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Differential Response of Estrogen Receptors α and β to SP500263, a Novel Potent Selective Estrogen Receptor Modulator

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ABSTRACT

We determined the differential response of a novel SERM, SP500263, on estrogen receptor (ER) α and the more recently cloned ER-β. Because of the high homology of amino acid residues in the ligand-binding domain of ER-α and ER-β, we were not surprised to find that SP500263 binds to both ERs equally well. In contrast, SP500263 acts as a strong estrogen agonist in a strictly ER-α–specific manner in U2OS osteosarcoma cell lines blocking the production of interleukin (IL) 6 and granulocyte macrophage colony-stimulating factor. SP500263 also blocked IL-6 production in primary bone cells. The mechanism of this inhibition is different from the classic estrogen stimulation involving an estrogen response element (ERE). SP500263 does not activate gene expression through an ERE. In contrast to the results observed in U2OS cells, SP500263 acts as a strong estrogen antagonist in an MCF-7 breast cancer proliferation assay. Therefore, SP500263 is a member of a series of next-generation SERMs with functional selectivity toward ER-α and a mixed agonist/antagonist profile in a bone cell assay versus a breast cancer assay. The panel of assays described herein allow for the development of receptor-specific ligands that may be further developed into novel pharmaceuticals with an improved profile for the treatments of osteoporosis and breast cancer.

Cytokines, in addition to hormones and neuronal signals, are part of an important communication system within eukaryotic cells. Because of their wide-ranging expression and effects, the inappropriate expression and modulation of the production of cytokines has important physiological consequences. IL-6 is a cytokine produced by many cell types, including fibroblasts, endothelial cells, keratinocytes, monocytes, T cells, mast and tumor cell lines, and cells of neural origins (Van Snick, 1990; Kishimoto et al., 1995; Sehgal et al., 1995; Keller et al., 1996). IL-6 is believed to be involved in the pathogenesis of numerous diseases, including inflammatory conditions (rheumatoid arthritis, inflammatory bowel disease, encephalitis), cancer (leukemia, renal cell carcinoma, prostate cancer, multiple myeloma), and osteoporosis (Tsukamoto et al., 1992; Eustace et al., 1993; Weissglas et al., 1997; Alonzi et al., 1998; Hobisch et al., 1998; Chung et al., 1999; Grey et al., 1999; Nishimoto et al., 1999; Sandhu et al., 1999). On the other hand, mice with a genetic knockout of the IL-6 gene have no major health defects (Kopf et al., 1994; Poli, 1998).

Therefore, IL-6 inhibitors may be useful for the treatment of a variety of significant diseases (Stein and Sutherland, 1998). A number of different approaches have been taken that not only established the role of IL-6 in a disease but were considered possible means by which the condition could be controlled. These include blocking or interfering with the action of IL-6 using antibodies against IL-6 itself or its receptor, small peptides capable of competing with IL-6 for binding to the receptor, antisense constructs to IL-6 mRNA, and small molecules that interfere with the production of IL-6 (Stein and Sutherland, 1998).

Estrogen is a well-known modulator of IL-6 gene expression (Jilka et al., 1992; Passeri et al., 1993; Ray et al., 1994). The classic way of estrogen action is through the binding of an estrogen receptor (ER) to an estrogen response element (ERE). However, IL-6 and an increasing number of other genes is regulated through a nonclassic pathway in which ER

ABBREVIATIONS: IL-6, interleukin-6; ER, estrogen receptor; ERE, estrogen response element; SERM, selective estrogen receptor modulator; FCS, fetal calf serum; GM-CSF, granulocyte macrophage colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay; SP500263, 7-hydroxy-3-phenyl-4-{[4-(2-piperidinylethoxy)phenyl]methyl}-2H-chromen-2-one; ICI-182,780, faslodex; DMSO, dimethyl sulfoxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; PBS, phosphate-buffered saline; RBA, relative binding affinity; ERE-LUC, estrogen response element-luciferase; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; C/EBPβ, CCAAT/enhancer-binding protein β.

indirectly binds to DNA by targeting other transcription factors such as NF-κB and C/EBPβ (Stein and Yang, 1995; Galien and Garcia, 1997; Kurebayashi et al., 1997; Ray et al., 1997).

We report here on the discovery of a novel nonsteroidal selective estrogen receptor modulator (SERM), SP500263, that specifically inhibits IL-6 and GM-CSF gene expression in the presence of ER-α but not ER-β. However, SP500263 binds with high affinity to both ER-α and ER-β. Furthermore, SP500263 represents a novel small-molecule compound that acts as an estrogen agonist on IL-6 in bone cells but does not activate genes through a classic response element. Also, SP500263 is a SERM that effectively antagonizes estrogen action in ER-α–expressing breast cancer cells. Therefore, SP500263 represents a novel class of SERMs that acts as selective ER-α agonists in bone, potentially preserving bone loss, and as antiestrogen in the breast, potentially preventing breast cancer progression.

