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Structural features underlying raloxifene's biophysical interaction with bone matrix

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ABSTRACT

Raloxifene, a selective estrogen receptor modulator (SERM), reduces fracture risk at least in part by improving the mechanical properties of bone in a cell- and estrogen receptor-independent manner. In this study, we determined that raloxifene directly interacts with the bone tissue. Through the use of multiple and complementary biophysical techniques including nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR), we show that raloxifene interacts specifically with the organic component or the organic/mineral composite, and not with hydroxyapatite. Structure–activity studies reveal that the basic side chain of raloxifene is an instrumental determinant in the interaction with bone. Thus, truncation of portions of the side chain reduces bone binding and also diminishes the increase in mechanical properties. Our results support a model wherein the piperidine interacts with bone matrix through electrostatic interactions with the piperidine nitrogen and through hydrophobic interactions (van der Waals) with the aliphatic groups in the side chain and the benzothiophene core. Furthermore, in silico prediction of the potential binding sites on the surface of collagen revealed the presence of a groove with sufficient space to accommodate raloxifene analogs. The hydroxyl groups on the benzothiophene nucleus, which are necessary for binding of SERMs to the estrogen receptor, are not required for binding to the bone surface, but mediate a more robust binding of the compound to the bone powder. In conclusion, we report herein a novel property of raloxifene analogs that allows them to interact with the bone tissue through potential contacts with the organic matrix and in particular collagen.

1. Introduction

Bone is a crucial tissue that provides protection to internal organs and support, as well as the ability to respond to environmental signals by adapting its geometry and mass. This unique combination of stability and adaptability is made possible by the composition of the bone matrix itself, where the mineral phase, the hydroxyapatite, provides stiffness, and the organic phase, mainly composed by collagen, provides the tensile strength. Moreover, throughout an individual's life, the bone matrix is constantly remodeled to meet metabolic and mechanical needs through the concerted effort of bone-forming cells, the osteoblasts, and bone-resorbing cells, the osteoclasts.

Selective estrogen receptor modulators (SERMs) have been shown to decrease bone resorption and increase bone mineral density[1,2] through direct interaction with the estrogen receptors followed by tissue selective transcriptional regulation.[3] Raloxifene is a clinically-validated SERM, which has been shown to mimic the protective effect of estrogen on the skeletal system without triggering the proliferative effects of estrogen on the breast and endometrium of the uterus.[4,5] Many clinical studies have confirmed the therapeutic effectiveness of raloxifene in reducing the risk of vertebral fractures in postmenopausal osteoporosis patients by significantly slowing bone turnover, preventing bone loss, and maintaining the structural integrity of cancellous bone.[6–9]

The ability of raloxifene to reduce fracture risk is far greater than what would be predicted based solely on its effect on bone mineral density (BMD).[10,11] Preclinical studies have documented that raloxifene leads to improved mechanical properties, most notably material-level toughness, despite little/no change in BMD or geometry/architecture.[12–14] Recent work has demonstrated that this effect of raloxifene is at least partly cell-independent.[15] Mechanistically, it has been proposed that raloxifene increases bound water within the matrix and this alters how strains are transferred between the organic and hydroxyapatite (HAP) portions of the

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matrix. Despite the biophysical evidence that this phenomenon is cell-independent, it remains unclear if this effect of raloxifene occurs through direct molecular interactions with bone and, if so, specifically what component of bone interacts with raloxifene.

Because a mechanism that is independent of the bone cells and the estrogen receptor (ER) pathway activation could represent an unexpected pharmacological approach to reducing osteoporotic fractures, we aimed to identify the structural features within the benzothiophene nucleus of the raloxifene that are responsible for this compound's interactions with bone tissue. We used multiple biophysical techniques including nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR) in order to quantify the interaction of benzothiophenes, estrogen and Alendronate with bone and its components, and to investigate the molecular requirements that mediate that interaction.

2. Materials

Ceramic hydroxyapatite (HAP) Type I, 20 μm, was from BioRad, Cat# 158-2000. 3D Collagen Culture System was from Millipore (Cat. No. ECM675). Poly D-lysine 48-well plates used for the binding exposure studies were obtained from BIOCOAT (Cat. No. 354509, Lot No. 217048). DPBS (—Ca, —Mg) (Cat. No. 14-190-136) was from Fisher Scientific. Deuterated water (D_2O) was from Sigma. SERMs (Table 1) were prepared as described.[16,17] The tested compounds were prepared as 10 mM solutions in 100% DMSO or DMSO-d_6 (Sigma Aldrich).

