

Hypocholesterolemic Activity of Raloxifene (LY139481): Pharmacological Characterization as a Selective Estrogen Receptor Modulator

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ABSTRACT

After once-daily oral dosing in ovariectomized rats, raloxifene (LY139481) hydrochloride produced dose- and time-dependent reductions in serum cholesterol and high-density lipoprotein-cholesterol. Paired-feeding studies demonstrated that effects of raloxifene on serum lipids were not secondary to effects on food consumption. Maximal reductions in serum cholesterol occurred within 4 days of raloxifene administration or sooner, depending on the administered dose. The ED₅₀ for 50% reduction in serum cholesterol by raloxifene was 0.13 ± 0.04 mg/kg/day (mean ± S.E.M., *n* = 17); maximal cholesterol reduction by raloxifene (68%) was significantly less than that produced by estrogen (17 α -ethinylestradiol; 89%) after 4 to 7 days of daily dosing. Dose-response curves for cholesterol lowering by raloxifene were generated in the presence of varying doses of 17 α -ethinylestradiol; two-way analysis of variance

revealed significant interactions between estrogen and raloxifene with respect to cholesterol lowering (*P* < .001). Furthermore, a high dose of raloxifene (10 mg/kg/day) prevented further reduction of serum cholesterol by estrogen (1–100 μ g/kg/day) beyond that produced by raloxifene alone. For a series of closely related structural analogs of raloxifene, log(ED₅₀) values for cholesterol lowering were highly correlated with log(relative binding affinity) for the estrogen receptor (*r* = 0.93; *P* < .0001). Thus, cholesterol lowering by raloxifene in ovariectomized rats is mediated primarily *via* partial agonist effects at estrogen receptors. Taken together with previous observations in uterine tissue of estrogen antagonism by raloxifene in the absence of significant agonism, the present findings support the classification of raloxifene as a selective estrogen receptor modulator.

Clinical and epidemiological studies have shown the postmenopausal state to be an important risk factor for at least two chronic disorders in women, *i.e.*, cardiovascular disease (Kannel *et al.*, 1976) and osteoporosis (Richelson *et al.*, 1984), major causes of morbidity and death in postmenopausal women. Although a large body of data supports the beneficial effects of estrogen therapy for prevention and treatment of both of these diseases (reviewed in Kauffman and Bryant, 1995), concerns relating to uterine bleeding and increased risk of cancer in reproductive tissues (breast and uterus) have adversely affected compliance with chronic replacement therapy (Hammond, 1994). Coadministration of a progestin during estrogen therapy effectively prevents occurrence of uterine cancer (Voigt *et al.*, 1991); however, such combination therapy may not be effective in reducing breast cancer risk (Colditz *et al.*, 1995), and the impact of combination therapy on cardiovascular disease remains largely uncharacterized. Consequently, the need exists for improved estrogens that

have beneficial effects on cardiovascular disease and osteoporosis in postmenopausal women without producing adverse effects on reproductive tissues.

The benzothiophene raloxifene (LY139481 or the hydrochloride salt, LY156758, previously referred to as keoxifene) was originally discovered and characterized as an estrogen antagonist with potential utility in the treatment of breast cancer. Thus, raloxifene was shown to bind with high affinity to the rat uterine ER (Black *et al.*, 1983), to inhibit estrogen-dependent proliferation of human MCF-7 breast cancer cells *in vitro* (Wakeling *et al.*, 1984; Sato *et al.*, 1995) and to inhibit development of carcinogen-induced mammary tumors in rats (Clemens *et al.*, 1983; Gottardis and Jordan, 1987; Anzano *et al.*, 1996). Subsequently, this compound was shown to preserve bone density and lower serum cholesterol during chronic treatment of OVX female rats (Black *et al.*, 1994; Turner *et al.*, 1994), activities consistent with agonist effects mediated *via* the ER. In contrast to the *in vivo* activity profile observed with synthetic or natural estrogens, raloxifene produced these agonist-like effects without causing significant

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ABBREVIATIONS: AEBS, antiestrogen binding sites; EE₂, 17 α -ethinylestradiol; ER, estrogen receptor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OVX, ovariectomized; RBA, relative binding affinity; SERM, selective estrogen receptor modulator.

uterotrophy (Black *et al.*, 1994; Turner *et al.*, 1994). Rather, raloxifene displayed prominent estrogen antagonism in the uterus together with minimal evidence of agonism in this reproductive tissue (Jones *et al.*, 1984; Black *et al.*, 1994; Turner *et al.*, 1994). Thus, the *in vivo* profile of raloxifene is distinct from that of estrogen, in that agonist-like effects are produced in bone and on cholesterol metabolism, whereas estrogen antagonism is the primary pharmacological effect in uterine tissue. As a result, raloxifene displays potential as a pharmacological alternative to the use of traditional estrogens for chronic postmenopausal therapy.

Based on the *in vivo* findings described above, the question arose whether estrogen agonist-like effects of raloxifene are in fact mediated by the ER. In other words, is the unique profile of raloxifene the result of tissue-selective agonist/antagonist actions mediated by a single receptor (ER) or is it the consequence of interaction with multiple pharmacological receptors including the ER? The importance of this question was emphasized by the previous demonstration that tamoxifen, a structurally dissimilar antiestrogen that also reduces serum cholesterol in estrogen-deficient rats (Gold *et al.*, 1994), inhibited cholesterol biosynthesis in MCF-7 cells *via* a mechanism independent of the ER (Cypriani *et al.*, 1988). Such an effect, were it to occur *in vivo* at pharmacological doses in a relevant tissue (*i.e.*, liver), could conceivably account for cholesterol reduction by this compound. Consequently, the present studies were carried out to evaluate the pharmacological mechanism of cholesterol lowering by raloxifene in OVX rats, with particular emphasis on the role of the ER in this activity.