Experimental Procedures

Materials. Fetal calf serum (FCS) and McCoy's 5A media were obtained from Hyclone Laboratories (Logan, UT). β-Mercaptoethanol, Dulbecco's modified Eagle's/Ham's F12 media, hygromycin B, sodium selenite, and zeocin were obtained from Invitrogen (Carlsbad, CA). Superfect was obtained from QIAGEN (Valencia, CA). IL-6 and GM-CSF ELISA kits were purchased from Endogen Corporation (Woburn, MA). The 96-well ELISA plate reader was manufactured by Molecular Devices (Menlo Park, CA). 17β-Estradiol, ethanolamine, tamoxifen, and 4OH-tamoxifen were obtained from Sigma (St. Louis, MO). [^{3}H]thymidine and [^{3}H]estradiol were obtained from Amersham Biosciences (Piscataway, NJ). Optiplate 96-well microtiter plates and the Cell Harvester were obtained from Packard Instruments (Meriden, CT). Recombinant ER-α and ER-β proteins were obtained from PanVera (Madison, WI). Raloxifene was isolated and purified from Evista tablets (Eli Lilly, Indianapolis, IN); arzoxifene, lasofoxifene, and levormeloxifene were synthesized by the Medicinal Chemistry group from Novartis Pharma AG (Basel, Switzerland); SP500263 (Fig. 1) and SPC-1 were synthesized by the Medicinal Chemistry group at Celgene Corporation (San Diego, CA); ICI-182,780 was custom-synthesized by Magellan Laboratories (Research Triangle Park, NC).

Solid-Phase [^{3}H]Estradiol Competition Assay. Methods used in this study have been adapted from those in the scientific literature (McGuire, 1978). Purified recombinant ER-α and ER-β (PanVera) were immobilized to an OptiPlate 96-well microtiter plate (Packard) via overnight incubation at 4°C in 100 μl/well PBS containing 10 nM ER-α or 30 nM ER-β (or 15 nM ER-α or 15 nM ER-β). Solutions in the plates were aspirated and washed three times with 250 μl/well PBS before the addition of 50 μl/well compound dilutions in 4% DMSO in binding buffer (99.5% PBS, 0.05% CHAPS, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) and 50 μl/well of 2 nM [2,4,6,7,16,17-^{3}H]estradiol (Amersham Biosciences) in binding buffer. "No compound" controls contained DMSO/binding buffer without compound, "background" controls contained 50 μl/well of 4 μM 17β-estradiol, and standards contained 50 μl/well of 2 nM 17β-estradiol. The final concentration of [^{3}H]estradiol was 1 nM in a total volume of 100 μl/well. The incubation occurred at 4°C overnight, after which solutions in the plates were aspirated and washed six times with 250 μl/well of PBS, followed by the addition of 250 μl/well of MicroScint-20 scintillation cocktail (Packard). The plates were sealed using transparent sealing tape and counted for radioactivity by use of the TopCount scintillation counter (Packard).

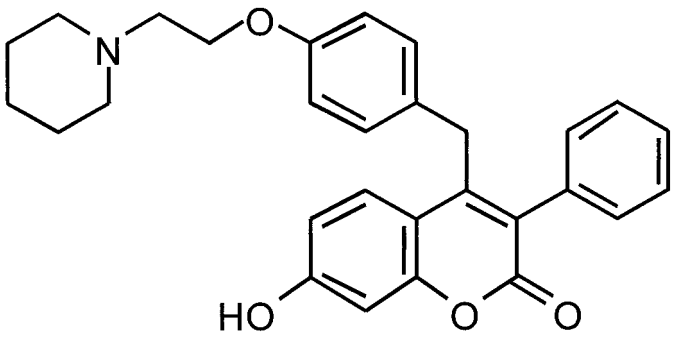

Fig. 1. Chemical structure of SP500263.

Compound IC_{50} values were determined through visual inspection of the graphs. K_i values were calculated using the formula $K_i = IC_{50} / \{1 + ([^3H\text{-ligand}] / K_d\ [^3H\text{-ligand}])\}$. K_d values of the [^{3}H]estradiol with ER-α and ER-β were determined previously. Relative binding affinity (RBA) was calculated using the formula RBA = (K_i [17β-estradiol] / K_i [compound]) × 100.

Homogenous In-Solution Steroid Hormone Receptor Competition Assays. Methods used in this study have been adapted from the scientific literature to maximize reliability reproducibility (MDS Panlabs, Bothell, WA) Briefly, purified ER-α, glucocorticoid receptor, progesterone receptor, or androgen receptor was incubated with [^{3}H]estradiol, [^{3}H]dexamethasone, [^{3}H]R-5020, and [^{3}H]mibolerone, respectively, in the absence or presence of SP500263 and reference compounds. Reference compounds were tested at five concentrations as an integral part of each assay to ensure the validity of the results obtained. The minimum concentration of SP500263 resulting in the specific inhibition of radioligand binding was determined. Semiquantitative follow-up was used to determine IC_{50} and K_i values. IC_{50} values were determined by a nonlinear, least-squares regression analysis. K_i values were calculated using the equation of Cheng and Prusoff (1973) by use of the observed IC_{50} value, the concentration of radioligand used, and the historic values for the K_d of the ligand (obtained experimentally at MDS Panlabs).