2.1. Mechanical testing

Skeletal tissue was obtained through a tissue sharing program at the Indiana University School of Medicine. All animals were on protocols that were approved by the Institutional Animal Care and Use Committee prior to their use. Femora from skeletally mature (15–24 mo/old) treatment naïve male hounds were machined into prismatic beams (N = 8–12 beams per experimental group) following the details described previously.[15] Beams were subjected to at least one freeze–thaw cycle to ensure eradication of cellular activity. Absence of any cellular activity after a single freeze–thaw was demonstrated by lactate dehydrogenase immunostaining of fresh and frozen–thawed bone and the results have been published in Ref. 15. Specified compounds were dissolved in dimethyl sulfoxide (DMSO) and then added to incubation media (1× PBS with 1% penicillin–streptomycin supplementation) at a 2 μM concentration. Solutions were changed every other day over the course of 2 weeks. Control bones were soaked in PBS with 0.04% DMSO v/v ratio. At the conclusion of soaking, bone beams were subjected to four-point bending as previously described.[15] Energy to fracture was measured as the area under the force displacement curve. As these beams were machined to similar sizes, the parameter of energy to toughness is analogous to the modulus of toughness of the bone tissue as a material, described in previous work.[15]

2.2. Brunauer, Emmett and Teller (BET) isotherms

Bone tissue from animals was ground using a mortar and pestle to produce a powder that was then sieved using a wire mesh to produce a distribution of differently-sized particles (<0.3 mm, >0.3 to <0.53 mm, >0.53 to <0.86 mm, and >0.86). These batches were placed into 3/8 inch diameter tubes and purged with nitrogen for 2 h at 40 °C. The samples, equilibrated to room temperature, were analyzed with 7-point BET (partial pressures of 0.05–0.2) using a Micromeritics Tristar 3000 with nitrogen adsorbate to assess surface area.[18] The batch with sample particle size of <0.3 mm failed the canonical acceptance criteria that require the plot of BET value versus P/P_0 to have the linear regression of $r < 0.9975$ due to an outlier in the data. This batch (<0.3 mm) was then tested on a Micromeritics ASAP 2020. Approximately 5 g of this batch (<0.3 mm) sample were placed into a 1/2 inch diameter surface tube and purged with nitrogen at 60 °C for approximately 6 h. After equilibrating to room temperature, the sample was analyzed with a 7 point BET, as described above, using a Micromeritics ASAP 2020 with krypton adsorbate. The results met the acceptance criteria ($r > 0.9975$) and are reported as BET SSA (specific surface area). Total surface area (TSA) is calculated by multiplying the BET SSA result by the sample weight.

2.3. Scanning electron microscopy (SEM)

For SEM, powder samples were sprinkled on 13 mm SEM stubs with carbon adhesive tabs using a stainless steel spatula. Stubs were then tapped to remove excess sample and sputter coated with Au/Pd (gold/palladium 60:40) target. FEI 200 Quanta ESEM Imaging was conducted in low vacuum mode, at 20 kv, with 3.0 spot size and magnifications of 50–2500×.

2.4. Energy dispersive spectroscopy (EDS)

SEM/EDS imaging was performed to assess the size heterogeneity of the bone powder and determine the most appropriate particle size to be used in further experiments. Bone powder (<0.3 mm sample only) was rinsed gently with PBS (3×) and then divided into 2 batches. One batch was dried for 1 h in a vacuum oven at 37 °C and then flushed with Argon. The other batch was air dried and flushed with Argon. Samples were sprinkled on 13 mm SEM stubs with carbon adhesive tabs using a stainless steel spatula. Stubs were then tapped to remove excess sample, but they were not sputter coated. SEM/EDS imaging was conducted at 15–25 kv, with magnifications of 30–500× using a FEI 200Quanta ESEM.[19]

2.5. Nuclear magnetic resonance (NMR)

A modified version of a protocol previously described[20] was used. In this version, a quantitative measurement is obtained, by calculating the integral of the NMR signal, which is directly proportional to the concentration of the compound in solution. Solutions of compounds (50 μM) mixed in deuterated water (D_2O) were divided into two equal aliquots. One aliquot (0.7 ml) was incubated with <0.3 mm bone powder (10 mg) or HAP (5 mg) in a 1.5 ml Eppendorf tube, for 1 and 16 h under rotation at room temperature, while the other aliquot (0.7 ml) was incubated without bone powder or HAP, but maintained under rotation at room temperature. After incubation, samples were centrifuged at 1000g for 5 min and the supernatant was transferred to a new tube and frozen at −20 °C until analysis. To evaluate binding to collagen, 3D collagen matrices (Merck-Millipore) were prepared following the manufacturer's instructions and 0.2 ml of collagen was added to each well of a 48-well plate (Biocoat) and allowed to polymerize at 37 °C. Incubations were performed with 0.7 ml of solution containing 50 μM of compounds (raloxifene, 88074 and 189005) at 37 °C for 1 or 16 h. Control solutions were incubated in wells without polymerized collagen. After the indicated incubation times, solutions were aspirated with a pipette and stored at −20 °C until analysis. All NMR data were obtained using a Bruker Avance III 600 MHz spectrometer equipped with a quadruple resonance ($^1H/^{19}F/^{13}C/^{15}N$) QCI CryoProbe. Spectra were acquired with a standard 1D proton sequence. Residual water signals were suppressed with presaturation at a pulse power of 50 Hz during the relaxation