Methods

Animal treatments. OVX female Sprague Dawley rats (200–250 g; approximately 80 days of age) were obtained from Charles Rivers Laboratories 1 week after surgery and were allowed to acclimate on a 12-hr light cycle (lights on from 6:00 A.M. to 6:00 P.M.) for 1 to 2 weeks, feeding *ad libitum* (except as noted) on rodent chow of defined calcium content (0.5%; Teklad no. TD89222). Alternatively, in one study (see table 2) male and female Sprague Dawley rats with intact gonads, in the same weight range, were obtained and were handled in an identical manner. After acclimation rats were randomly assigned to test groups consisting of six animals per group, three rats per cage (unless noted otherwise), and oral administration of drug or vehicle was initiated. All groups were dosed by gavage once daily, in the morning, with test compounds or an equivalent volume of vehicle (either 20% hydroxypropyl- β -cyclodextrin or 1.5% carboxymethylcellulose). Dosing solutions were stored at 4°C during the studies, conditions under which pharmacological activity and structural stability of raloxifene were both shown to be preserved (data not shown). After either 4 or 7 days of dosing, rats were fasted overnight. On the following morning rats were anesthetized with ketamine (80 mg/kg *i.m.*) plus rompun (16 mg/kg *i.m.*) and exsanguinated by cardiac puncture. Blood was allowed to clot at room temperature, and serum was prepared by low-speed centrifugation. The time course for effects of raloxifene on serum lipids was examined by using a slight variation in the standard protocol. In this study, blood was drawn for serum lipid analysis after administration of either drug or vehicle for a variable number of days (0–7 days). Paired-feeding studies were carried out using metabolic cages, by including an extra vehicle-treated group of rats that were fed an amount of food identical to that consumed, on average, by drug-treated rats on the prior day. For this study, rats were housed separately and were not acclimated to the metabolic cages before initiation of the study.

Serum lipid analyses. Cholesterol was determined spectrophotometrically, using a commercial assay kit (Boehringer Mannheim Diagnostics, Indianapolis, IN) calibrated with cholesterol standards. Assays were carried out in 96-well plates and were semiautomated, using a Beckman Biomek 1000 laboratory workstation. For total cholesterol determinations, whole-serum samples (fresh or stored frozen at -70°C) were used in the assay described above. For rat HDL-cholesterol measurements, HDL was separated from lower density lipoproteins in serum by ultracentrifugation, as described previously (Hatch and Lees, 1968). Subsequently, cholesterol content of the HDL fraction was determined spectrophotometrically by the technique described above. For dose-response analyses, ED₅₀ values (defined as the dose at which a 50% reduction in serum cholesterol was observed) were estimated by graphical analysis of log dose-response curves.

ER binding analysis. Lysates of MCF-7 human adenocarcinoma cells were prepared in 50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 400 mM KCl, 10% glycerol, 0.5 mM 2-mercaptoethanol, 10 mM sodium molybdate (TEG buffer), containing protease inhibitors (1 $\mu\text{g}/\text{ml}$ pepstatin A, 2 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 mM phenylmethylsulfonylfluoride) (TEGP buffer). Cells were resuspended in TEGP buffer at 0–4°C (1 ml of TEGP buffer/100-mg pellet) and sonicated for 30 sec using a Branson Sonifier 450. Lysates were pelleted by centrifugation at $10,000 \times g$ for 15 min at 4°C, and supernatant fluids were either used immediately or stored at -70°C . For competitive binding studies, the buffer was TEGP buffer containing ovalbumin (1 mg/ml), in which 400 mM KCl was replaced with 50 mM NaCl. Displacement curves were generated using final concentrations of 0.5 nM 17β -[^3H]estradiol as radioligand, 0.15 mg protein/ml MCF-7 lysate and $\frac{1}{2}$ -log unit increments of competing ligands, ranging from 0.1 to 100 nM. Incubation time was 24 hr at 4°C, after which dextran-treated charcoal (0.5 \times volume of binding solution) was added, with vigorous shaking for 8 min at 4°C. The suspension was then centrifuged at $1500 \times g$ for 10 min at 4°C, and radioactivity in the supernatant fluid was determined by scintillation counting. Percent binding was determined in triplicate at each concentration of displacing ligand, after correction for background levels of radioactivity not extractable with dextran-treated charcoal. IC₅₀ values for 50% inhibition of 17β -[^3H]estradiol binding were determined by graphical analysis of log concentration-displacement curves, and RBA was determined as the ratio IC₅₀(unlabeled 17β -estradiol)/IC₅₀(displacing ligand).

Statistical analysis. Dunnett's two-tailed test (Dunnett, 1955) was used to analyze differences between values from the control group *vs.* multiple drug treatment groups. Differences between drug treatment groups were analyzed for statistical significance by Fisher's protected least significant differences test (Fisher, 1949). For analysis of interactions between raloxifene and EE₂, two-way analysis of variance was used; where significant interactions were observed, significance of differences between drug treatment groups was evaluated by contrast analysis (JMP Software, version 2.0; SAS Institute, Inc.). Correlations between $-\log(\text{ED}_{50})$ values for cholesterol lowering and $-\log(\text{RBA})$ values for binding to ER were determined by linear regression analysis. P values of $<.05$ were taken to indicate statistical significance.

Sources of compounds. Raloxifene hydrochloride (LY139481-HCl) and structural analogs thereof (shown in fig. 6) were synthesized and characterized within Lilly Research Laboratories, Eli Lilly and Co. The purity of test articles was $>99\%$ for raloxifene and $>95\%$ for all other compounds in figure 6. EE₂ and carboxymethyl-cellulose were obtained from Sigma Chemical Co. (St. Louis, MO). Ketaset (ketamine) and rompun were obtained from Aveco (Fort Dodge, IA) and Mobay Corp. (Shawnee, KA), respectively. Hydroxypropyl- β -cyclodextrin was purchased from Aldrich (Milwaukee, WI). 17β -[^3H]estradiol was obtained from New England Nuclear (Boston, MA). The MCF-7 human adenocarcinoma cellline was purchased from the American Type Culture Collection (Rockville, MD).