Plasmid Constructs. The 1.8-kb cDNA for human wild-type estrogen receptor-α (Green et al., 1986) was subcloned from its original plasmid KCR2-HEG0 (Metzger et al., 1995) into the mammalian expression vector pSG5 (Green et al., 1988). The 1.6-kb cDNA for human wild-type estrogen receptor-β was isolated through polymerase chain reaction amplification from a testis cDNA library and cloned into the mammalian expression vector pSG5. Expression of the presumed full-length ER-β protein of approximately 60 kDa, corresponding to the 530-amino acid protein, was confirmed through in vitro transcription/translation using the T7 promoter of the SG5 plasmid. The ERE-LUC vector was generated by cloning a 3× repeat of the ERE binding site AGGTCAGCGTGACCT into the simian virus 40 promoter luciferase vector pGL2 (Promega, Madison, WI).

Hygromycin resistance was achieved through transfection of the pCB7 plasmid. The zeocin resistance vector was engineered by replacing the coding sequence for neo from the original pLXSN vector by a cDNA fragment encoding for zeocin resistance.

Transient ERE-LUC Transfections and Luciferase Determination. U2OS cells were placed in 24-well plates at a concentration of 10,000 cells per well. Twenty-four hours later, cells were transiently transfected with 0.5 μg of ERE-LUC reporter plasmid using Superfect according to the manufacturer's recommendation (QIAGEN). Twenty-four hours after this transfection, luciferase activity was measured using a luciferase reagent kit (Promega) according to the manufacturer's recommendations.

Generation of Stable ER-α and ER-β U2OS Cell Lines. U2OS cells were transfected with the drug resistance vector for hygromycin B and the ER-α expression vector, the drug resistance vector for zeocin and the ER-β expression vector, or the corresponding empty control vectors using Superfect according to the manufacturer's recommendation (QIAGEN). Hygromycin- and zeocin-resistant clones were selected and analyzed for ER-α and ER-β expression levels by mRNA analysis. Clones with the highest ER-α and ER-β mRNA

TABLE 1
Solid-phase ER binding assay with SP500263, tamoxifen, raloxifene, and estradiol

Receptor Assay	K_i (RBA[%])			
	SP500263 · HCl	Tamoxifen · citrate	Raloxifene · HCl	17β-Estradiol
	nM (%)			
ER-α	1.4 (57)	72 (1)	0.4 (200)	0.8 (100)
ER-β	7.3 (34)	173 (1)	13 (19)	2.5 (100)

TABLE 2
Homogenous steroid hormone receptor binding assays with SP500263

Receptor Assay	SP500263 · HCl			
	Concentration	% Inhibition	IC_{50}	K_i
	μM			
ER-α	0.01	89	5.5nM	211.5pM
Glucocorticoid	10	0	N.A.	N.A.
Progesterone	10	12	N.A.	N.A.
Androgen	10	0	N.A.	N.A.

N.A., not applicable.

levels, respectively, were used for subsequent functional studies. ER-α mRNA levels were slightly higher than ER-β mRNA levels and easily detectable by RNase protection analysis using 20 μg of RNA. However, small differences can be caused by differences in RNase protection probe-labeling efficiency.

Preparation of Primary Human Osteoblasts. The generation of osteoblastic cells from normal adult femoral trabecular bone from patients without evidence of metabolic bone disease was carried out as described previously (Sutherland et al., 1995). The Regional Tissue Bank (San Diego, CA) supplied the bone fragments. Bone fragments were cultured in Ham's F12 medium supplemented with 28 mM HEPES, pH 7.4, 10% FCS, 1.1 mM $CaCl_2$, 2 mM glutamine, and 1% antibiotic-antimycotic agent. The primary cells were shown to differentiate in culture to form mineral deposits as assessed by von Kossa silver stain followed by staining with 1% alizarin red and calcium analyses of acid extracts of the cells (Sigma) (data not shown). Biochemical characterization further demonstrated parathyroid hormone-responsive adenylate cyclase production, 1,25-dihydroxyvitamin D-responsive alkaline phosphatase activity, and synthesis of type I collagen (data not shown).

ELISA Procedure. Three osteosarcoma cell lines were used in these experiments: U2OS-ER-negative (parental cell line) and two lines engineered to express ER: U2OS-ERα (stably expresses ER-α) and U2OS-ERβ (stably expresses ER-β). Cells were grown in McCoy's 5A media without phenol red with 10% FCS (Hyclone). In addition, the U2OS-ERα cell media included 0.2 mg/ml hygromycin B (Invitrogen) as a selection agent, and the U2OS-ERβ cell media included 0.2 mg/ml zeocin (Invitrogen) as a selection agent.

