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Bone selective effect of an estradiol conjugate with a novel tetracycline-derived bone-targeting agent

Jason R. Neale [a], Natali B. Richter [b], Kevyn E. Merten [c], K. Grant Taylor [b], Sujan Singh [b], Leonard C. Waite [a], Nicole K. Emery [a], Ned B. Smith [a], Jian Cai [a], William M. Pierce Jr. [a,b,c,*]

[a] Department of Pharmacology and Toxicology, University of Louisville, Louisville, KY 40292, USA
[b] Department of Chemistry, University of Louisville, Louisville, KY 40292, USA
[c] Pradama, Inc., Louisville, KY 40202, USA

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ABSTRACT

In this study a novel bone-targeting agent containing elements of the tricarbonylmethane system of ring A of tetracycline was developed and was shown to bind to the mineral constituent of bone, hydroxyapatite. Conjugation of this bone-targeting agent to estradiol resulted in a bone-targeted estrogen (BTE_2-A1) with an enhanced ability to bind to hydroxyapatite. In an ovariectomized rat model of osteoporosis a partial separation of the skeletal effects of estradiol from the uterine effects was observed following subcutaneous administration of BTE_2-A1. This novel bone-targeting estradiol delivery system has the potential to improve the safety profile of estradiol in the treatment of osteoporosis.

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Numerous agents including acidic oligopeptides and bisphosphonates display a high affinity for the mineral constituent of bone (hydroxyapatite) and these agents have been used as bone-targeting moieties.[1–5] Chemical conjugation of these agents to estradiol has been investigated as a means to improve the therapeutic index of this compound in the treatment of osteoporosis.[6,7] These osteotropic estradiol delivery systems exhibit preferential distribution of estradiol to bone and show pharmacological activity in bone comparable to estradiol without the systemic adverse effects of estradiol.[6–8] Clinically, however, these targeting agents are less than ideal due to the inherent biological activities, potential side effects, and routes of administration of the bone-targeting moieties themselves.[9,10] The polycyclic naphthacene carboxamide, tetracycline, is known to avidly bind to hydroxyapatite and has previously been evaluated as a bone-targeting moiety following conjugation with therapeutic agents, including estradiol.[11–13] As a bone-targeting agent, tetracycline offers the advantages of oral bioavailability and of being relatively non-toxic, however, the clinical utility of tetracycline conjugates is questionable due to the intrinsic antibiotic activity of tetracycline.[11] Furthermore, the complex chemical structure and poor stability of tetracycline during chemical conjugation limit the feasibility of using tetracycline as an osteotropic agent.[14]

Structure–activity relationship (SAR) studies indicate that it may be possible to separate the hydroxyapatite binding domain from the minimum structural requirements for the biological activity of tetracycline. These studies have revealed that many changes can be made at positions 5, 6, and 7 of tetracycline without affecting the antibacterial properties.[15] However, the remaining structural and stereochemical features of tetracycline, including the dimethylamino group, the phenolic β-diketone system, and the tricarbonylmethane system, are essential for in vivo antibacterial activity.[15,16] On the other hand, the structural requirements for hydroxyapatite binding appear much less extensive as the tricarbonylmethane grouping of ring A of tetracycline (Fig. 1) has been suggested to be sufficient to allow binding to hydroxyapatite.[17] In this study we synthesized and characterized a bone-targeting agent modeled after the tricarbonylmethane grouping of ring A of tetracycline. This novel bone-targeting agent was shown to possess significant hydroxyapatite binding affinity. A bone-targeted estrogen was subsequently synthesized via chemical conjugation of estradiol and the pharmacological effects of this conjugate were evaluated in vitro and in vivo in an ovariectomized rat model of osteoporosis.

The bone-targeting agent was synthesized in four steps, as depicted in Scheme 1, starting with 2,6-dihydroxybenzoic acid (**1**).

* Corresponding author. Tel.: +1 502 852 7424; fax: +1 502 852 7868.
E-mail address: pierce@louisville.edu (W.M. Pierce).