Results

Cholesterol lowering by raloxifene was initially demonstrated during chronic (5-week) studies of bone metabolism in OVX rats (Black *et al.*, 1994). The time dependence for reductions in serum cholesterol was examined to characterize effects of raloxifene (1 mg/kg/day) over shorter time intervals (fig. 1). As can be seen, no significant change in control values for serum cholesterol was observed when OVX rats were dosed daily with vehicle for 7 days. In the raloxifene-treated rats, a significant reduction in serum cholesterol was seen 24 hr after the first dose; maximal reduction occurred after 2 to 3 days of dosing. At a lower dose of raloxifene of 0.1 mg/kg/day, maximal reduction of serum cholesterol occurred after 3 to 4 days of daily dosing (data not shown). Based on these findings, subsequent studies of cholesterol lowering by raloxifene were carried out for a period of 4 or 7 days.

Similarly to previous findings with estrogen (*e.g.*, Staels *et al.*, 1989), raloxifene decreases food consumption and produces weight loss in OVX rats. Studies with EE_2 showed that cholesterol reduction was not secondary to decreases in dietary intake or weight (Staels *et al.*, 1989). Consequently, pair-fed control studies were carried out for 7 days to assess the role of food consumption and weight loss in effects of raloxifene on serum cholesterol (table 1). On day 3 and thereafter, raloxifene (1.0 mg/kg/day) produced an average 36% reduction (maximal effect) in daily food consumption (data not shown); average food consumption during the entire 7-day period was reduced by 29% in the raloxifene treatment group. Food intake for the pair-fed control group was slightly, but not significantly, lower than for the raloxifene-treated group, because one rat in this group failed to consume all of the chow provided on several days during the study. In this experiment, vehicle-treated control rats lost weight, probably as a result of individual housing without acclimation in the metabolic cages. Raloxifene administration resulted in sig-

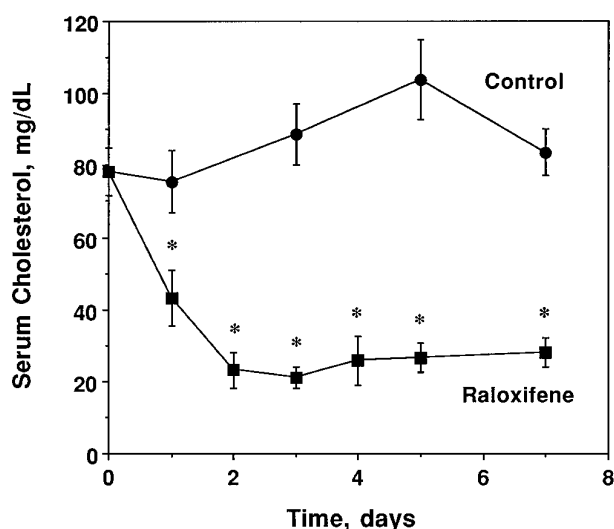


Fig. 1. Time course for effects of raloxifene on serum cholesterol in OVX rats. Rats (groups of six) were administered raloxifene (1 mg/kg/day) or vehicle (20% hydroxypropyl- β -cyclodextrin) once daily and sacrificed after the number of days indicated. Serum cholesterol was determined as described in "Methods," using blood samples taken at sacrifice after an overnight fast. Data represent mean \pm S.E.M.; $n = 6$. * $P < .05$ vs. day 0 control value.

TABLE 1
Effects of raloxifene on serum cholesterol and food consumption: paired-feeding studies

Rats were administered raloxifene or vehicle (20% cyclodextrin) once daily for 7 days. Serum cholesterol was determined as described in "Methods." Pair-fed animals were provided an amount of food identical to that consumed, on average, by the raloxifene-treated group on the previous day of the study. Food intake was determined daily for each group; average values for the entire study period are reported. Serum cholesterol values and body weights were determined at the beginning and end of the study. Base-line serum cholesterol was determined using blood samples obtained from the retroorbital sinus under light anesthesia with CO_2 . Terminal blood samples were obtained by cardiac puncture. Significant changes in serum cholesterol values from day 0 to day 7 occurred only in the raloxifene-treated group; consequently, only day 7 cholesterol values are reported here. Results are mean \pm SEM of five or six rats per group, except for food intake, which was averaged over time.

Group	Food Intake	Weight Change	Serum Cholesterol
	g/day	g	mg/dl
Control	18.8 \pm 0.8	-17 \pm 1	102 \pm 15
Raloxifene (1 mg/kg/day)	13.1 \pm 0.9*	-37 \pm 3*	29 \pm 6*
Pair-fed control	12.3 \pm 0.9*	-51 \pm 6*	97 \pm 9

* $P < .05$ vs. control group.

nificantly greater weight loss than that seen in the control group. Similarly, pair-fed control rats lost significantly more weight than did the control group and slightly more than that observed in the raloxifene-treated group ($P = .06$; see above). Raloxifene treatment produced a significant 71% reduction in serum cholesterol, compared with the control group, whereas serum cholesterol was not significantly altered in the pair-fed control group (table 1). Therefore, reductions in serum cholesterol by raloxifene were not secondary to effects on dietary intake or weight.