For the experiment, cells were plated in the media described above except that 10% charcoal/dextran-treated FCS (Hyclone) was used. Cells were plated at 25,000 cells/well in a 96-well plate and incubated for 24 h at 37°C in 5% CO_2. The compound was dissolved in DMSO at 30 mM. Appropriate dilutions of the compound were added to cells in duplicate wells for a final DMSO concentration of 0.2%. After incubation at 37°C in 5% CO_2 for 30 min, TNF-α and IL-1β were added to each well for final concentrations of 2.5 ng/ml and 1 ng/ml, respectively. Cells were incubated for 24 h at 37°C in 5% CO_2. The supernatant was removed, and 50 μl was used for IL-6 or GM-CSF ELISAs (Endogen) using the standard protocols. Plates were read at 450 nm in a plate reader (Molecular Devices).

MCF-7 Proliferation Assay. MCF-7 cells were cultured in phenol red-free Dulbecco's modified Eagle's media/Ham's F12 (Invitrogen) containing 5% fetal calf serum (Hyclone), 3 mM β-mercaptoethanol (Invitrogen), 43 ng/ml sodium selenite (Invitrogen), and 20 nM ethanolamine (Sigma) at 37°C in 5% CO_2. Cells were plated in 96-well plates at 3000 cells/well in the above media with charcoal/dextran-treated FCS (Hyclone). The next day, media were removed by aspiration, and 200 μl of fresh media was added. The compound was dissolved in DMSO at 30 mM. Appropriate dilutions of the compound in DMSO were added to cells in duplicate wells for a final DMSO concentration of 0.2%. 17β-Estradiol was also dissolved in DMSO and added to cells where indicated to a final concentration of 100 pM in 0.2% DMSO.

Cells were incubated at 37°C in 5% CO_2 for 48 h. [^{3}H]Thymidine (Amersham) was added to each well (final concentration, 0.15 μCi/μl). Cells were incubated at 37°C in 5% CO_2 for 6 h. Media were then removed, 200 μl of H_2O was added to each well, and the plate was incubated for 15 min. Cells were harvested on a Millipore filter plate using a cell harvester (Packard). Scintillation fluid (50 μl) was added to each well, and counts per well were determined by the use of the TopCount scintillation counter (Packard).

Results

Affinity to ER-α and ER-β. Binding to human ER-α and ER-β was evaluated in a solid-phase [^{3}H]estradiol competition assay using recombinant full-length protein. Results (K_i values and percentage of RBA) presented in Table 1 are the average from at least three different experiments. Reference compounds were tested concurrently as an integral part of each assay to ensure the validity of the results obtained. Binding of SP500263 (Fig. 1) to ER-α and ER-β was compared with binding of 17β-estradiol, tamoxifen, and raloxifene. SP500263 bound with high affinity to both estrogen receptors with an RBA, compared with estrogen, of 57% for ER-α and 34% for ER-β (Table 1). Tamoxifen was the weakest binder to both receptors.

Binding of SP500263 to human ER-α, human glucocorticoid, progesterone, and androgen receptors was evaluated in homogenous in-solution steroid hormone receptor competition assays using a single concentration of SP500263 and the corresponding radiolabeled ligand. Results presented in Table 2 are the average of duplicate determinations. Reference compounds for each receptor were tested concurrently as an integral part of each assay to ensure the validity of the results obtained (data not shown). SP500263 bound only to ER-α with no significant binding measurable to glucocorticoid, progesterone, and androgen receptors. Binding to ER-α was followed up in a full dose-response curve with SP500263 to determine IC_{50} and K_i values.

Therefore, SP500263 binds with high affinity to ER-α and ER-β. As with other SERMs tested concurrently, binding to ER-β was slightly weaker. The affinity of SP500263 for ER was in the range observed for other ligands of ER such as estradiol and raloxifene. No significant binding to other steroid hormone receptors such as the glucocorticoid, progesterone, and androgen receptors was found.

Effect on IL-6 and GM-CSF Gene Expression. The production of cytokines such as IL-6 is blocked by estrogen through interference with the transcription of IL-6 (Girasole et al., 1992; Kassem et al., 1996) via a novel nonclassic mechanism that does not involve direct binding of ER to DNA. The ligand-bound ER has been reported to bind directly to NF-κB and C/EBPβ, two transcription factors critical for the regulation of IL-6 (Stein and Yang, 1995; Galien et al., 1996; Galien and Garcia, 1997; Kurebayashi et al., 1997; Ray et al., 1997). Estrogen blocked IL-6 production equally well in bone cell lines engineered to overexpress ER-α or

