased tumor number per animal that have developed tumors during treatment with CHF 4227. Thus, these results show that CHF 4227 may be of interest in the prevention of estrogen-dependent tumors. A treatment study will be performed to test also the efficacy of CHF 4227 on the growth of established mammary tumors. Whether being used as a selective estrogen for prevention and treatment of osteoporosis or as an estrogen antagonist for breast cancer, it is critical that CHF 4227 does not induce a significant estrogen agonist effects on uterine tissue.

In line with the results observed in the immature rats assay, also in aged OVX rats CHF 4227 has minimal stimulatory effects on the uterus. In fact, after 4 weeks of treatment, histological analysis of the uterine tissue showed that CHF 4227, like raloxifene, has nonsignificant effects on endometrium epithelia thickness. In contrast, EE2 increased epithelial thickness, demonstrating significant uterine hypertrophic effects. This trend was reproduced in analysis of uterine eosinophil peroxidase activity to show that CHF 4227 is much less stimulatory in the uterus than EE2. CHF 4227 differed substantially also from others benzopyran estrogens agonist/antagonist such as levormeloxifene, which, as detailed previously (Galbiati et al., 2002), significantly increased uterine epithelial thickness and uterine eosinophil peroxidase in OVX rats. It is worth noting that the clinical development of levormeloxifene was discontinued after reports of endometrial thickening side effects in postmenopausal women (Mitlak and Cohen, 1999).

After 4 weeks of treatment, CHF 4227 caused 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 4227’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). Thus, CHF 4227 is expected to have a tissue selectivity profile in postmenopausal women similar to the one of raloxifene and strictly different compared with those of others SERMs such as tamoxifen and levormeloxifene. The full estrogen antagonist effect of CHF 4227 in the uterine tissue of immature rats indicates that its tissue selectivity is not simply due to a selective tissue distribution.

In conclusion, the new benzopyran derivative CHF 4227 is a selective estrogen receptor modulator that compares favorably in efficacy and potency with raloxifene in preventing bone loss, lowering serum cholesterol levels, and antagonizing EE2 stimulation of the uterus. Moreover, the results observed in the DMBA-induced mammary tumors model indicate that CHF 4227 has significant chemopreventive effects. This profile along with the minimal uterine stimulation suggests a therapeutic advantage to CHF 4227 over estrogens, raloxifene, or tamoxifen for the prevention and/or treatment of osteoporosis and estrogen-dependent tumors. In particular, the lack of a significant agonist activity on uterine tissue may avoid some of the potential estrogen-related clinical side effects that may arise with the use of long-term estrogens and tamoxifen therapy, whereas the higher efficacy and potency of CHF 4227 on bone tissue indicate that this compound may be superior to raloxifene as antiosteoporotic drug. Clinical studies with CHF 4227 will be performed to confirm its preclinical profile in postmenopausal women.

References
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