Initial dose-ranging studies for cholesterol lowering by raloxifene were carried out in OVX rats at 1-log unit dose intervals (0.01–10 mg/kg/day), with daily dosing for a period of 1 week (fig. 2A). As can be seen, serum cholesterol and HDL-cholesterol values for OVX rats did not differ from those of sham-operated female rats under the conditions of these studies. Onset of cholesterol reduction was observed at a raloxifene dose of 0.01 mg/kg/day in this experiment. Maximal reduction of serum cholesterol (78%) occurred at 1.0 mg/kg/day; no further effect was observed at the highest dose of 10 mg/kg/day. With fractionation of serum lipoproteins by ultracentrifugation, HDL-cholesterol was shown to be reduced by raloxifene in OVX rats with a dose dependence similar to that of total serum cholesterol (fig. 2A). From an extensive series of *in vivo* studies, the maximal reduction of serum total cholesterol by raloxifene ranged from 45 to 80% in OVX rats, with an average value \pm S.E.M. of $67.9 \pm 1.4\%$ ($n = 36$).

After establishment of the range of doses over which effects on serum cholesterol occur, a more precise dose-response relationship for reduction of serum total cholesterol and HDL-cholesterol by raloxifene was assessed at $\frac{1}{2}$ -log unit dose increments. After once-daily dosing by oral gavage for 1 week, raloxifene produced dose-dependent reductions in serum cholesterol, with half-maximal effects occurring at approximately 0.03 mg/kg/day and maximal reduction of 73% occurring at 1 mg/kg/day (fig. 2B). The ED_{50} for reduction of serum cholesterol in this experiment was estimated to be 0.07 mg/kg/day; the ED_{50} (mean \pm S.E.M.) from a series of dose-response studies was 0.13 ± 0.04 mg/kg/day ($n = 17$). Dose-response curves for serum cholesterol and HDL-choles-

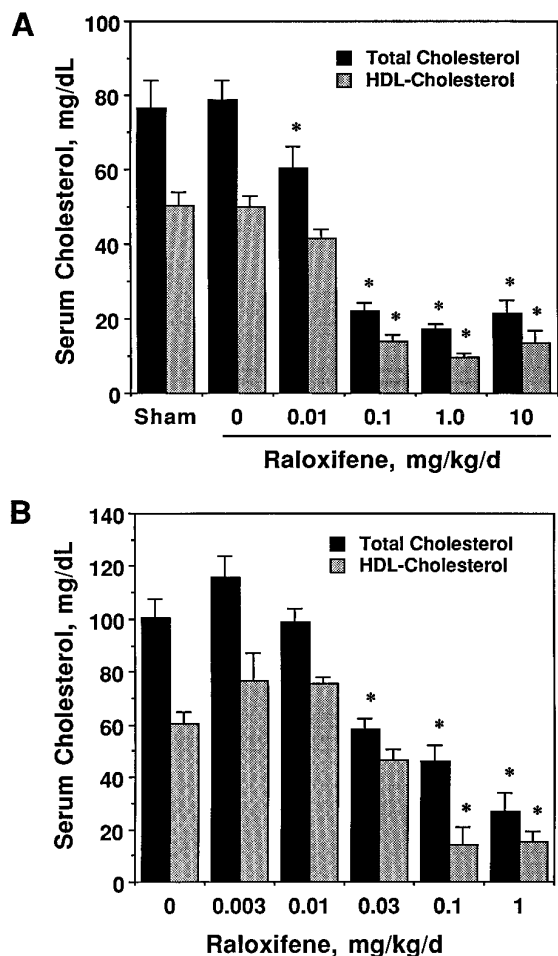


Fig. 2. Dose-response relationships for reductions in serum cholesterol and HDL-cholesterol by raloxifene in OVX rats. OVX rats were administered raloxifene at the indicated daily oral doses, by gavage, for 1 week; blood was then drawn, after an overnight fast, for determination of total serum cholesterol and HDL-cholesterol, as described in "Methods." A, dose-response relationship at 1-log unit dose increments. Sham, sham-OVX rats. B, refined dose-response relationship at 1/2-log unit dose increments. Data are presented as mean \pm S.E.M.; $n = 6$. * $P < .05$ vs. corresponding OVX control group in the absence of raloxifene.

terol in figure 2B were not identical, although the separation was prominent only at the dose of 0.1 mg/kg/day.

Maximal cholesterol reductions by raloxifene and EE₂ in OVX rats were compared side by side after 1 week of drug administration, and the results are presented in figure 3. Similarly to previous observations (Staels *et al.*, 1989), EE₂ at a dose of 0.1 mg/kg/day substantially reduced serum total cholesterol and HDL-cholesterol; both of these parameters were reduced by approximately 95% in this experiment. From a series of *in vivo* studies, the average maximal reduction of serum total cholesterol by EE₂ was $88.6 \pm 1.9\%$ (mean \pm S.E.M., $n = 30$). In contrast, a maximally effective dose of raloxifene (1 mg/kg/day) (fig. 2) reduced both serum total cholesterol and HDL-cholesterol by 77%, a value that was significantly less than the maximal effect of EE₂ (fig. 3). Similar results were obtained from an extensive analysis of comparative effects of EE₂ and raloxifene on serum total cholesterol at maximally effective doses of 0.1 and 1.0 mg/kg/day, respectively. In these studies, the maximal cholesterol