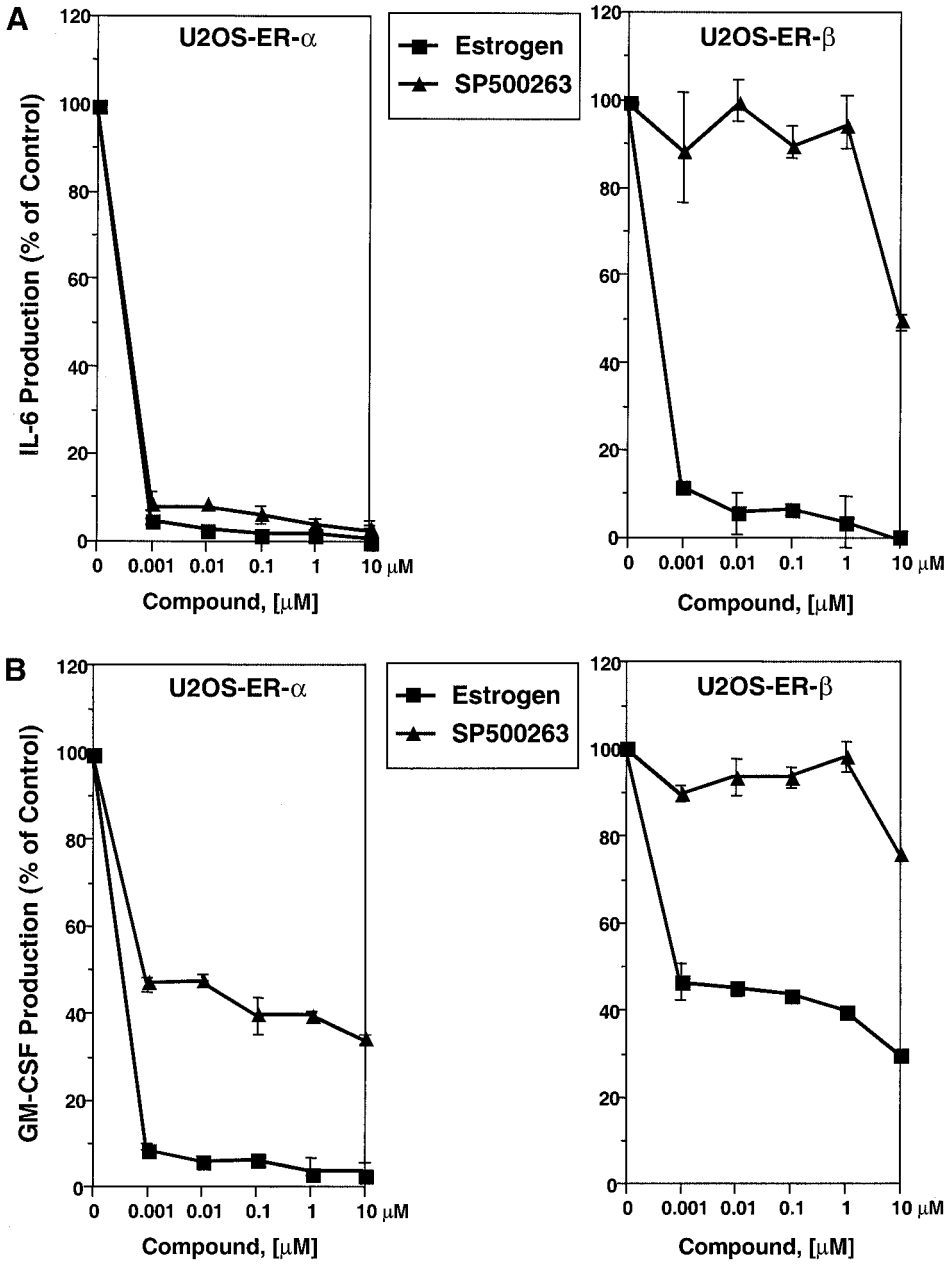

Fig. 2. Dose-response to estrogen and SP500263 in the U2OS/ER-α and U2OS/ER-β IL-6 and GM-CSF cytokine release assay. The different genetically engineered U2OS clones were exposed to the indicated concentrations of compound for 24 h before assaying for IL-6 production (A) or GM-CSF production (B) by ELISA. In both cases, cytokine production was stimulated by IL-1β/TNF-α 30 min after compound addition. Values given are the means with error bars for S.D. from triplicate determinations for each concentration of compound. For some concentrations, error bars are not visible because they are smaller than the symbol.

TABLE 3
Inhibition of IL-6 production (IC_{50}) in U2OS cell lines

	U2OS/ER-α	U2OS/ER-β	U2OS
			μM
17β-Estradiol	50 pM	17 pM	>1
SP500263	0.67 nM	>1 μM	>1
Raloxifene	3.1 nM	>1 μM	>1
Arzoxifene	0.40 nM	>1 μM	>1
Lasofoxifene	0.80 nM	>1 μM	>1
Levormeloxifene	245 nM	>1 μM	>1
Tamoxifen	455 nM	>1 μM	>1
4OH-Tamoxifen	1.9 nM	>1 μM	>1
SPC-1	278 nM	0.15 nM	>1
ICI-182,780	>1 μM	>1 μM	>1

ER-β (U2OS/ER-α and U2OS/ER-β, respectively) (Fig. 2) but has no effects in the ER-negative U2OS cell line (data not shown). In contrast, SP500263 blocks IL-6 production only in the presence of ER-α with an average IC_{50} value of approximately 0.67 nM (Fig. 2A and Table 3). Similar results were obtained using GM-CSF production as a readout (Fig. 2B). We also included other well-known SERMs in the IL-6 cytokine release assay (Table 3). With the exception of estrogen, SPC-1, and ICI-182,780, all SERMs tested seem to be ER-α selective IL-6 inhibitors. SPC-1 is a proprietary Celgene compound with a novel core structure different from all other SERMs tested. This compound, which binds to both ER-α and ER-β (data not shown), is a potent ER-β selective IL-6 inhibitor. ICI-182,780, which is well known as a pure antiestrogen, had no effects in the U2OS cytokine release assay.