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Alkylation of compound **1** with iodomethane via an intermediate silver salt yielded the corresponding methyl ester **2**. Treatment of the ester with ammonium hydroxide led to the amide product **3**. Nitration at the 3-position was accomplished using the standard combination of glacial acetic and concentrated nitric acids to yield **4**. Subsequently, compound **5** was attained by reduction of the 3-nitro group.

The ability of compounds **1**, **3**, 4, and **5** to bind to the mineral constituent of bone (hydroxyapatite, $Ca_{10}(PO_4)_6{\cdot}OH_2$) was evaluated using a hydroxyapatite (HA) binding assay.[18] For comparative purposes tetracycline was included as a control and was defined as having a HA binding index of 100. Compound **1** did not bind to hydroxyapatite (data not shown), while compounds **3** and **4** showed little to no appreciable binding (Fig. 2). Compound **5**, 3-amino-2,6-dihydroxybenzamide, on the other hand, bound to hydroxyapatite approximately 50% as well as tetracycline. Interestingly, neither 1-carboxamidocyclohexane-2,6-dione (data not shown) nor 2,6-dihydroxybenzamide (**3**), both similar in structure to the tricarbonylmethane system of ring A of tetracycline, bound to hydroxyapatite in vitro indicating that this system may not be sufficient in and of itself to allow binding to bone mineral. Our data suggest that in addition to the tricarbonylmethane system of tetracycline, an amino group at position 3 of compound **5** (position 4 of the A ring of tetracycline) is necessary to allow binding to hydroxyapatite. This conclusion is further supported by the finding that the corresponding 3-nitro analog (**4**) does not appreciably bind to hydroxyapatite.

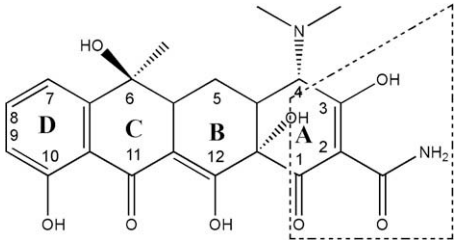


Figure 1. Structure of tetracycline. The region outlined is the tricarbonylmethane region of tetracycline and is the template for synthesis of the novel bone-targeting agent.

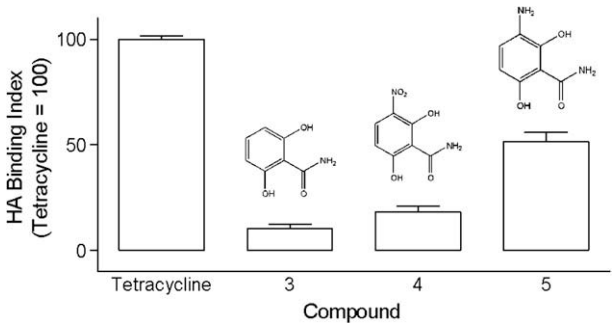
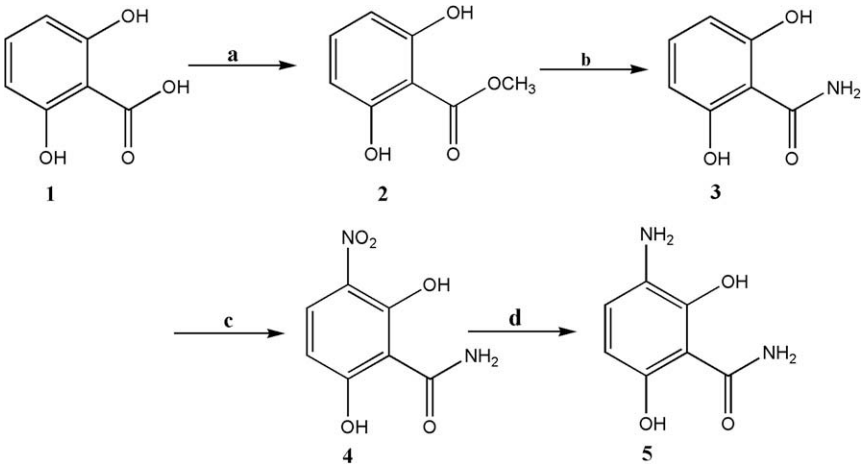