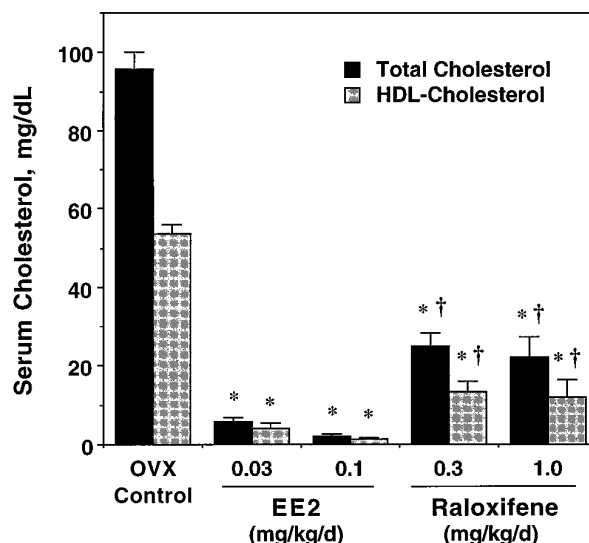


Fig. 3. Maximal reductions in total serum cholesterol and HDL-cholesterol by EE₂ and raloxifene in OVX rats. OVX rats were administered EE₂ or raloxifene at the indicated daily oral doses, by gavage, for 1 week; blood was then drawn, after an overnight fast, for determination of total serum cholesterol and HDL-cholesterol, as described in "Methods." Data are presented as mean \pm S.E.M.; $n = 6$. * $P < .05$ vs. corresponding OVX control group. † $P < .05$ vs. corresponding cholesterol fractions at EE₂ dose of 0.1 mg/kg/day.

reduction by raloxifene, expressed as a percentage of the maximal effect of EE₂, was $77 \pm 2\%$ (mean \pm S.E.M., $n = 30$). Thus, raloxifene was slightly less effective than EE₂ at lowering cholesterol in OVX rats.

To test for interactions between raloxifene and EE₂, dose-response curves for cholesterol lowering by raloxifene were generated in the presence of varying doses of EE₂ (fig. 4). The effects of EE₂ on raloxifene dose-response curves were complex, suggesting pharmacological interactions between these compounds with respect to effects on serum cholesterol. In support of this interpretation, two-way analysis of variance revealed a highly significant interaction between raloxifene and EE₂ with respect to cholesterol lowering ($P < .001$).

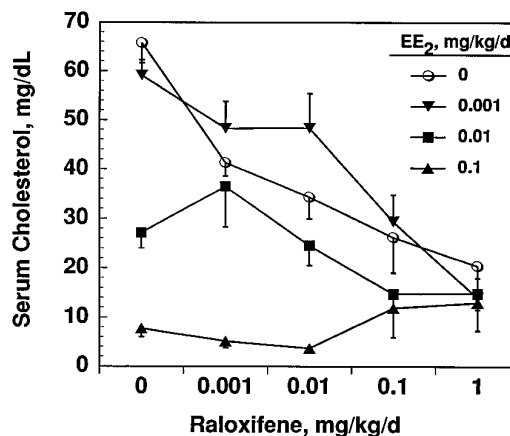


Fig. 4. Interactions between EE₂ and raloxifene with respect to serum cholesterol lowering in OVX rats. Dose-response curves for serum cholesterol reduction by raloxifene were determined in the presence of variable doses of EE₂, as indicated. Rats were dosed daily for a period of 4 days; blood was then drawn, after an overnight fast, for determination of total serum cholesterol, as described in "Methods." Data represent the mean \pm S.E.M. from groups of six rats at each dose of raloxifene.

Furthermore, regardless of the dose of EE₂, dose-response curves for raloxifene converged, at high doses, at a common cholesterol value not significantly different from that of raloxifene in the absence of EE₂, suggesting a partial estrogen agonist effect of raloxifene.

To test further for partial estrogen agonism by raloxifene, dose-response curves for serum cholesterol reduction by EE₂ were determined side by side in the presence or absence of a high dose of raloxifene (10 mg/kg/day). This high dose was used to test for effective competition *vs.* estrogen at a maximally efficacious dose of the latter. The results of this experiment are presented in figure 5. In this experiment, maximal serum cholesterol reduction by raloxifene was approximately 50%. Similarly to published findings (Staels *et al.*, 1989), EE₂ produced potent extensive reduction of serum cholesterol, with half-maximal effects occurring at approximately 5 µg/kg/day in the absence of raloxifene. However, in the presence of the high dose of raloxifene, EE₂ over this same dose range failed to significantly reduce serum cholesterol below the control serum cholesterol value observed in the presence of 10 mg/kg/day raloxifene alone. Comparison of cholesterol reduction by the combination of 10 µg/kg/day EE₂ plus 10 mg/kg/day raloxifene *vs.* that produced by each compound alone at these doses demonstrated further that the effects of raloxifene and EE₂ were not additive. Furthermore, contrast comparison of serum cholesterol values at the maximally efficacious dose of 100 µg/kg/day EE₂ (with or without raloxifene) revealed that raloxifene produced partial antagonism of cholesterol lowering by EE₂.

For a series of closely related structural analogs of raloxifene, RBA values for the soluble ER from MCF-7 breast cancer cells were determined and related to *in vivo* ED₅₀ values for cholesterol lowering in OVX rats. The structures of raloxifene analogs used in this analysis are presented in figure 6. This set of compounds was selected on the basis of their possessing a wide range of affinities for the ER (300-

fold), while displaying relatively minor deviations in structure from the parent molecule raloxifene. This strategy for compound selection was adopted at the outset to minimize alterations in *in vivo* parameters such as absorption, distribution, metabolism and elimination across the entire set of analogs. As can be seen in figure 7, $-\log(\text{ED}_{50})$ values were highly and significantly correlated with $-\log(\text{RBA})$ values for the series of raloxifene analogs ($r = 0.93$, $P < .0001$). The slope of the regression line was -1.09 , consistent with a functional relationship between ER binding and cholesterol lowering.

EE₂ was previously shown to produce extensive reductions in serum cholesterol in both male and female rats with intact gonads (Weinstein *et al.*, 1986). Accordingly, the effects of raloxifene on serum cholesterol were determined in intact male and female rats, and the results are presented in table 2. In both sexes, raloxifene significantly reduced serum cholesterol by approximately 50% at the dose of 0.1 mg/kg/day, in good agreement with the ED₅₀ observed in OVX rats. Higher doses of raloxifene in males produced further decrements in serum cholesterol to a maximal reduction of about 75%. In contrast, in intact females higher doses failed to produce further reductions in serum cholesterol beyond that which occurred at 0.1 mg/kg/day.