To confirm that SP500263 has similar effects in primary bone cells, we treated bone cells isolated from human trabecular bone with SP500263 or estrogen and measured IL-6 production (Fig. 3). Similar to the results obtained with the U2OS osteosarcoma cell line, estrogen as well as SP500263

potently down-regulated IL-6 gene expression. RNase protection analysis confirmed the expression of high levels of ER-α mRNA and no significant amounts of ER-β mRNA in those cells (data not shown). We further confirmed that the inhibitory effect of SP500263 on IL-6 expression was at the transcriptional level by transfection analysis of IL-6 promoter luciferase plasmids (data not shown). In summary, these in vitro evaluations revealed that SP500263, a novel SERM with high affinity to ER-α and ER-β, acts as a selective ER-α estrogen agonist in bone cells on IL-6 and GM-CSF expression.

Activation of a Classic ERE. We were next interested in evaluating the effect of SP500263 in U2OS cells on a classic ERE. U2OS cells expressing either ER-α or ER-β were transiently transfected with an ERE-luciferase reporter construct and then treated with increasing doses of either estrogen or SP500263. As expected, the estrogen dose dependently increased transcription derived from the ERE (Fig. 4). In contrast, SP500263 had no effect on this classic ERE, indicating that it is not an estrogen agonist in this assay system.

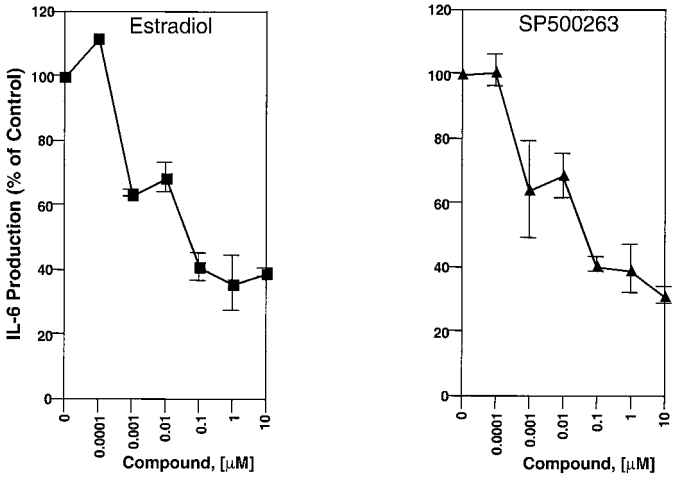

Fig. 3. Dose-response to estrogen and SP500263 in the primary human osteoblast IL-6 cytokine release assay. The primary bone cells were exposed to the indicated concentrations of compound for 24 h before assaying for IL-6 production by ELISA. Cytokine production was stimulated by IL-1β/TNF-α 30 min after compound addition. Values given are the means with error bars for S.D. from triplicate determinations for each concentration of compound. For some concentrations, error bars are not visible because they are smaller than the symbol.

Estrogen Antagonist in MCF-7. To evaluate the effect of SP500263 as an estrogen antagonist, MCF-7 breast cancer cells were treated with estrogen to stimulate proliferation. A 30-min pretreatment with SP500263 effectively down-regulated estrogen-dependent MCF-7 proliferation with IC_{50} values of approximately 8 nM, indicating that SP500263 acts as an estrogen antagonist in breast cancer cell lines (Fig. 5). Similar results were observed with tamoxifen treatment. Both compounds seem to act as cytostatic agents because the cells looked healthy after microscopic inspection and no increase in lactate dehydrogenase or 3-[4,5-dimethylthiazol-2-yl-5[3-carboxymethoxyphenyl]-2-]4-sulphenyl]-2H-tetrazolium activity was detectable (data not shown).

Discussion

In this study, we showed that ER-α and ER-β respond to ligands in very specific ways depending on the assay system used. Overall, likely because of the high homology of the ligand binding domain between ER-α and ER-β (58% amino acid identity), the majority of ligands described in the literature have very similar affinity for both receptors, with slightly higher affinity usually observed for ER-α (Tong et al., 1997). However, a few ligands with higher affinity for ER-α than for ER-β, and vice versa, have been described (Kuiper et al., 1997; Barkhem et al., 1998; Meyers et al., 1999; Sun et al., 1999).