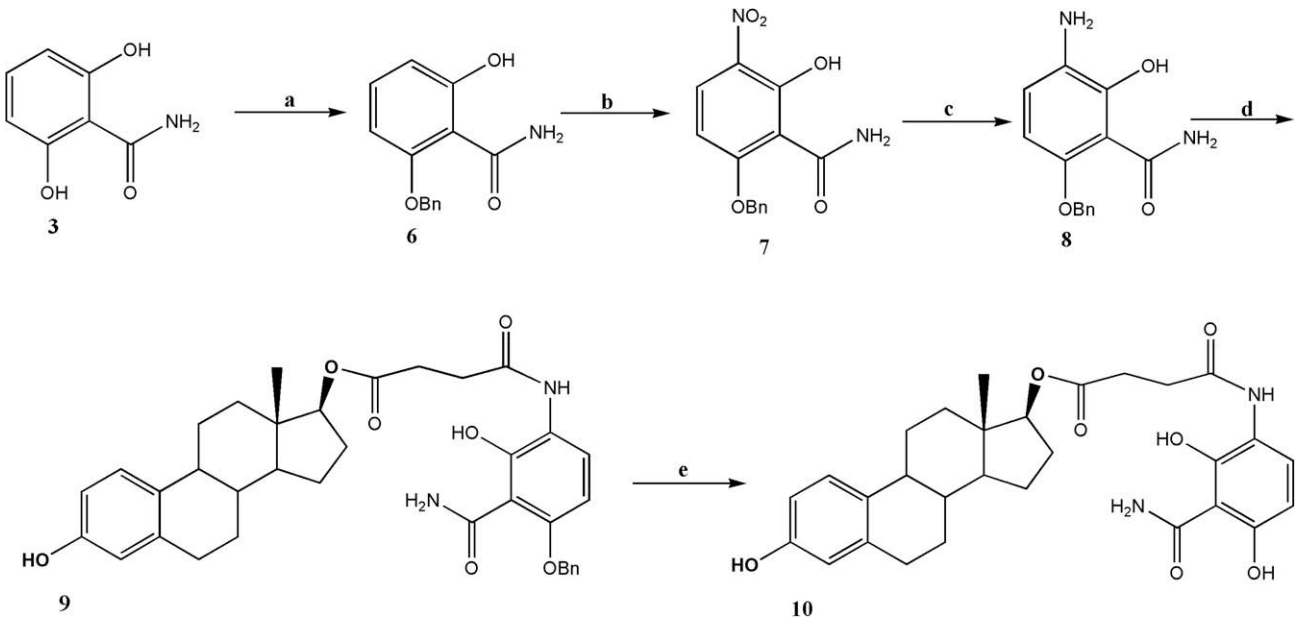
Figure 2. Hydroxyapatite binding capacity. The ability of tetracycline and compounds **3**, **4**, and **5** to bind to hydroxyapatite was evaluated using a hydroxyapatite binding assay. Compound **5**, similar to the A ring of tetracycline, has approximately 50% of the binding ability of tetracycline in this surrogate bone-targeting system.

As indicated above, 3-amino-2,6-dihydroxybenzamide (**5**) binds to hydroxyapatite approximately 50% as well as tetracycline. Compound **5** was therefore chosen as the prototype bone-targeting agent and was subsequently conjugated with estradiol to produce a bone-targeted estrogen, BTE_2-A1. The synthesis of BTE_2-A1 was carried out as shown in Scheme 2. Prior to nitration, compound **3** was protected asymmetrically using one molar equivalent of benzyl bromide under basic conditions to give product **6**. The compound was nitrated at the 3-position, yielding **7**, which was then selectively reduced with hydrazine, ferric chloride hexahydrate, and activated carbon to give compound **8**. Carbodiimide-mediated coupling of the bone-targeting moiety to estradiol-17-*O*-hemisuccinate (Steraloids, Inc., Newport, R.I.) yielded product **9**. Finally, catalytic hydrogenation of **9** gave the desired conjugate **10**, (13*S*,17*S*)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[*a*]phenanthren-17-yl 4-(3-carbamoyl-2,4-dihydroxyphenylamino)-4-oxobutanoate. Detailed synthetic protocol, as well as NMR and mass spectral data, for compounds **5** (Scheme 1) and **10** (Scheme 2) can be found in the Supplementary material.