Discussion

The major finding in the present studies is that raloxifene is characterized pharmacologically as a partial estrogen agonist with respect to cholesterol metabolism in OVX rats. This conclusion is based on the following observations: 1) raloxifene was slightly, but significantly, less effective at reducing serum cholesterol and HDL cholesterol in OVX rats than is exogenous estrogen (EE₂); 2) significant interaction between estrogen and raloxifene with respect to cholesterol lowering was observed in OVX rats dosed with varying doses of both compounds; 3) effects of raloxifene and EE₂ on serum cholesterol were not additive; 4) a high dose of raloxifene prevented further cholesterol reduction by EE₂ beyond that produced by raloxifene alone; and, 5) for a series of structural analogs of raloxifene, ED₅₀ values for cholesterol lowering were highly and significantly correlated with RBA values for the ER. Taken together, these pharmacological data provide strong support for a critical role of the ER in mediating estrogen agonist-like effects of raloxifene on serum cholesterol. Although these data do not eliminate the possibility that both estrogen and raloxifene reduce serum cholesterol by interacting with a common receptor distinct from the ER (but possessing a binding site with topography and binding interactions highly similar to those of the ER), this possibility was considered unlikely.

Additional support for the role of estrogen agonism in the ability of raloxifene to reduce serum cholesterol is derived from studies in hypophysectomized rats. In good agreement with literature observations (Steinberg *et al.*, 1967), Bryant *et al.* (1994) observed that cholesterol lowering by estrogen was dramatically reduced by hypophysectomy in OVX rats. Importantly, the ability of raloxifene to reduce serum cholesterol was similarly attenuated by hypophysectomy in this study. These findings demonstrate a common requirement of pituitary-dependent hormonal factors for cholesterol reduction by both estrogen and raloxifene. In addition, they sug-

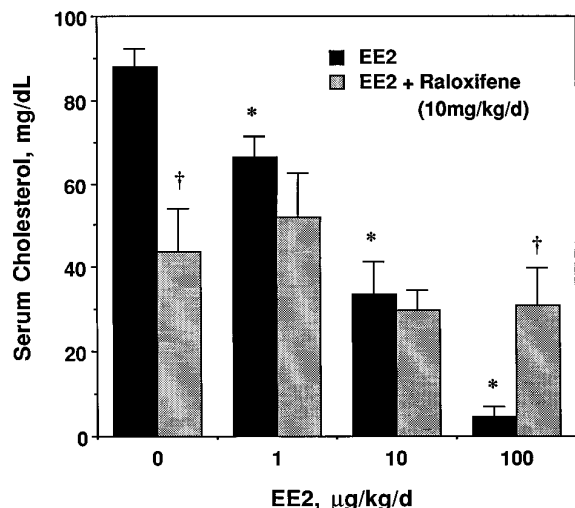


Fig. 5. Antagonism of EE₂-induced cholesterol lowering by raloxifene in OVX rats. Dose-response relationships for EE₂ were determined in the presence or absence of raloxifene (10 mg/kg/day), as indicated. Rats were dosed daily for a period of 7 days; blood was then drawn, after an overnight fast, for determination of total serum cholesterol, as described in "Methods." Data are presented as mean \pm S.E.M.; $n = 6$. * $P < .05$ vs. corresponding control in the absence of EE₂. † $P < .05$ vs. corresponding EE₂ dose group in the absence of raloxifene.

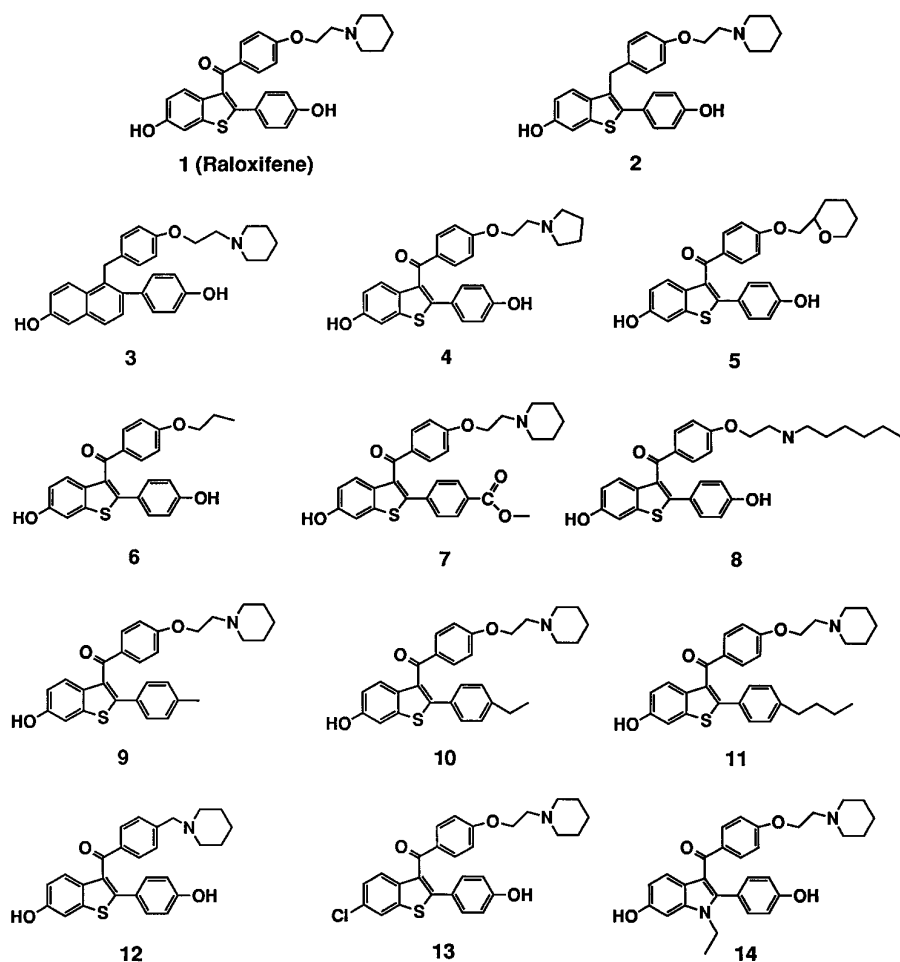


Fig. 6. Chemical structures of raloxifene and analogs used in figure 7.