We discovered a novel SERM, SP500263, using a functional cell-based assay with ER-α. Extensive in vitro characterization revealed that SP500263 bound with high affinity to ER-α and ER-β but not to other steroid hormone receptors

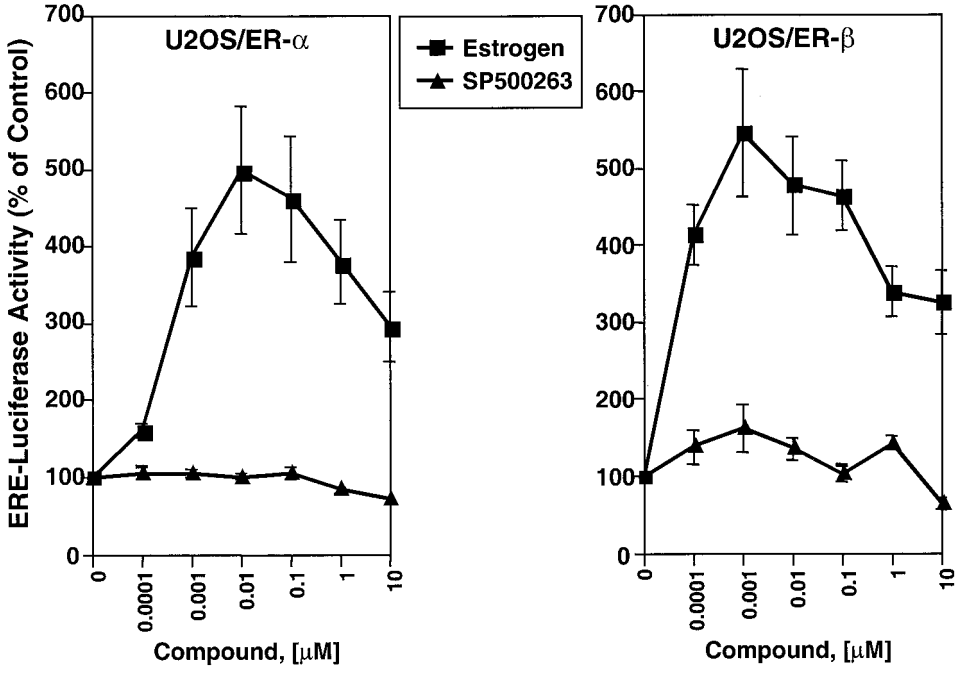

Fig. 4. Dose-response to estrogen and SP500263 in the U2OS/ER-α and U2OS/ER-β ERE-LUC assay. The different genetically engineered U2OS clones were transiently transfected with the ERE-LUC reporter plasmid. Twenty-four hours after transfection, cells were exposed to the indicated concentrations of compound for 24 h before assaying for luciferase activity. Values given are the means with error bars for S.D. from triplicate determinations for each concentration of compound. For some concentrations, error bars are not visible because they are smaller than the symbol.

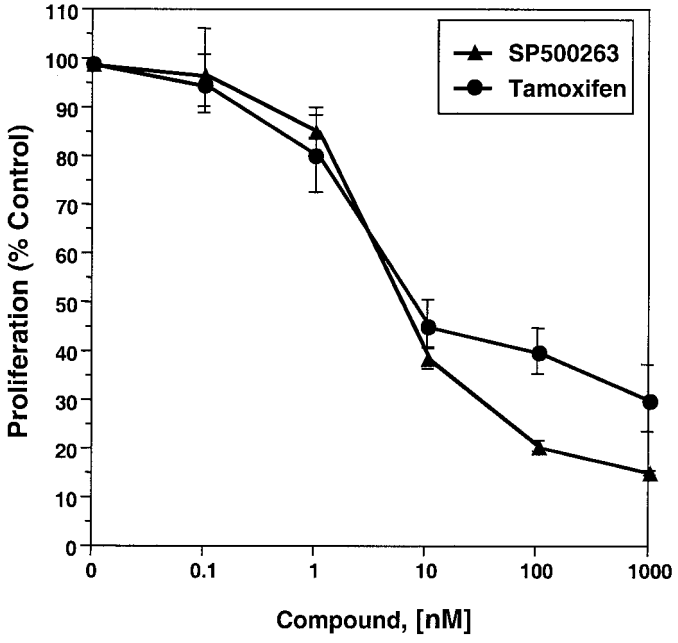

Fig. 5. Dose-response to tamoxifen and SP500263 in the MCF-7 proliferation assay. MCF-7 cells were exposed to the indicated concentrations of compound for 30 min before the addition of 100 pM estradiol to stimulate proliferation. Proliferation compared with the vehicle-treated cells was evaluated 72 h later using [^{3}H]thymidine incorporation. Values given are the means with error bars for S.D. from triplicate determinations for each concentration of compound. For some concentrations, error bars are not visible because they are smaller than the symbol.

such as the glucocorticoid, progesterone, and androgen receptors (Tables 1 and 2). The affinity of SP500263 was similar to the affinity observed for raloxifene and estradiol and was significantly higher than that for tamoxifen. Tamoxifen is reported to have low affinity to ER until its conversion to 4OH-tamoxifen.