The ability of BTE_2-A1 (**10**) to bind to crystalline hydroxyapatite in vitro was used as a predictive measure of this compounds ability to bind to bone in vivo. Tetracycline, estradiol, and BTE_2-A1 (**10**) were evaluated using the hydroxyapatite binding assay.[18] Surprisingly, BTE_2-A1 (**10**) had a HA binding index of 105 (tetracycline = 100), indicating that the addition of the estrogenic group to the bone-targeting agent (**5**) enhanced the ability of this compound to bind to the mineral constituent of bone. This data lends support to our bone-targeting strategy, as non-targeted estradiol did not bind to hydroxyapatite (HA binding index = 0).



Scheme 1. Synthesis of bone-targeting agent. Reagents: (a) NH_4OH (28% NH_3), $AgNO_3$, CH_3I; (b) NH_4OH (28% NH_3); (c) glacial acetic acid, concentrated HNO_3; (d) 10% Pd/C, H_2.



Scheme 2. Synthesis of bone-targeted estrogen. Reagents: (a) K_2CO_3, benzyl bromide; (b) glacial acetic acid, concentrated HNO_3; (c) activated carbon, $FeC1_3{\cdot}6H_2O$, $NH_2NH_2{\cdot}H_2O$; (d) estradiol-17-*O*-hemisuccinate, HOBT, DCC; (e) 10% Pd/C, H_2.

Aspartic acid conjugated estradiol has previously been shown to have a significantly lower affinity for the estrogen receptor than free estradiol.[6] A competitive estrogen receptor binding assay was performed using purified recombinant ERα and ERβ in order to determine the binding affinity of BTE_2-A1.[19] Data for BTE_2-A1 and an estradiol (E_2) standard curve were plotted as percent [^{3}H]-E_2 bound versus molar concentration and the IC_{50} (50% inhibition of [^{3}H]-E_2 binding) for each was determined. The relative binding affinity (RBA) of BTE_2-A1 was calculated as IC_{50} E_2/IC_{50} BTE_2-A1 × 100%. BTE_2-A1 was found to have a RBA of 16% for ERα and 9.4% for ERβ indicating that BTE_2-A1 has the ability to bind to both estrogen receptors though with a significantly lower affinity than free estradiol.

The ovariectomized rat is commonly used as a model of postmenopausal bone loss as the two systems share many characteristics, including similar skeletal and systemic responses to estrogen therapy.[20] This model was therefore used to determine whether or not BTE_2-A1 demonstrates selective effects on bone, in comparison to 17β-estradiol. Three month old, bilaterally ovariectomized (OVX) or sham-operated Sprague–Dawley female rats were administered vehicle, 17β-estradiol or BTE_2-A1 three times per week via subcutaneous injection. Drug administration was initiated 4 days following surgery and lasted for 6 weeks. Uterine and femoral mass were determined directly using excised tissue and normalized to body mass. Ovariectomy significantly decreased the normalized femoral mass (femoral mass/body weight) by 17.1% and the normalized uterine mass (uterine mass/body weight) by 90.5% in comparison to sham-operated controls. Subcutaneous administration of 17β-estradiol suppressed the OVX-induced decrease in normalized femoral mass and normalized uterine mass in a dose-dependent manner (Fig. 3A). 17β-Estradiol appeared to be equipotent in both bone and uterus as demonstrated by the overlapping dose–response curves in these tissues (Fig. 3A). In fact, the calculated uterine and femoral ED_{50} of 17β-estradiol were 9 and 10 nmol/kg, respectively. On the other hand, subcutaneous administration of BTE_2-A1 resulted in a partial separation of the bone and uterine effects of this compound (Fig. 3B) with calculated uterine and femoral ED_{50} of 90 and 8 nmol/kg, respectively. The separation of skeletal and systemic effects following subcutaneous administration of BTE_2-A1 is similar to the dose-dependent suppression of bone loss and mitigation of uterine stimulation previously observed with other osteotropic estradiol delivery systems.[6,7] Collectively, the preservation of femoral mass and the partial separation of uterine and skeletal effects achieved by BTE_2-A1 indicate that this compound may produce its effects through the selective delivery and accumulation of estradiol in bone. Alternatively, the partial separation of skeletal and uterotrophic effects following adminis-