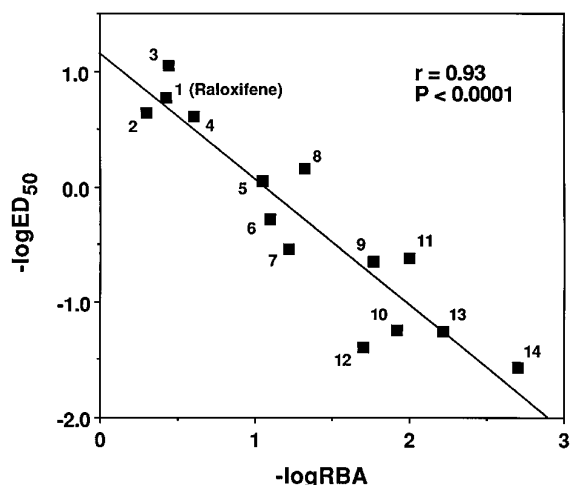


Fig. 7. Correlation between cholesterol lowering in OVX rats and ER binding affinity for a series of raloxifene analogs. Dose-response curves for reduction of serum cholesterol were generated from three or more doses (spanning at least 2 log units) of test compounds administered orally once daily for 4 days, and ED_{50} values were determined as described in "Methods." RBA values (vs. 17β -estradiol) for the ER were determined as described in "Methods." The line drawn through the data points represents the linear regression fit to logarithmically transformed ED_{50} and RBA values. The numbers adjacent to each data point represent compound numbers from figure 6.

TABLE 2

Effects of raloxifene on serum cholesterol in intact female and male rats

Rats were administered raloxifene or vehicle (20% cyclodextrin) by oral gavage once daily for 4 days. After an overnight fast after the last dose, serum cholesterol was determined at sacrifice, as described in "Methods." Results are presented as mean \pm S.E.M. of six rats per group.

Group	Dose	Serum Cholesterol	
		Males	Females
	mg/kg/day	mg/dl	
Control		71 \pm 3	87 \pm 7
Raloxifene	0.1	39 \pm 7*	41 \pm 4*
Raloxifene	1.0	25 \pm 4*	46 \pm 5*
Raloxifene	10	19 \pm 2*	52 \pm 7*

* $P < .05$ vs. control group.

gest a common role for liver ERs in cholesterol reduction by both estrogen and raloxifene, because hypophysectomy results in an approximately 10-fold reduction in liver ERs (Thompson *et al.*, 1983). By analogy with previous studies of estrogen (Brown and Goldstein, 1980; Ma *et al.*, 1986), the mechanism of cholesterol lowering by raloxifene probably involves ER-mediated induction of hepatic LDL receptors, resulting in enhanced clearance of serum lipoproteins containing apolipoproteins B or E.

Both estrogen and raloxifene produced reductions in HDL-cholesterol in OVX rats, whereas neither compound produced

an analogous effect in humans (Walsh *et al.*, 1991; Draper *et al.*, 1996). In the case of rats, reductions in HDL-cholesterol are presumably due to the presence of apolipoprotein E, an apolipoprotein with high affinity for LDL receptors, on HDL particles (Chao *et al.*, 1979). The fact that HDL-cholesterol is the predominant serum lipoprotein in rats would thus explain the extensive reductions in total serum cholesterol produced by estrogenic compounds in this species. The lack of a similar effect in humans is probably explained by the relative lack of apolipoprotein E in human HDL particles (Chao *et al.*, 1979).

In addition to binding to ERs, raloxifene, like tamoxifen (Miller *et al.*, 1983), binds to the class of low-affinity, high-capacity sites referred to as AEBS, which are present in a variety of cells and tissues (A. L. Glasebrook and D. L. Phillips, unpublished data). Biological consequences of binding to AEBS are unclear and controversial (Jordan, 1984). Using ^3H -labeled raloxifene, RBA values for AEBS in MCF-7 cell lysates were determined for the raloxifene analogs shown in figure 6. In contrast to results obtained with ER, no significant correlation was found between $\log(\text{RBA})$ values for AEBS and $\log(\text{ED}_{50})$ values for cholesterol lowering ($r = 0.19$, $P > .55$; data not shown). Thus, AEBS do not appear to play a role in the hypocholesterolemic effects of raloxifene and related benzothiophenes.

Serum cholesterol values from OVX rats differed little from values observed in sham-operated females during the time course of these studies (fig. 2A) (Black *et al.*, 1994). Thus, the extensive reductions of cholesterol seen with estrogen in this model appear to be pharmacological in nature, rather than physiological. The ability of EE_2 to produce extensive reductions in serum cholesterol in intact female or male rats confirms the pharmacological nature of this effect (Weinstein *et al.*, 1986). Similarly, raloxifene apparently exploits this same pharmacological mechanism mediated by ERs to produce reductions in serum cholesterol in this model. In the present studies, raloxifene also produced reductions in serum cholesterol in intact female and male rats analogous to those seen in OVX females. Consequently, the ER-mediated mechanism for cholesterol lowering does not appear to be specific for sex or hormone status.