Increasing evidence is accumulating that the affinity of a ligand for a particular receptor does not correlate with its activity observed in a cell-based assay. Differences in receptor-selective affinity were not always mirrored in activity in cell-based assays with cell lines expressing ER-α or ER-β (Paech et al., 1997; Watanabe et al., 1997; Pennie et al., 1998; Sun et al., 1999).

Estrogen was an extremely potent inhibitor of IL-6 and GM-CSF production in U2OS cells expressing either ER-α or ER-β. Surprisingly, SP500263 acted like estrogen in this assay system but in a strictly ER-α–selective manner (Figs. 1 and 2). Therefore, SP500263 is a SERM demonstrating that selectivity in receptor-binding assays does not predict selective functional activity. SP500263 was approximately 10 times less potent than estrogen in the U2OS/ER-α assay, although its binding affinity is only approximately 2 times lower (compare Table 1 with Table 3). This illustrates that affinities for the receptor in vitro do not correlate with functional activity. IC_{50} values in the U2OS IL-6 assay were derived from dose-response curves similar to the ones shown in Fig. 2, but compounds were titrated and tested at concentrations that allowed for the accurate determination of IC_{50} values. A number of other SERMs tested in the U2OS IL-6 ELISA also displayed ER-α functional selectivity (Table 3). The two weakest inhibitors, levormeloxifene and tamoxifen, both lack the critical A-ring hydroxyl group, which results in low ER binding affinity. Therefore, the U2OS assay is predictive also for compounds with very low binding affinity. Not all ER ligands in the U2OS system were ER-α-selective. SPC-1 is an example of a structurally different SERM, which bound with relatively good affinity to both ER-α and ER-β (data not shown) but showed strong preference (1800-fold) for ER-β in the U2OS system. In contrast, ICI-182,780, a high-affinity binder to ER-α (RBA = 12%) and to ER-β (RBA = 21%), was totally inactive in the U2OS assay. The lack of activity of ICI-182,780 is not caused by a lack of stability or lack of penetration of cells because under these conditions, it was able to reverse the inhibition of IL-6 expression by estrogen (data not shown).

Although estrogen blocked IL-6 and GM-CSF production nearly 100% at concentrations greater than 1 nM, SP500263 failed to block GM-CSF production in the presence of ER-α more than 60%. Conversely, estrogen failed to block GM-CSF production in the presence of ER-β more than 60%. We currently have no explanation for this observation, but it may suggest that other signaling pathways not responsive to ER are involved.

Estrogen blocks IL-6 gene expression at the transcriptional level through the interaction of ER with NF-κB and C/EBPβ (Stein and Yang, 1995). We confirmed that SP500263 works through a similar mechanism (data not shown). Our studies with SP500263 in primary human osteoblasts expressing endogenous levels of ER-α (Fig. 3) suggest that SP500263 will be a potent inhibitor of IL-6 gene expression in bone. IL-6 is a critical osteotropic factor involved in the differentiation and activation of osteoclasts leading to increased bone resorption and as such may be an integral factor in the pathogenesis of osteoporosis (Grey et al., 1999; Sandhu et al., 1999). Therefore, SP500263 represents a member of a novel series of SERMs that may have clinical use in treating and preventing postmenopausal osteoporosis. SP500263 was demonstrated to be an orally active SERM that acts in rats as an estrogen agonist on bone without causing uterine stimulatory effects (M. K. Sutherland, H. Brady, L. M. Gayo-Fung, J. Leisten, S. Lipps. J. A. McKie, E. O'Leary, N. Patnaik, N. Sakurai, T. Takagi, et al., manuscript in preparation).

The classic way of regulating genes through an ERE has been used historically to measure the estrogen agonist activity of a particular compound. However, SP500263 is an example of a compound that, although classified as an estrogen agonist (i.e., acts like estrogen) in the U2OS/IL-6 assay, is inactive in the U2OS ERE assay (Fig. 4). This exemplifies the importance of testing a particular SERM in a cellular assay system that is relevant for its functional activity.

Along the same lines, estrogen is growth-stimulating in hormonally responsive breast cancer cells. In contrast, SP500263 behaved as antiestrogen and antagonized the stimulatory effect of estrogen in breast cancer proliferation assays (Fig. 5) and in murine models of breast cancer (Brady et al., 2002). Therefore, SP500263 is an example of a compound that combines the positive effects of estrogen on bone cells with prevention of the negative effects of estrogen on breast cancer cells.

In summary, SP500263 is a member of a novel series of SERMs that is structurally different from other SERMs currently on the market or in development. SP500263 acts as an estrogen agonist in bone cells and an estrogen antagonist in breast cancer cells, and it does not activate genes through a

classic ERE. Further in vivo efficacy and safety studies should demonstrate how suitable this class of SERMs is for development as antiosteoporosis and anticancer agents.

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