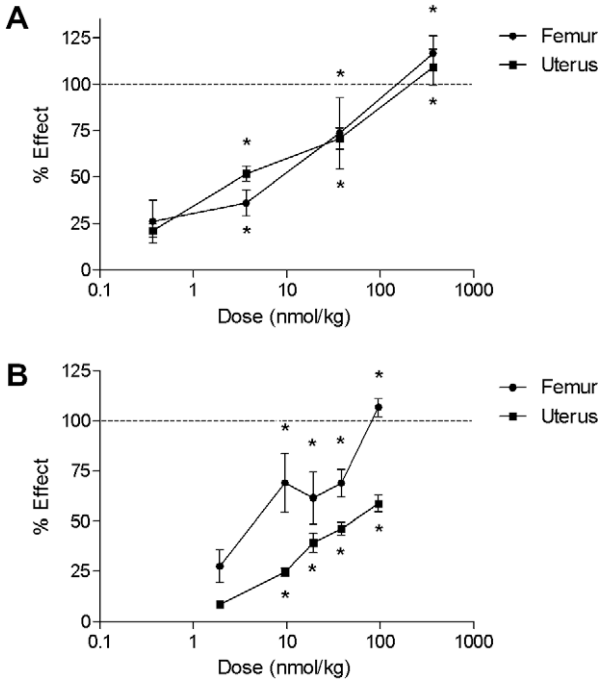


Figure 3. Effects of subcutaneous administration of 17β-estradiol (A) or BTE2-A1 (B) on normalized femoral mass and normalized uterine mass in OVX rats. Percent effect is expressed as the ratio of the difference between treatment and OVX control groups to the difference between sham-operated and OVX control groups (n = 6–10). 0% effect represents control OVX rats and 100% effect represents sham-operated control rats. The data are expressed as mean ± SEM. Significantly different from OVX rats: $^*p < 0.05$.

tration of BTE_2-A1 could be attributable to a differential selectivity for ER subtypes as BTE_2-A1 has an ERα selectivity (ERα RBA/ERβ RBA) of 1.74. However, these data appear to support this bone-targeting strategy, as the bone protective effects of BTE_2-A1 and estradiol were observed at similar ED_{50} despite the fact that BTE_2-A1 binds to the estrogen receptor with a much lower affinity than estradiol.

Additional studies will be required to determine the mechanism of action of BTE_2-A1. The ester bond linking the bone-targeting agent to estradiol may be hydrolyzed to generate free estradiol following the selective delivery of the compound to bone. Alternatively, intact BTE_2-A1 may mediate the selective effects on bone. Future SAR studies will be performed in which the bond linking the bone-targeting agent to estradiol is modified from an ester to a less labile linkage such as an amide or ether. Additionally, drug activation mechanisms which will allow for the generation of estradiol specifically at bone will be explored. Modifications will also be made to the bone-targeting agent in order to determine the effects that steric hindrance may have on the ability of the moiety to bind to hydroxyapatite. Additional studies will also be required to demonstrate that our bone-targeting agent is biologically inactive and devoid of the inherent antibacterial properties of tetracycline. However, from this study it is apparent that our single ring bone-targeting agent maintains an affinity for hydroxyapatite similar to that of the complete four ring system of tetracycline and that conjugation of the bone-targeting agent to estradiol results in a compound with selectively efficacious effects on bone.