The finding that raloxifene produces prominent estrogen agonism with respect to cholesterol metabolism is somewhat surprising, in view of previous demonstrations of estrogen antagonism together with minimal or no intrinsic agonism in uterine tissue (Jones *et al.*, 1984; Black *et al.*, 1994; Turner *et al.*, 1994). Furthermore, raloxifene displayed estrogen antagonist effects in both *in vitro* (Wakeling *et al.*, 1984; Sato *et al.*, 1995) and *in vivo* (Clemens *et al.*, 1983; Gottardis and Jordan, 1987; Anzano *et al.*, 1996) models of breast cancer. Taken together with these previous findings, the present results demonstrate tissue selectivity for expression and/or degree of estrogen agonism *vs.* antagonism by raloxifene. Thus, raloxifene displays an antagonist profile in reproductive tissues such as the breast and uterus, whereas prominent partial agonist effects are observed with respect to liver cholesterol metabolism.

Similarly to effects on cholesterol metabolism, raloxifene also produces an estrogen agonist-like effect in preventing loss of bone mass and bone strength in OVX rats (Black *et al.*, 1994; Turner *et al.*, 1994). Analogously to estrogen, raloxifene was shown by histomorphometric analysis to be an antiresorptive agent with respect to effects on bone mass in OVX rats (Evans

et al., 1993). Furthermore, like estrogen, the protective effect of raloxifene on bone was markedly attenuated by hypophysectomy (Bryant *et al.*, 1994). Therefore, although definitive information on the mechanism underlying bone effects of raloxifene is not yet available, data obtained thus far support a role for estrogen agonism in this effect.

As discussed above, the *in vivo* profile for raloxifene displays estrogen agonism or antagonism, depending on the particular tissue examined. Based on currently available data, the primary difference in *in vivo* profiles of estrogen and raloxifene lies in differential effects on reproductive tissues (agonism *vs.* antagonism, respectively). Therefore, this compound represents a novel pharmacological class distinct from either classical agonists (*e.g.*, 17β -estradiol) or "pure" antagonists (*e.g.*, ICI-164,384). Because the unique *in vivo* profile for raloxifene is apparently mediated through interactions with a single receptor (ER), rather than with multiple receptors, we have referred to this compound as a "SERM" (Sato *et al.*, 1995). The SERM profile of raloxifene (*i.e.*, estrogen agonistic effects on bone and cholesterol metabolism together with estrogen antagonism in uterus and breast) suggests therapeutic utility in postmenopausal women, as an alternative to estrogen or hormone replacement therapy that lacks reproductive tissue side effects associated with traditional hormonal approaches.

Tamoxifen, a structurally distinct antiestrogen used in breast cancer therapy, displays potential for inclusion in the SERM class because it also produces estrogen agonist-like effects on bone (Gotfredsen *et al.*, 1984; Moon *et al.*, 1991) and cholesterol metabolism (Bruning *et al.*, 1988; Gold *et al.*, 1994). However, with respect to cholesterol lowering, available *in vitro* and *in vivo* data raise the possibility that the mechanism for this effect may be independent of the ER (Cypriani *et al.*, 1988; Gylling *et al.*, 1992). Consequently, inclusion of tamoxifen in the SERM class is considered tentative until more conclusive data on the *in vivo* mechanism(s) underlying agonist-like effects become available. Additional compounds with potential for inclusion in the SERM class have also been reported in the literature (for review, see Kauffman and Bryant, 1995).

The mechanism for tissue-selective effects of raloxifene remains the subject of intense research efforts at this time. In ER protease protection assays, raloxifene binding protected a different peptide sequence than did 17β -estradiol, suggesting that raloxifene produced a unique conformation of the ER-ligand complex (McDonnell *et al.*, 1995). Furthermore, raloxifene displayed an *in vitro* profile of activity distinct from those of 17β -estradiol, tamoxifen and ICI 164,384 in a cell line transiently transfected with the ER or mutants thereof (McDonnell *et al.*, 1995). Thus, McDonnell *et al.* proposed that a unique ligand-induced receptor conformation is responsible for the differential *in vitro* profile of raloxifene. As an additional consequence of a unique conformation, the possibility arises that the raloxifene-ER complex may also bind to a sequence of DNA distinct from the classical estrogen response element in tissues where raloxifene exerts estrogen agonistic effects. Indeed, such a raloxifene-inducible element, distinct from the estrogen response element, has been identified in the transforming growth factor- β 3 promoter, which, when activated by the raloxifene-ER complex, leads to a marked stimulation of transcription (Yang *et al.*, 1996). Clearly further work is needed to understand the

detailed basis for tissue-selective agonist/antagonist actions of raloxifene.

Initial clinical data suggest that the preclinical SERM profile of raloxifene is expressed in postmenopausal women. After 8 weeks of raloxifene administration (200 or 600 mg/day) to healthy postmenopausal women in a placebo-controlled double-blind study, serum and urinary biochemical markers of bone metabolism were altered in direction and extent, similarly to changes produced by conjugated estrogens (Draper *et al.*, 1996). Serum cholesterol and LDL-cholesterol were lowered by raloxifene in that study, whereas HDL-cholesterol was unaffected by raloxifene. Importantly, and in sharp contrast to results seen in the estrogen-treated group, raloxifene had no stimulatory effect on histology scores for estrogenicity of uterine biopsy samples obtained during the study. Therefore, these limited clinical data suggest that raloxifene acts as a SERM in postmenopausal women, with a tissue selectivity profile similar to that observed in preclinical studies. The ultimate utility of raloxifene as an alternative for chronic estrogen replacement therapy is currently being evaluated in long-term clinical trials in postmenopausal women.

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