In summary, we have successfully synthesized and characterized a novel bone-targeting agent modeled after the tricarbonylmethane grouping of ring A of tetracycline. This bone-targeting agent, 3-amino-2,6-dihydroxybenzamide (**5**), was shown to possess significant hydroxyapatite binding affinity. A bone-targeted estrogen, BTE_2-A1 (**10**), was subsequently synthesized via chemical conjugation of estradiol and the pharmacological effects of this conjugate were evaluated in an ovariectomized rat model of osteoporosis. Using this model, a partial separation of the skeletal effects of estradiol from the uterine effects was observed following subcutaneous administration of BTE_2-A1, indicating that our novel osteotropic estradiol delivery system has the potential to improve the safety profile of estradiol in the treatment of osteoporosis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.051.

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18. Hydroxyapatite binding assay. A 10^{-3} M solution for each analyte was made in 100% dimethylsulfoxide (DMSO). A 100-fold dilution was then made to form a 10^{-5} M solution in 50 mM Tris–HCl buffer, pH 7.4, 1% DMSO. Tetracycline was used as a reference analyte and approximately 50% was adsorbed to HA at the concentration of 10^{-5} M. The HA slurry was 0.5 g/100 mL 50 mM Tris–HCl buffer, 1% DMSO. For each analyte, two samples were prepared. For one sample, 1 mL of 10^{-5} M analyte and 100 μL 50 mM Tris–HCl buffer, 1% DMSO was pipetted into a microcentrifuge tube. For the second sample, 1 mL of 10^{-5} M analyte and 100 μL of the HA slurry was pipetted into a microcentrifuge tube. The samples were mixed gently by inversion for 4 min and then centrifuged at 12,000g for 3 min to sediment the HA contained in those samples. The supernatant was transferred to another microcentrifuge tube. An electronic spectral scan (ultraviolet–visible) from 220 to 520 nm was obtained for each analyte using a Varian Cary 300 Bio Scan. The blank was 50 mM Tris–HCl buffer, 1% DMSO. The wavelength of maximum absorbance (λ_{max}) was determined, and the extinction coefficient (ε) was calculated using the Beer–Lambert Law. The absorbance of the samples incubated with HA was measured at λ_{max}, and the molar concentration of the analyte was then determined using the Beer–Lambert Law and the previously calculated extinction coefficient. The fraction adsorbed to HA for each sample was calculated.
19. Estrogen receptor competition binding assays. Purified ERα and ERβ were obtained following expression in Sf21 insect cells and were generously provided by Dr. Carolyn Klinge of the University of Louisville. Reaction mixtures containing 5 nM ERα or ERβ, 5 nM [^{3}H]-estradiol, and increasing concentrations of competitor were added to 40 mM Tris buffer, pH 7.4, containing 1 mM ethylenediamine tetraacetic acid (EDTA), 111 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT). Total reaction volume was 60 μL. A 200-fold excess of unlabeled estradiol was used to determine non-specific binding (0% [^{3}H]-estradiol bound) and 0 nM competitor represented total binding (100% [^{3}H]-estradiol bound). Each sample was performed in triplicate and incubations were conducted for 16 h at 4 °C. Bound and free radioligand were separated by a hydroxyapatite (HA) slurry consisting of 10% (w/v) HA, 40 mM Tris buffer, pH 7.4, 111 mM KCl, 0.5 mM PMSF, and 1 mM DTT. A volume of 50 μL of the HA slurry was added to the reaction mixture and vortexed briefly to precipitate the ER and stop the binding reaction. The reaction mixture containing the HA solution and the precipitated ER with bound ligand was centrifuged, the supernatant was discarded, and the HA pellet was resuspended in buffer to rinse the HA pellet of any unbound [^{3}H]-estradiol. Scintillation cocktail was added to the HA pellet. A scintillation counter was used to determine the amount of [^{3}H]-estradiol present.
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